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"*ACINETOBACTER* SPECIES IN CLINICAL ISOLATES AND DETECTION OF A NEW BIOTYPE OF *ACINETOBACTER BAUMANNII*"

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SUMMARY

Acinetobacter infections have stirred interest because of a rise in frequency, severity, and difficult therapeutic approach. Notwithstanding, studies have been hampered by uncertainties about the taxonomic position of the genus. 255 *Acinetobacter* strains, isolated from clinical material from both inpatients and outpatients, were identified through 28 phenotypic tests belonging to a recent scheme of taxonomic reorganization. *A. baumannii* was the most often found species (81.6%), followed by *A.* genospecies 3 (4.3%), *A. haemolyticus* and *A. junii* (2.3%); 5 other species were isolated less often while 5.5% of the strains were not identified. A biotyping system for *A. baumannii* based on the use of 6 carbon sources led to the identification of 13 out 19 defined biotypes. Biotypes 2 (42%), 6 (20.2%) and 9 (18.3%), mostly isolated from the patients' respiratory tract secretions predominated. A new biotype characterized by the utilization of L-tartarate only was described and named biotype 20. The combination of identification schemes for *Acinetobacter* species and *A. baumannii* biotyping allowed us to know the prevalence of *A. baumanni* species biotype 2 in the area and during the time of study, showing the epidemiological usefulness of these methods.

Key words: *Acinetobacter, Acinetobacter baumanni*, biotypes.

INTRODUCTION

Bacteria of the genus *Acinetobacter* are short rods or coccoid forms, frequently occurring in pairs or in chains of variable length, Gram-negative, nonmotile, strictly aerobic, catalase positive and oxidase negative. They are ubiquitous in nature and may be found in water, soil, sewage, and as commensals in humans and animals, especially on skin, upper respiratory tract and genital tract (7,17).

Since the 1970's, in several countries, *Acinetobacter*, despite its low virulence, has been recognized as an emerging opportunist pathogen frequently involved in severe nosocomial outbreaks. Usually they infect patients on antibiotic or immu-

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nosuppressive therapy, as well as those with neoplasias, tracheal or intravascular interventions, and prolonged treatment in intensive care units. These infections constitute a serious therapeutic problem due to an increase in occurrence as well as dissemination of multiple resistance to antibiotics (1,2,17,19,21,22).

The first description of *Acinetobacter* organisms was made by Beijerinck, in 1911, who named them *Micrococcus calcoaceticus*. Since then, they have been known by a number of different names, making their nomenclature extremely misleading: *Diplococcus, Bacterium, Achromobacter, Mima, Herellea* and *Moraxella* are some examples.

The genus *Acinetobacter* was described by Brisou and Prévot, in 1954 (8). Only two species (*A. calcoaceticus* and *A. iwoffii*) appear in Approved Lists of Bacterial Names (24), and only one species (*A. calcoaceticus*) is described in Bergey's Manual of Systematic Bacteriology (7).

Recently, Bouvet and Grimont (3) changed extensively the taxonomy of the genus *Acinetobacter*. Through DNA-DNA hybridization 12 genomic groups (genospecies) were discriminated, 11 of them could been biochemically differentiated through 28 phenotypical tests. Four genomic groups (2,4,5 and 7) were recognized as new species and named *A. baumannii, A. haemolyticus, A. junii* and *A. johnsonii*, respectivelly. Species previously known as *A. calcoaceticus* and *A. iwoffii* were redefined and allocated in groups 1 and 8, respectivelly. Tjemberg and Ursing (25) presented three additional DNA groups, coded 13 through 15 and Bouvet and Jeanjean (5) described five DNA groups of proteolytic *Acinetobacter* strains numbered 13 through 17.

Due to these taxonomic changes, several typing methods such as biotyping (4), phage typing (6), determination of antibiotic susceptibility patterns (9,11), serotyping (26), cell envelope protein profiles (10), enzyme profiles (23), plasmid analysis (29), restriction patterns of chromosomal DNA (16) and ribotyping (9,12) have been proposed for epidemiologic studies if infections by *Acinetobacter*.

The aims of our study were to classify, using the methodology proposed by Bouvet and Grimont (3), strains of *Acinetobacter* isolated from hospitals and outpatients clinics, and to employ biotyping as a method of discrimination of strains classified as *Acinetobacter baumannii*, a prevalent species in clinical specimens.

MATERIALS AND METHODS

Bacteria: A total of 255 clinical isolates of *Acinetobacter* species were studied. They were isolated from hospitalized (186 strains) and non-hospitalized patients (69 strains) in 1990 and 1991 from different departments of two hospitals of Ribeirão Preto City, São Paulo, Brazil.

The isolates were identified presumptively as *Acinetobacter* according to the methodology of Gilardi (15) and maintained on Tryptic Soy Agar (TSA) slants at 20°C and also lyophilized.

The following reference strains served as controls: *A. baumannii* CIP 70.34, A. genospecie 3 CIP 70.29, *A. haemolyticus* CIP 64.3, *A. junii* CIP 64.5, *A.* genospecie 6 CIP A 165, *A. johnsonii* CIP A 64.6, *A. iwoffii* CIP 64.10, *A. calcoaceticus* CIP 81.08, *A. genospecie* 10 CIP 70.12, *A. genospecie* 11 CIP 63.46 and A. genospecie 12 SEIP 12.81. All strains were kindly provided by Dr. Philippe J.M. Bouvet and Dr. Patrick A.D. Grimont (Service des Entérobactéries, Institut Pasteur, Paris, France). For the transformation assay we included the reference strain BD 413 TrpE27-ATCC 33308.

Identification of isolates: All the isolates had the properties that define the genus *Acinetobacter* short rods, sometimes diplococcal forms, occurring in pairs or chains of variable size. Gram-negative but sometimes difficult to destain. Nonmotile. Strictly aerobic, catalase positive and oxidase negative. All were confirmed as *Acinetobacter spp* by the transformation assay of Juni (20).

The following phenotypical tests were used for identification at the species level. All were done at 30°C unless otherwise indicated.

Growth at 37°C, 41°C and 44°C: Two drops of an overnight Tryptocasein Soy Broth - TSB (Difco) culture were inoculated in tubes containing 10 ml of TSB. Incubation were done in a water bath at the temperatures above and the growth verified after 24 and 48 hours. A control-test was done at 30°C.

Production of acid from glucose: Petri dishes containing Purple Agar Base (Difco) supplemented with 1% (wt/vol) D-glucose were inoculated (spots around 5 mm). The acid production was indicated for yellow zone around growth, verified after 24 and 48 hours.

Hemolysis: A clear zone of hemolysis was recorded after 48 hours of growth in Petri dishes containing TSA supplemented with 5% (vol/vol) sheep blood.

Gelatin hydrolisis: Overnight cultures in TSA slants were spot-inoculated (around 5 mm) in Petri dishes containing TSA supplemented with 4% (wt/vol) bacteriological gelatin (Difco) and incubated for 48 hours. Gelatin hydrolisis was verified after flooding the plates with Frazier reagent (15% [wt/vol] $HgCl_2$ in HCl 2N) and examined for clear zones around spots.

Carbon source utilization tests: We employed as a minimal medium the M70 defined medium without agar according Véron (28). Filter-sterilized solutions containing each carbon source were added at a final concentration of 0.1% and three ml of each were dispensed in 12 X 120 mm tubes. The following substrates (Sigma) were used: acetate, L-aspartate, L-arginine, azelate, ß-alanine, 2,3-butanediol, citratre, DL-4-aminobutyrate, L-phenylalanine, glutarate, histamine, L-histidine, DL-lactate, L-leucine, D-malate, malonate, L-ornithine, and trans-aconitate. The tubes were inoculated with 0.1 ml of a bacterial suspension in distilled water containing 10^8 cells/ml. Growth was verified after 2 and for up 6 days.

Biotyping of *Acinetobacter baumannii* strains: The strains identidied as *A. baumannii* were biotyped according to Bouvet and Grimont (4). The methodology was the same employed in carbon source urilization tests.

Six carbon sources were tested: levulinate, citraconate, L-phenylalanine, phenylacetate, 4-hydroxybenzoate and L-tatarate, which made possible to delineate 19 biotypes of *A. baumannii*.

RESULTS AND DISCUSSION

In epidemiological assessment of infections the right species definition is the basic step towards typing for subsequent procedures and effective recognition of the strain involved.

The scheme of Bovet and Grimont (3) enabled us to correctly identify 242 strains (94.5%) belonging to 10 out of 17 genomic groups described. A small percentage of strains (5.5%) belong to non-identified strains (Table 1). These results indicate the usefulness of the proposed scheme to the differentiation of new species of *Acinetobacter*.

In general the strains behaved typically and homogeneously within their groups (Table 2). Exceptions were noticed as regards growth at 41°C in 4 of the 6 strains of *A. haemolyticus* and as regards hemolysis in 3 of the 6 strains of *A. junii*, features considered negative by Bouvet and Grimont (3), but noticed by Tjernberg and Ursing (25) and Gerner-Smidt et al (13). Low percentage of positivity for the use of transaconitate (76%) and L-ornithine (64%) in strains of *A. baumannii* and of DL-4-aminobutyrate (66%) in strains of *A. haemolyticus* (test regarded as useful in its separation from other hemolytic species) was also detected.

TABLE 1 - Occurrence of *Acinetobacter* genospecies in clinical specimens obtained from impacients and outpatients at two hospitals of Ribeirao Preto city, Sao Paulo, Brazil

Species	Origin		Total
	Inpacients	Outpatients	
A. *baumannii*	155 (60,8)a	53 (20,8)	208 (81,6)
A. genospecies 3	3 (1,2)	8 (3,1)	11 (4,3)
A. *haemolyticus*	4 (1,6)	2 (0,8)	6 (2,3)
A. *junii*	6 (2,3)	0	6 (2,3)
A. genospecies 6	1 (0,4)	0	1 (0,4)
A. *johnsonii*	2 (0,8)	0	2 (0,8)
A. genospecies 10	1 (0,4)	1 (0,4)	2 (0,8)
A. genospecies 11	2 (0,8)	1 (0,4)	3 (1,2)
A. group 13	1 (0,4)	0	1 (0,4)
A. group 14	1 (0,4)	0	1 (0,4)
Acinetobacter spp	10 (3,9)	4 (1,5)	14 (5,5)
Total	186 (72,9)	69 (27,1)	255 (100,0)

a - (%)

The simplified classification scheme proposed by Bouvet and Grimont (4) was adequate for the identification of the main *Acinetobacter* species. Notwithstanding, as was recently noticed Tjernberg and Ursing (25) and Gerner-Smidt et al (13), we report difficulty at separating groups 1,2,3 and 13, owing to the similarity of response to assimilation tests. In the context, we agree with Gerner-Smidt et al (13) who think it more appropriate to join these 4 groups in an "*Acinetobacter calcoaceticus- Acinetobacter baumannii* complex".

A. baumannii was the most prevalent species during the study period, making up 81.6% of the strains, figures close to those detected in clinical material by Bouvet and Grimont (4), 83.8%, and Joly-Guillou et al (18), 85.5%. A lower percentage was found by Bouvet et al (6) and Vaz et al (27) (60.8% and 42.2%, respectively). Other species were less frequent: *A.* genospecies 3 with 11 strains (4.3%), *A. haemolyticus* and *A. junii* with 6 strains each one (2.3%), *A.* genospecies 11 with 3 strains (1.2%), *A. johnsonii* and *A.* genospecies 10 with 2 strains each one (0.8%), *A.* group 13 and *A.* group 14, both with 1 strain (0.4%) (Table 1).

TABLE 2 - Phenotypic characteristics of *Acinetobacter* genospecies isolated from clinical scpecimens.

Tests	Acinetbacter genospecies									
	A. *baumannii* (208)[a]	A. gs 3 (11)	A. *haemolyticus* (6)	A. *junii* (6)	A. gs 6 (1)	A. *jonhsonii* (2)	A. gs 10 (2)	A. gs 11 (3)	A. grupo 13 (13)	A. grupo 14 (1)
Growth at:										
44° C	100[b]	0	0	0	0	0	0	0	0	0
41° C	100	100	66	100	0	0	0	33	0	0
37° C	100	100	100	100	100	0	100	100	100	100
Gelatin hydrolysis	0	0	66	0	100	0	0	0	100	100
Hemolysis	0	0	100	50	100	0	0	0	100	100
Acid from glucose	99	100	50	0	100	0	100	0	100	100
Urease	82	82	16	16	100	0	100	33	100	0
Utilization of										
DL-lactate	100	100	16	100	0	100	100	100	100	100
Glutarate	97	82	0	0	0	50	100	100	0	100
L-Phenylalanine	98	91	16	0	0	50	50	0	100	100
Malonate	92	73	0	0	0	0	0	0	0	100
Histidine	100	100	100	100	100	0	100	100	100	100
Azelate	97	91	0	0	100	50	50	100	0	100
D-malate	93	91	100	83	100	50	100	100	100	100
L-asplpartate	100	100	33	33	100	50	100	100	0	0
L-leulcine	98	82	33	0	100	0	0	0	0	100
Histamine	0	0	0	0	0	0	50	100	0	0
b-alanine	90	82	0	16	100	0	100	33	0	100
2,3-butanediol	100	100	0	16	0	100	100	100	0	0
Trans-aconitate	76	100	33	0	0	0	0	0	0	100
L-arginine	99	100	83	100	0	0	0	0	0	100
L-ornithine	64	82	0	0	0	50	0	0	0	100
DL-4-minobutyrate	100	100	66	66	0	50	100	100	0	100
Citrate	100	100	66	50	100	50	100	100	100	100
Acetate	100	100	100	100	100	100	100	100	100	100
Transformation test	100	100	100	100	100	100	100	100	100	100

a) nº of strains tested b) % of positive strains

These results confirm *A. baumannii* as the most prevalent species of the genus *Acinetobacter* in our clinical material as well highlighting the importance of the species in hospital-acquired infections. Conversely, *A.* genospcies 3, the species most often isolated in inpatients after *A. baumannii*, was the most prevalent in this study in outpatients.

The fact that *A. calcoaceticus* (genospecies 1) was not isolated can be explained because this species has the soil as its natural habitat (17). Likewise, A. *iwoffi* and *A.* genospecies 12 strains inhabit the skin of healthy subjects and are found in several places in hospital environment, only rarely being associated with infections (2,3,4,17), which may explain why they were not found. The 4 strains from the environment were *A. baumannii.* These data lend support to the need of a proper identification of *Acinetobacter* species so as to avoid wrong epidemiological conclusions to be drawn.

Of the 208 strains of *A. baumannii*, the scheme of Bouvet and Grimont (4) identified 13 out of 18 biotypes previously established for the species.

Atypical behavior regarding the 6 substrats, based on the use of L-tartarate only led to the description of a new biotype, named biotype 20, corresponding to 273/91 strain, isolated from tracheal secretion of a intensive care unit patient.

Biotypes 2,6 and 9 were most frequently found (169 out of 208 strains = 81.2%), biotype 2

TABLE 3 - Occurrence of Acinetobacter baumannii biotypes in clinical specimens inpatients and outpatients of two hospitals of Riberao Preto City, Sao Paulo, Brasil

Biotypes	Origin		Total
	Inpacients	Outpatients	
1	6	2	8
2	67	22	89
5	3	0	3
6	33	9	42
7	0	1	1
8	3	1	4
9	29	9	38
10	2	5	7
11	5	0	5
12	0	1	1
13	1	1	2
16	1	0	1
19	4	2	6
20	1	0	1
Total	155	53	208

predominating with 42% followed by biotypes 6 (20,2%) and 9 (18.3%) (Table 3). Additionally, 6 strains (2.9%) of recently described biotype 19 (6) were noticed. The finding of biotype 2 in both inpatients and outpatients is noteworthy and demonstrates the prevalence of this biotype in the study area. Literature data report the frequent isolation of biotypes 1, 2, 6, 9 and 18 (4, 6, 9, 11, 18, 23, 27) with the relative predominance ranging according to geographical distribution and source of isolates. Vaz et al (27), in Brazil, have recently reported the prevalence of biotype 9 (46%) in *A. baumannii* strains mostly isolated from blood and cerebrospinal fluid in the city of São Paulo. Bouvet and Grimont (4) have also demonstrated an association between *A. baumannii* biotypes and site of isolation, highlighting greater yields of biotype 1 strains from skin and upper respiratory tract secretions, of biotypes 2 and 6 from blood and urine and of biotype 9 from blood.

Table 4 lists biotype distribution regarding the source of isolates. Biotypes 2, 6 and 9 significantly predominate in clinical material from the respiratory tract, site where *Acinetobacter* infections are often reported (1,2,19,21,22). From the 4 strains isolated from blood, 3 are biotype 2 and so are the 2 only strains isolated from cerebrospinal fluid. Among the 4 environmental strains, 2 are biotype 6, 1 is biotype 2 and 1 biotype 11.

The use in our strains of the identification schemes to *Acinetobacter* species and of biotyping of those classified as *A. baumannii* has, therefore, provided evidence for the relatively long lasting presence of *A. baumannii* species, biotypes 2,6 and 9, mainly biotype 2 in both, inpatients and outpatients.

Thus, given the ease of execution and owing to the growing interest in *Acinetobacter* infections, it seems possible to implement carbon source assimilation tests and growing assessment at different temperatures, techniques which will allow the characterization of new genus species with biotyping as a useful screening method for *A. baumannii* strains. These data coupled with other typing systems may provided important epidemiological information for the control and prevention of such infections.

TABLE 4 - Distribuition of *Acinetobacter baumannii* biotypes in relation to clinical sources.

Source	Biotype														
	1	2	5	6	7	8	9	10	11	12	13	16	19	20	Total
Respiratory Tract	2	29	3	15	1	2	15	2	2	0	1	0	2	1	75
Urine	3	10	0	8	0	1	4	3	0	0	1	0	3	0	33
Wound	2	13	0	5	0	1	5	1	1	1	0	0	0	0	29
Catheter	1	8	0	3	0	0	5	0	0	0	0	1	1	0	19
Abscess	0	6	0	3	0	0	2	0	0	0	0	0	0	0	11
Surgical Wound	0	2	0	1	0	0	1	0	0	0	0	0	0	0	4
Blood	0	3	0	0	0	0	1	0	0	0	0	0	0	0	4
Cerebrospinal Fluid	0	2	0	0	0	0	0	0	0	0	0	0	0	0	2
Vaginal Discharge	0	1	0	1	0	0	0	0	0	0	0	0	0	0	2
Ears	0	1	0	0	0	0	0	1	0	0	0	0	0	0	2
Environment	0	1	0	2	0	0	0	0	1	0	0	0	0	0	4
Others	0	13	0	4	0	0	5	0	1	0	0	0	0	0	23
Total	8	89	3	42	1	4	38	7	5	1	2	1	6	1	208

RESUMO

Espécies de *Acinetobacter* em materiais clínicos e detecção de um novo biotipo de *Acinetobacter baumanii*

As infecções por *Acinetobacter* têm despertado interesse face ao aumento de sua frequência, gravidade e dificuldade terapêutica. No entanto, estudos epidemiológicos sempre foram dificultados em razão da incerta posição taxonômica do gênero. Um total de 255 cepas de *Acinetobacter*, isoladas de material clínico de pacientes hospitalizados e não hospitalizados, foram identificadas através de 28 testes fenotípicos constantes de recente reorganização taxonômica. *A. baumannii* foi a espécie mais frequentemente encontrada (81,6%), seguida de A. genoespécie 3 (4,3%), *A. haemolyticus* e *A. junii* (2,3%); 5 outras espécies foram isoladas em menor número, enquanto 5,5% das cepas não foram identificadas. Um sistema de biotipagem para A. baumannii baseado na utilização de 6 fontes de carbono nos levou à identificação de 13 dentre 19 biotipos determinados. Houve o predomínio dos biotipos 2 (42%), 6 (20,2%) e 9 (18,3%), isolados, em sua maioria, de secreções do trato respiratório dos pacientes de ambas origens. Um novo biotipo, caracterizado pela utilização somente do L-tartarato, foi descrito e denominado biotipo 20. A combinação dos esquemas de identificação de espécies de *Acinetobacter* e de biotipagem de *A. baumannii* nos permitiu evidenciar a prevalência, na área e período analisados, da espécie A. baumannii biotipo 2, demonstrando a aplicabilidade desses métodos para fins epidemiológicos.

Palavras-chave: *Acinetobacter*, *Acinetobacter baumannii*, biotipos.

REFERENCES

1. Anstey, N.M.; Currie, B.J. & Withinall, K.M. - Community-acquired *Acinetobacter* pneumonia in the northern territory of Australia. *Clin. Infect. Dis.*, **14**: 83-91, 1992.
2. Bergogne-Bérézin, E. & Joly-Guillou, M.L. - Hospital infection with *Acinetobacter spp*: an increasing problem. *J. Hosp. Infect.*, **18** (Suppl.A): 250-5, 1991.
3. Bouvet, P.J.M. & Grimont, P.A.D. - Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii sp.* nov., *Acinetobacter haemolyticus sp.* nov., *Acinetobacter johnsonii sp.* nov. and *Acinetobacter junii sp.* nov. and emended description of *Acinetobacter calcoaceticus* and *Acinetobacter iwoffii*. *Int. J. Syst. Bacteriol.*, **36**: 228-40, 1986.
4. Bouvet, P.J.M. & Grimont, P.A.D. - Identification and biotyping of clinical isolates of *Acinetobacter*. *Ann. Inst. Pasteur/Microbiol.*, **138**: 569-78, 1987.
5. Bouvet, P.J.M. & Jeanjean, S. - Dekineation of new proteolytic genomic species in the genus *Acinetobacter*. *Res. Microbiol.*, **140**:291-9, 1989.
6. Bouvet, P.J.M. ;Jeanjean, S.; Vieu, J.F. & Dijkshoorn, L. - Species, biotype, and bacteriophage type determinations compared with cell envelope protein profiles for typing *Acinetobacter* strains. *J. Clin. Microbiol.*, *28*: 170-6, 1990.
7. Bovre, K. - Family *Neisseriaceae* - In: Krieg, N.R. & Holt, J.C., eds. - Bergey's manual of systematic bacteriology. Baltimore, Willians & Wilkins, v.1, p. 288-309, 1984.
8. Brisou, J. & Prévot, A.R. - Études de systématique bacteriénne. X. Révision des espécies réunies dans le genre *Achromobacter*. *Ann. Inst. Pasteur*, **86**: 722-8, 1954.
9. Dijkshoorn, L.; Auken, H.M.; Gerner-Smidt, P.; Kaufmann, M.E.; Ursing, J. & Pitt, T. - Correlation of typing methods for *Acinetobacter* isolates from hospital outbreaks. *J. Clin. Microbiol.*, **31**: 702-5, 1993.
10. Dijkhoorn, L.; Wubbels, J.L.; Beunders, A.J.; Degener, J.E. & Boks, A.L. - Use of protein profiles to identify *Acinetobacter calcoaceticus* in a respiratory care unit. *J. Clin. Pathol.*, **42**: 853-7, 1989.
11. Gerner-Smidt, P. - The epidemiology of *Acinetobacter calcoaceticus*: biotype and resistance pattern of 328 strains consecutively isolated from clinical specimens. *APMIS* (Sect. B), **95**: 5-11, 1987.
12. Gerner-Smidt, P. - Ribotyping of the Acinetobacter calcoaceticus - *Acinetobacter baumannii* complex. *J. Clin. Microbiol.*, **30**: 2680-5, 1992.
13. Gerner-Smidt, P.; Tjernberg, I. & Ursing, J. - Reliability of phenotypic tests for identification of *Acinetobacter* species. *J. Clin. Microbiol.*, 29: 277-82, 1991.
14. Giammanco, A.; Vieu, J.F.; Bouvet, P.J.M.; Sarnaza, A. & Sinatra, A. - A comparative assay of epidemiological markers for *Acinetobacter* strains isolated in a hospital. *Int. J. Med. Microbiol.*, **272**: 231-41, 1989.
15. Gilardi, G.L. - Identification of miscellaneous glucose non-fermenting Gram-negative bacteria. - In: Gilardi, G.L. - Glucose non-fermenting Gram-negative bacteria in clinical microbiology. West Palm Beach, CRC Press, p. 45-69, 1978.
16. Gouby, A.; Carles-Nurit, M.; Bouziges, N.; Bourg, G.; Mesnard, R. & Bouvet, P.J.M. - Use of pulsed-field gel electrophoresis for investigation of hospital outbreaks of *Acinetobacter baumannii*. *J. Clin. Microbiol.*, **30**: 1588-91, 1992.
17. Henriksen, S.D. - *Moraxella, Acinetobacter* and the *Mimeae*. *Bacteriol. Rev.*, **37**: 522-61, 1973.
18. Joly-Guillou, M.L.; Bergogne-Bérézin, E. & Vieu, J.F. - A study of the relationships between antibiotic resistance phenotypes, phage-typing and biotyping of 117 clinical isolates of *Acinetobacter* spp. *J. Hosp. Infecct.*, **16**: 49-58, 1990.
19. Joly-Guillou, M.L.; Bergogne-Bérézin, E. & Vieu, J.F. - Épidémiologie et résistance aux antibiotiques des *Acinetobacter* en milieux hospitalier. *Presse Méd.*, **19**: 357-61, 1990.
20. Juni, E. - Interspecies transformation of *Acinetobacter*: geneti evidence for a ubiquitous genus. *J. Bacteriol.*, **112**: 917-31, 1972.

21. Le Pennec, M.P.; Pean, Y.; Boisivon, A.; Bejot, T.; Berardi-Grassias, L.; Eme, A.; Hacquard, B.; Morice, J. & Rouchon, M. - Épidémiologie et résistance aux antibiotiques d'*Acinetobacter* dans 9 hôpitaux généraux d'Ile-de-France. *Presse Méd.*, **19**: 1505, 1990.
22. Muller-Seriyes, C.; Lesowoy, J.B.; Perez, E.; Fichelle, A.; Boujeois, B.; Joly-Guillou, M.L. & Bergogne-Bérézin, E. - Infections nosocomiales à *Acinetobacter*. Épidémiologie et difficultés thérapeutiques. *Presse Méd.*, **18**: 107-8, 1989.
23. Poüedras, P.; Gras, S.; Sire, J.M.; Mesnard, R.; Donnio, P.Y.; Picard, B. & Avril, J.L. - Esterase electrophoresis compared with biotyping for epidemiological typing of *Acinetobacter baumannii* strains *FEMS Microbiol. Lett.*, **96**: 125-8, 1992.
24. Skerman, V.B.D.; McGowan, V. & Sneath, P.H.A. - Approved list of bacterial names. *Int. J. Syst. Bacteriol.*, **30**: 225-420, 1980.
25. Tjernberg, I. & Ursing, J. - Clinical strains of *Acinetobacter* classified by DNA-DNA hybridization. *APMIS*, **97**: 595-605, 1989.
26. Traub, W.H. - *Acinetobacter baumannii* serotyping for delineation of outbreaks of nosocomial cross-infection. *J. Clin. Microbiol.*, **27**: 2713-6, 1989.
27. Vaz, T.M.I.; Gonçalves, C.R.; Irino, K.; Tavechio, A.T.; Dias, A.M.G. & Fernandes, S.A. - Espécies de *Acinetobacter* associadas às infecções humanas. *Rev. Microbiol.*, São Paulo, **23** (Supl. 1): 153, 1991.
28. Véron, M. - Nutrition et taxonomie des *Enterobacteriaceae* et bactéries voisines. *Ann. Microbiol.* (Inst. Pasteur), **126A**: 267-74, 1975.
29. Vila, J.; Almela, M. & Jimenez de Anta, M.T. - Laboratory investigation of outbreaks caused by two different multiresistant *Acinetobacter calcoaceticus* subsp. *anitratus* strains. *J. Clin. Microbiol.*, **27**: 1086-9, 1989.

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PRODUCTION OF MONOCLONAL ANTIBODIES WITH DISTINCT NEUTRALIZING ACTIVITY AGAINST VIRULENT AND THE ATTENUATED BARTHA STRAIN OF AUJESZKY'S DISEASE VIRUS

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ABSTRACT

Three monoclonal antibodies (MoAbs) with *in vitro* neutralizing activity against virulent Aujeszky's disease virus (ADV) were produced. MoAbs 2E9 and 1H1 neutralized the virus only in the presence of exogenous complement, whereas MoAb G4 neutralized the virus in the absence of complement. However, none of the MoAbs were able to neutralize the naturally attenuated Bartha strain of ADV even in the presence of complement. Western blot analysis against virulent VDA proteins, resolved by SDS-PAGE under non-reducing conditions, showed that these MoAbs react against a 138K protein, and against a 68K protein under reducing conditions. A very faint reaction was noticed also against a 116K protein under reducing conditions. These results indicate a reaction against a disulfide linked protein, analogous to protein gII of ADV. This distinct neutralizing activity to a disulfide linked complex protein suggests differences in neutralizing epitopes of this protein from virulent and attenuated virus. These monoclonal antibodies may be good candidates for the use as makers for comparative analysis of virulent and attenuated strains of ADV.

Key words: Aujeszky's disease virus, monoclonal antibodies, neutralizing monoclonal antibodies

INTRODUCTION

Aujeszky's disease virus (ADV) is a member of the family Herpesviridae, subfamily Alphaherpesvirinae, also known as Pseudorabies virus (PrV), which causes an economically important disease of swine. The pig is the natural host, and infections may run an acute or subclinical course depending on the virulence of the virus and the age of the pig. Abortions and stillbirth in pregnant sows, neurological disorders in piglets with mortality approaching 100% and respiratory signs in fattening pigs are the most prominent disease symptoms (5). In ADV-infected cell cultures, there are eight glycoproteins and more than 30 non-glycosylated viral proteins (3). Glycoproteins gI, gII and gIII are the major components of the viral envelope and are expressed on the membranes of virus-infected cells (16, 2). Glycoprotein gI (molecular weight of 130K), gIV (M.W. 98K), and gVI (M.W. 62K) form a noncovalently linked complex with a 115K non-glycosylated protein. Glycoprotein gIII (98K) is not complexed to any other protein, whereas gII (140K) is a complex of three related proteins linked through disulfide bonds (125K, 74K and 58K) (4, 10). The virulence of ADV is multigenically controlled, and deletion mutants defective in the genes encoding either gI

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or gIII have reduced virulence. The naturally attenuated Bartha vaccine strain is deleted in the regions coding for gI and gp63 (9, 13, 14), and carries reduced amounts of gIII (1, 18). The role of ADV glycoproteins in inducing an immune response vary widely. Monoclonal antibodies (MoAbs) specific for gII and gIII can neutralize ADV without complement, while MoAbs for gI neutralize the virus only in the presence of complement (16).

This paper describes the production of complement dependent and non-complement dependent neutralizing monoclonal antibodies against a disulfide linked protein virulent ADV and the *in vitro* neutralization of these monoclonal antibodies against virulent and naturally attenuated virus.

MATERIALS AND METHODS

Cells: A continuous pig kidney cell line, SK-6 (6) was used to propagate ADV. Cultures were grown at 37°C, in a mixture of medium 199 and F-10 (Labor. Interlab, São Paulo SP) containing 5% heat inactivated bovine serum and 200 units of penicillin, 15 µg of neomicin and 25 µg of fungizon per ml. Mieloma cell lines for fusion were P3X63.Ag8.653 maintained in RPMI 1640 media (Labor. Interlab) supplemented with 15% non-heat inactivated fetal calf serum (Labor. Cultilab, Campinas, SP) antibiotics, L-glutamine and sodium pyruvate (complete RPMI).

Virus: PrV 261/83 is a virulent isolate of VDA from an outbreak of Aujeszky's disease in a swine herd in the State of Santa Catarina, with 9 passages in SK-6 cells. The virus stock was propagated in SK-6 cells maintained with F10-199 medium without sera. When cytopathic effect reached almost 100%, cells and supernatant were freeze-thawed twice and clarified by slow speed centrifugation, aliquoted and frozen. The virus was titrated by ten-fold dilutions to determine number of infectious dose 50% (TCID 50)/ml. The naturally attenuated Bartha strain was kindly provided to EMBRAPA by Dr. J. B. MacFerran (Stormont Laboratories, Belfast, Northern Ireland) and was propagated and titrated in the same manner.

Immunization of mice: Balb-C mice were immunized with an average 10^6 TCID 50 of whole tissue culture propagated virus PrV 261/83 in a first subcutaneous injection with an equal amount of complete Freund's adjuvant and two more injections in three week intervals with incomplete Freund's adjuvant. Final boost injection was given intraperitoneally 4 days prior to fusion, without adjuvant. The inoculated virus was inactivited with 0.15% glutaraldehyde for 2 hours at 37°C and full inactivation was assessed by tissue culture inoculation. Mouse serum was obtained by tail bleeding and immune response of mice was monitored by ELISA.

Fusion: Four days after the last immunization mieloma cells were fused in a ratio of 5:1 spleen cells from immunized mice, with 50% polyethylene glycol M.W. 1400 (Sigma, Chemical Co., St. Louis, MO. USA), according to the method of Letchworth & Appleton (1984) (8). Hybridoma cultures were grown in 96 well plastic sterile tissue culture plates (Falcon, Oxnard, CA, USA) and kept in complete RPMI media containing HAT (100 µM hypoxanthine, 16 thymidine and 0.4 µM aminopterin). Cultures were reefed every 3 to 4 days with 100 µl of HAT containing media for a week and later reefed in the same way in complete-HT media, also to assure diluting out antibodies secreted by unfused mouse spleen cells. Fully grown hybridoma culture supernatants were screened by ELISA at 10 and 14 days after fusion.

ELISA: The ELISA assay was performed according to the method of Rowe et al. 1988 (17) with few modifications. SK-6 cells were infected (positive antigen) and mock infected (negative antigen) with virulent PrV 261/83 or Bartha strain to prepare the ELISA antigens. Hybridoma supernatants were tested at 1:2 dilution and hyperimmune and negative mouse sera at 1:20 dilution in PTN buffer. Incubation was for 2 hours at 37°C, followed by incubation with 100 µl of a 1:10.000 dilution in PTN-3% horse serum of goat anti-mouse IgG (heavy and light chain) peroxidase conjugate (KPL- Kirkegard & Perry Laboratories Inc., Gathesburg, Maryland, USA). Absorbance indexes (obtained by dividing the absorbance readings for positive and negative antigen) higher than 3 were considered positive. ELISAs against the Bartha strain were carried out in the same manner and in parallel to virulent VDA ELISAs.

Serum-neutralization (SN) assays: Hybridoma supernatants were tested in serial twofold dilutions from 1:2 to 1:256 against virulent PrV 261/83 as well as against the Bartha strain in 96 well polyestyrene plastic plates. A volume of 25µl of each supernatant dilution was incubated for 1 hour at 37°C with an equal volume of virus diluted to contain 100 TCID 50. Selected hybridoma culture supernatants (which contained non-heat inactivated fetal calf serum) were heat inactivated at 56°C for 30 minutes and tested in parallel in the absence and in

the presence of 5% fresh rabbit serum as a source of exogenous complement, and the virus-supernatant-complement mixture incubated for 1 hour/37°C prior to addition of 100µl of SK-6 cells in F10-199 media supplemented with heat inactivated bovine serum and antibiotics. Plates were read after 3 and 5 days and the neutralization titer determined as the highest culture supernatant dilution that inhibited appearance of virus CPE. Known positive reference porcine serum, negative porcine serum and mieloma culture media (complete RPMI) were always included as assay controls.

Cloning: Selected positive hybridomas were cloned 2 on 3 times by limiting dilution in 96 well tissue culture plates, in the presence of complete RPMI media and 50% mouse macrophage conditioned culture media (MCM). MCM was prepared by culturing mouse peritoneal macrophages for 4 days in RPMI complete media. Supernatants from cloned hybrids were screened by ELISA, and selected hybridomas further expanded, frozen and recloned in the same way.

Western Blot: SK-6 cells were infected with PrV 261/83 as already described. Cells and culture supernatants from infected (positive antigen) and mock infected(negative antigen) cultures were centrifuged at 100.000 X g/ 2 hours in a Sorvall T 568 fixed angle rotor in a Sorvall OTD75B model ultracentrifuge and the pellets were treated with TGT buffer (0.038 M Tris, 0.01 M glycine and 0.5% Triton X-100) for 1 hour at 5°C and DNA pelleted by centrifugation at 100.000 X g for 1 hour (4, 10). Protein samples of approximately 0.8 mg were mixed with an equal volume of eletrophoresis sample buffer (65 mM Tris, pH 6.8, 2% SDS, 15% glycerol, 0.01% bromophenol blue) in the presence of 25 mM DTT (reducing conditions) or without DTT (non-reducing conditions) and boiled for 3 minutes. Polyacrylamide gel eletrophoresis (SDS-PAGE) was performed in a discontinuous system (7), with 3% stacking gel and 10% concentration in the resolving gel. Molecular weight markers were Sigma 6H kit (Sigma Chemical Co., USA) containing a 205K, 116K, 97.4K, 66K, 45K, and 29K proteins. After eletrophoresis proteins were electroblotted on a 0.45 µm pore size nitrocellulose paper (Sigma, Chemical Co., St. Louis, MO. USA) according to the method of Towbin et al. (1979) (19). Membranes were blocked for 2 hours at room temperature in phosphate buffered saline (PBS) containing 0.05% tween 20 and 5% horse serum (PBS-THs) and then incubated for 2 hours with undiluted hybridoma supernatants. After 30 minutes washes in PBS the membranes were incubated with a 1:500 dilution in PBS-THs of anti mouse IgG (H & L chain) peroxidase conjugate (Sigma Chemical Co., USA) for 1 hour at room temperature. After a further 30 minutes washes in PBS the reaction was revealed with 60 mg of 4-chloronaphtol (4CN) (Sigma Chemical Co., USA) substrate diluted in 20 ml cold methanol and 100 ml PBS, and 60 µl of a stock 30% H_2O_2. Reaction was stopped by rinsing the membranes in distilled water. Transferred reference molecular weight markers were separately stained with 0.25% amido black stain (Sigma Chemical Co., St. Louis, MO. USA).

RESULTS

ELISA and SN ASSAYS: Tissue culture supernatants from monoclonal antibodies' secreting hybridomas were screened by ELISA 14 days after fusion and randomly selected best growing colonies with high ELISA positive results were expanded and cloned by limiting dilution. After first and second cloning hybrids were always screened by ELISA and then tested by SN against virulent and attenuated Aujeszky's disease virus. Out of seven cloned and expanded monoclonal antibody (MoAb) secreting hybrids, only MoAb G4 had neutralizing activity against virulent virus without

TABLE 1 - Absorbance indexes from ELISA assays and SN results of seven monoclonal antibodies produced, rested against virulent PrV 261/83 and the attenuated Bartha strain of ADV.

	ELISA*		SN Titer*			
MoAb	PrV 261/8	Bartha	PrV 261/83		Bartha	
	Index(1)	*Index*	*+C*(2)	*-C*	*+C*	*-C*
G4	7.7	4.7	1:128	1:64	neg	neg
2E9	10	3.2	1:32	neg	neg	neg
1H1	10	6.4	1:32	neg	neg	neg
4D4	4.7	4.3	neg	neg	neg	neg
2F8	19	22	neg	neg	neg	neg
4D10	17	20	neg	neg	neg	neg
1G8	3.9	16	neg	neg	neg	neg

* All assays included plain tissue culture media and porcine negative serum as negative controls, and a porcine positive serum with SN titers higher than 1:128 against PrV 261/83 and 1:64 against the Bartha strain. (1)Indexes were calculated as the ratio of the absorbance readings from the reactions against positive (viral) antigen / negative antigen (uninfected cells) as described in materials and methods.

(2) SN assays performed in the presence or not of exogenous rabbit complememt: +C: with complement; -C: without complement

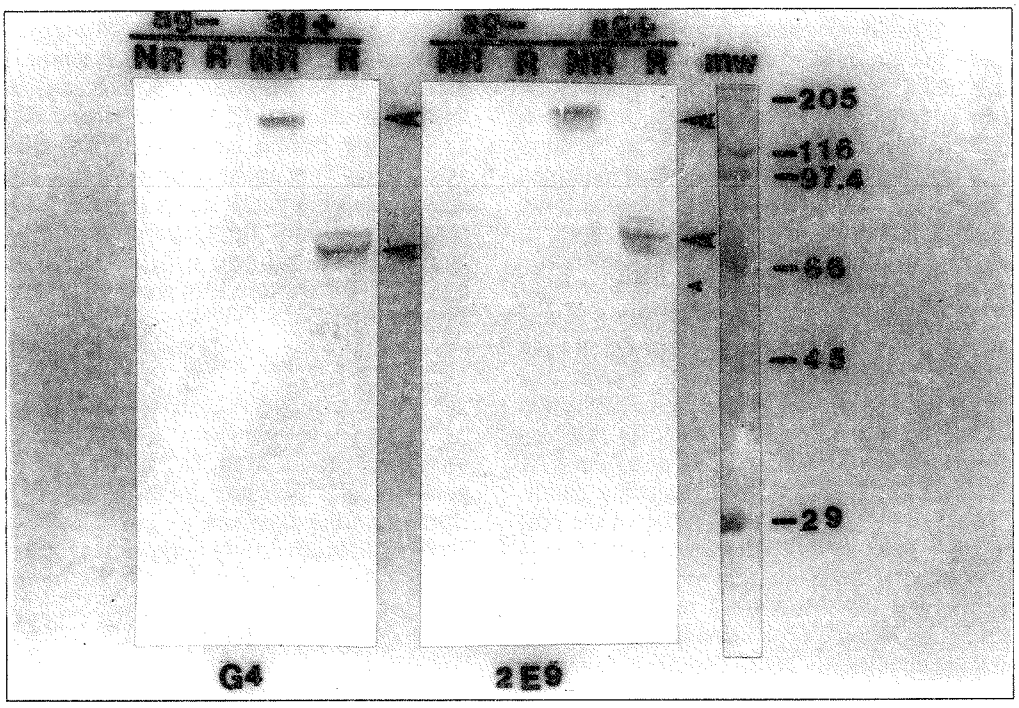

FIGURE 1 - Western Blot analysis of MoAbs G4 and 2E9 against Triton X-100 extracted proteins from cells infected with the PrV 261/83 virulent strain of ADV (Ag+: positive antigen) and against extracted proteins of non-infected cells (Ag-: negative antigen). Proteins were resolved by SDS-PAGE under reducing (R) and non-reducing conditions (NR) and electrotranferred to a nitrocellulose membrane. The arrows indicate the virus-specific reactions against a 138 K protein (Ag+ lanes) resolved under non-reducing contitions, and against a 68 K protein under reducing condition (R lanes.) Amido black stained molecular weight markers (MW x 10^{-3}) are indicated. The immune reaction was revealed with the chromogen 4-chloronaphtol and H_2O_2 as substrate.

complement, while two other MoAbs (2E9 and 1H1) neutralized the virus only in the presence of exogenous complement (Table 1). None of these neutralizing MoAbs had neutralizing activity against the Bartha strain even in the presence of exogenous complement.

WESTERN BLOT ANALYSIS: The SN positive MoAbs G4, 2E9 (Figure 1) and 1H1 were tested by Western Blot. All three MoAbs reacted against a 138K protein of virulent PrV 261/83, resolved by PAGE under non-reducing conditions (Ag+, NR lanes). When proteins were resolved by PAGE under reducing conditions, the three MoAbs had a strong reaction against a protein of 68K (Ag+, R lane). A very faint reaction against a 116K protein was seen after substrate development of the blots and was not well indicated in the picture. None of the three MoAbs tested reacted with the negative antigen, prepared from non-infected cells and resolverd by PAGE under the prepared from non-infected cells and resolverd by PAGE under the same conditions (Ag- lanes). Figure 1 shows representative results for two of the MoAbs: G4 (non-complement dependent) and 2E9 (complement dependent).

DISCUSSION

Glycoproteins gI, gII and gIII are major components of the virion envelope of Aujeszky's disease virus. Glycoprotein GII is the only so far reported as a complex of the three related proteins linked by disulfide bonds, of approximately 120 K, 67K and 58K (10) or 125K, 74K and 58K (4). These complexes can be disrupted by treatment with reducing agents such as DTT or 2-mercaptoethanol, that break disulfide bridges. Removal of the viral envelope with non-ionic detergent (Triton x-100) selectively removes from the virion all of the glycoproteins as well as several non-glycosylated proteins (4, 11). In order to test the specificity of the developed neutralizing MoAbs against envelope proteins of ADV, the

Western blot technique was performed with detergent extracted proteins. The reaction of MoAbs G4, 2E9 and 1H1 against a 138K protein under non-reducing conditions, and to a 68K protein under reducing conditions indicates a reaction against a subunit of a disulfide linked complex protein. This result is not unexpected, since these MoAbs may recognize only specific epitopes of the complex protein, taking also in consideration that one of the drawbacks of the western blot technique is that it does not allow the detection of tertiary structure of the proteins due to denaturing conditions. Lukacs et al. (10) have shown that MoAbs against a disulfide linked complex protein of ADV, characterized as gII, reacted in Western Blot only with the 125K and 67K, or to the 67K and 58K subunits resolved under reducing conditions. Nakamura et al., 1990 (15) reported the production of a complement dependent neutralizing MoAb against a 140K protein characterized as protein gII, and this MoAb did not react in the Western Blot when the proteins were resolved under reducing conditions. These comparative results give evidence that MoAbs G4, 2E9 and 1H1 react against a protein analogous to gII.

The role of different ADV proteins in inducing immune responses vary widely. MoAbs to gII have been demonstrated to neutralize ADV in the presence or not of exogenous complement. Most of the reported MoAbs to gII have complement-dependent neutralizing activity against virulent strains of ADV (5,12,15,16), and have not been characterized against attenuated virus. Our results show the production of complement dependent MoAbs as well as a non-complement dependent neutralizing MoAb, all with distinct neutralizing activity against virulent and attenuated virus, indicating that these disulfide-linked complexed protein posses differences in neutralizing epitopes between virulent an attenuated virus. Although the Bartha strain has been shown to carry large amounts of protein gII, probably as a compensatory mechanism to maintain functional glycoprotein complexes (1), there might be differences in gII protein from attenuated and virulent virus. Further tests against other virulent and attenuated strains of ADV could prove MoAbs G4, 2E9 and 1H1 as good candidates for the use as markers for comparative analysis between virulent and attenuated strains of ADV.

It is noteworthy that the SN test is of great value as a functional selective type of assay to screen MoAbs against viral envelope proteins, indicating differences between virus strains which can not be detected by ELISA. MoAbs 2F8, 4D4, 4D10 and 1G8 need further characterization, more particularly MoAb 1G8 which has consistently resulted in higher ELISA absorbance readings against the Bartha strain in repeated assays of 1G8 clone. This result does not allow any sort of speculation, but yet, it could indicate some differences in affinity or specificity of this MoAb against Bartha antigen, which could be indicated by the future western blot analysis using a Bartha strain antigen.

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RESUMO

Produção de anticorpos monoclonais com diferentes atividades neutralizantes contra cepas virulentas e atenuadas Bartha do vírus da doença de Aujeszky

Foram produzidos três anticorpos monoclonais contra cepa virulenta do vírus da doença de Aujeszky (VDA). Anticorpos monoclonais 2E9 e 1H1 neutralizam o vírus *in vitro* apenas na presença de complemento exógeno enquanto G4 neutraliza o vírus mesmo na ausência de complemento. Contudo, nenhum destes anticorpos possui atividade neutralizante contra a cepa Bartha, naturalmente atenuada. Análise por Western Blot contra proteinas do VDA, separadas por SDS-PAGE sem agente redutor, demonstrou que estes anticorpos monoclonais reagem contra uma proteina de peso molecular de 138K, revelada como uma proteina de 68K quando eletroforese foi com agente redutor. Uma reação muito tênue foi também observada contra uma proteina de 116K em condições de redução. Estes resultados indicam especificidade contra uma proteina complexa ligada por pontes disulfidicas, análoga à proteina gII do VDA. A distinta atividade neutralizante destes anticorpos sugere a presença de diferenças em epitopes neutralizantes desta proteina no vírus atenuado e virulento. Estes anticorpos monoclonais podem vir a ter potencial uso como marcadores para análise comparativa de cepas virulentas e atenuadas do VDA.

Palavras-chave: vírus da doença de Aujeszky, anticorpos monoclonais, anticorpos monoclonais neutralizantes.

REFERENCES

1. Ben-Porat, T., DeMarchi, J.M., Lomniczi, B. and Kaplan, A.S. Role of glycoproteins of pseudorabies virus in eliciting neutralizing antibodies. *Virol.ogy*, **95**: 285-295, 1986.
2. Eloit, M., Fergaud, D., L'Haridon, R., Toma, B. Identification of the pseudorabies virus glycoprotein gp50 as a major target of neutralizing antibodies. *Arch. Virol.*, **99**: 45-46, 1988.
3. Hahn, E.C. and Hahn P.S. Induced proteins in cells infected with pseudorabies virus. *Arch. Virol.*, **94**: 247-257, 1987.
4. Hampl, H., Ben-Porat, T., Erlicher, L., Habermehl, K-O. and Kaplan, A.S. Characterization of the envelope proteins of pseudorabies virus. *J. Virol.*, **52**(3): 583-590, 1984.
5. Gustafson, D.P. Pseudorabies, p 391-410. In H.W. Dunne and A.D. Lemman (editors) *Disease of Swine*. The Iowa State University Press, Ames, Iowa. USA., 1975.
6. Kasza, L. and Shadduck, J.A. Establishment of viral susceptibility and biological characteristics of a swine kidney cell line SK-6. *Res. Vet. Sci.*, **13**: 46-51, 1972.
7. Laemli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature., London., **227**: 680-685, 1970.
8. Letchworth, G.J III and Appleton, J.A. Methods for production of monoclonal antibodies. *United States Department of Agriculture, Agricultural Research Service.*, Agricultural Handbook, 630, 1984.
9. Lomniczi, B., Watanabe, S., Ben-Porat, T. and Kaplan, A. Genome location and identification of functions defective in the Bartha strain of pseudorabies virus. *J. Virol.*, **61**(3): 796-801, 1987.
10. Lukacs, N., Thiel, H.J., Mettenleiter, T.C. and Rziha, H.J. Demonstration of three major species of pseudorabies virus glycoproteins and identification of a disulfide-linked glycoprotein complex. *J. Virol.*, **53**(1): 166-173, 1985.
11. Lupton, H.W. and Reed, D.E. Evaluation of experimental subunit vaccines for infectious bovine rhinotracheitis. *Am. J. Vet. Res.*, **41**(3): 383-390, 1980.
12. Matsuda, A., Okada, N., Katayama, S., Okabe, T. and Sasaki, N. Characterization of proteins for pseudorabies infection. *Jpn. J. vet Sci.*, 737-741, 1991.
13. Mettenleiter, T.C., Schreurs, C., Thiel, H.J., Rziha, H.J. Variability of pseudorabies virus glycoprotein gI expression. *Virol.ogy*, **158**: 141-146, 1987.
14. Mettenleiter, T.C., Schreurs, C., Zickermann, F., Ben-Porat, T. and Kaplan, A. Role of glycoprotein gII of pseudorabies virus in virulence. *J. Virol.*, **62**(8): 2712-2717, 1988.
15. Nakamura, T., Jhara, T., Nagata, T., Ishizma, A. and Ueda, S. A complement-dependent neutralizing monoclonal antibody against protein gII of pseudorabies virus. *Vet Microbiol.*, **24**: 193-198, 1990.
16. van Oirschot, J.T., Gielkens, A.L.J., Moormann, R.J.M., Berns A.J.M. Marker vaccines, virus-protein specific antibody assays and the control of Aujeszky's disease. *Vet. Microbiol.*, **23**: 85-101, 1990.
17. Rowe, C.A., Muller, I., Guidoni, A.L. and Romero, C.H. An index enzyme linked immunosorbent assay (I-ELISA) for antibodies to Aujeszky's disease virus. *Arq. Biol. Tecnol.*, **32**(4): 719-731, 1989.
18. Robbins, A.K., Ryan, J.P., Whealy, M.E. and Enquist, L.W. The gene encoding the gIII envelope protein of pseudorabies virus vaccine strain Bartha contains a mutation affecting protein localization. *J. Virol.*, **63**: 250-258, 1989.
19. Towbin, H., Stahelin, T. and Gordon, G. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA*, **76**(9): 4350-4353, 1979.

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PURIFICATION OF STAPHYLOCOCCAL ENTEROTOXIN A BY DYE LIGAND CHROMATOGRAPHY

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ABSTRACT

Enterotoxin A was purified from 400 ml of *Staphylococcus aureus* strain FRI-722 culture supernatant fluid. The sac culture method was used to produce the enterotoxin. Ten milligrams (36%) of purified enterotoxin A was obtained from the 28 mg present in the culture supernatant fluid. The purified enterotoxin was homogeneous when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The procedures used are adequate for purifying a satisfactory amount of enterotoxin and can be used in laboratories where the necessary equipment is not available for handling large volumes of culture supernatant fluid.

Key words: staphylococcal enterotoxin A; purification; chromatography.

INTRODUCTION

The staphylococcal enterotoxins are responsible for staphylococcal food poisoning which is characterized by vomiting and diarrhea a short time after ingestion of food containing the enterotoxin. A number of antigenically different enterotoxins (SEs, SEA-SEE) have been identified and purified (1). Of the SEs, SEA is the one most commonly involved in staphylococcal food poisoning (16). The methods used for detection of SEs are based on the use of specific antisera to each of the SEs (2), several mg of the purified SEs are needed to prepare the specific antisera (14). Purification of SEB and the SECs are relatively easy because these SEs are produced in relatively large amounts, > 100 μg/ml of culture by the shake flask method, whereas SEA, SED, and SEE are produced in relatively small amounts, < 5 μg/ml of culture by the same method (15). The amount of SEs can be increased by up to 10-fold with the sac culture method of Donnelly et al. (8). A modification of this method has been used for production of increased amounts of SEA, SED and SEE (11). Although a number of methods have been used for the purification of SEA, the use of dye ligand chromatography resulted in the recovery of > 50% of highly purified SEA in one step (5, 13).

The purpose of this study was to purify SEA by dye ligand chromatography on a small scale for use in the preparation of specific antibodies to SEA.

MATERIALS AND METHODS

Microorganism *Staphylococcus aureus.* strain FRI-722, a mutant strain developed from

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strain FRI-100 that produces a relatively large amount of SEA (9), was provided by one of us (MSB). The culture was stored on nutrient agar (Difco Laboratories, Detroit, MI) at 4°C.

Culture medium. The culture medium used for the production of SEA contained 6% Biosate peptone (Becton Dickinson Microbiology Systems, Cockeysville, MD) plus 2% yeast extract (Difco Laboratories) pH 6.6 (11).

Production of SEA. The sac culture method of Donnelly et al. (8) was used with modifications (11). The bacterial cells were removed by centrifugation at 12,000 X g for 30 minutes and the supernatant fluid sterilized by filtration with a Millipore filter (o.25μM). The culture filtrates containing the SEA were combined and concentrated overnight at 4°C with polyethylene glycol 15-20,000 (PEG) (Sigma Chemical Co., St. Louis, MO). The sacs containing the concentrated SEA were washed several times with distilled water and 20 mM $KHPO_4$, pH 6.8 buffer before the solution was further concentrated by filtration through an XM 10 filter (Amicon Corp., Lexington, MA).

Dye ligand chromatrography. A volume of 15 ml of Red A gel (Amicon Corp.) was washed with two volumes of 8 M urea containing 0.5 M NaOH, followed by several volumes of 20 mM $KHPO_4$, pH 6.8 buffer, and placed in a chromatographic tube (0.7 x 24 cm). The concentrated culture fluid containing the SEA was passed onto the column, the column washed with two to three volumes of the 20 mM $KHPO_4$, pH 6.8 buffer, and the SEA eluted with a stepwise increase in the molarity of the $KHPO_4$, pH 6.8 buffer (20 to 500 mM) at a flow rate of 7 to 8 ml/hour. Fractions (1 ml) were collected and their protein content measured by absorption at 280 nm. The SEA content was determined by radial immunodiffusion and the fractions composing the SEA peak were pooled. The chromatography was performed at 4°C.

TABLE 1 - Purification of SEA from 400 ml culture supernatant fluid

Fraction	Volume (ml)	SEA (mg)	Protein (mg)	Recovery of SEA (%)
Culture supernatant fluid	400	28	60	
Concentrated, dialysated culture supernatant	11	20	63	71
Red A gel pooled fractions	88	10	19	36

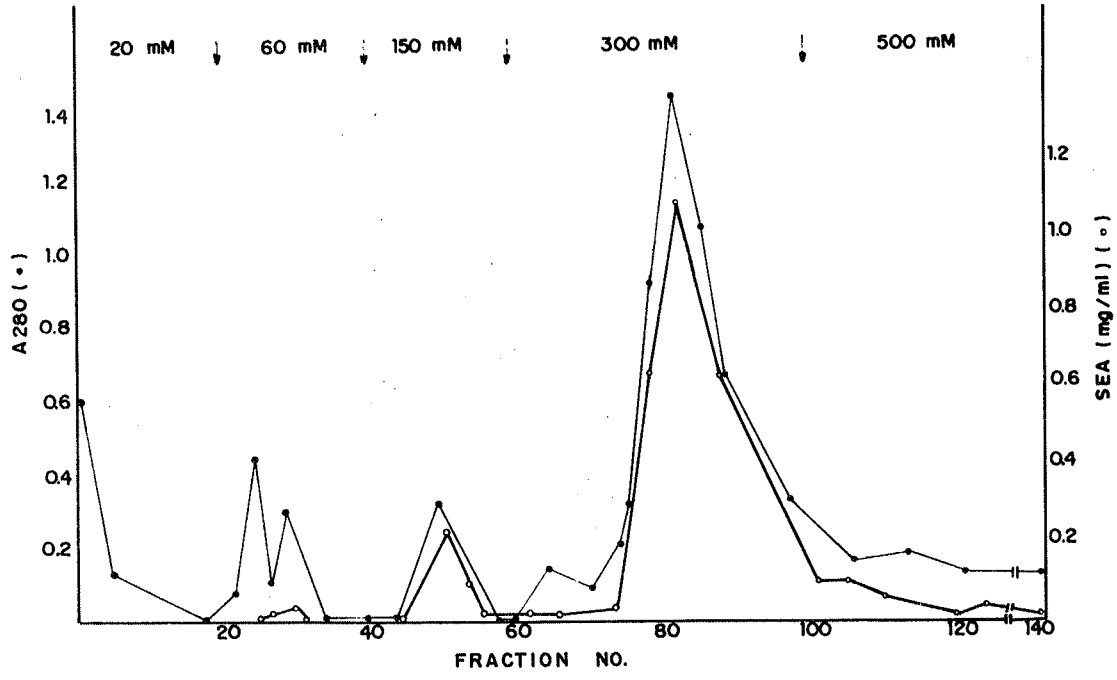

FIGURE 1 - Dye ligand chromatography of SEA from 400 ml of *S. Aureus* strain FRI-722 culture supernatant fluid on Red A gel column, equilibrated with 20 mM $KHPO_4$, pH 6.8 buffer, and elution stepwise with 20 to 500 mM $KHPO_4$, pH 6.8 buffer. Fractions (1.0 ml) were collected at a flow rate of 7.5 ml/hour during elution.

Enterotoxin assay. SEA was assayed by a modification of the radial immunodiffusion assay described by Meyer and Palmieri (12). A 1:70 dilution of SEA antiserum (provided by MSB) was prepared in 0.85% NaCl at 56°C and added to an equal volume of melted 2% Noble Agar (Difco Laboratories) (wt/vol) at the same temperature. The agar-serum (3.5 ml) was poured into petri dishes (50 x 9 mm). The SEA-containing fractions (10µl) were placed in wells (3 mm diam) cut into the agar and incubated overnight at 37°C in a moist chamber. The concentration of SEA was determined by comparing the diameters of the precipitate rings with those of standards of known SEA concentrations (20 to 100 µg/ml).

Protein assay. The protein content was determined by the Coomassie brillant bluc mcthod (4) using bovine albumin as a standard.

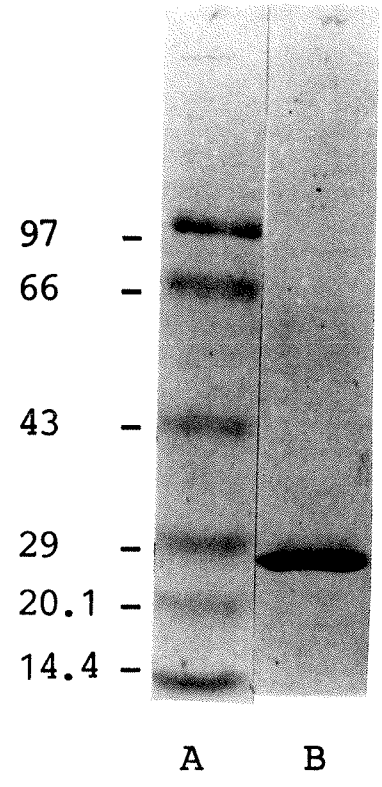

FIGURE 2 - SDS-polyacrylamide gel electrophoresis of SEA. Lane A, molecular mass markers (in kilodaltons): phosphorylase b (97), albumin (66), ovalbumin (43), carbonic anhydrase (29), trypsin inhibitor (20.1), and α-lactalbumin (14.4). Lane B, 20 µl of material eluted with 300 mM $KHPO_4$, pH 6.8 buffer.

SDS-PAGE. The purity of the SEA was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in polyacrylamide 15% gels according to the method of Laemmli (10).

RESULTS

The SEA was purified from 400 ml of culture supernatant fluid in a single step by chromatography on a Red A gel column. The amount of SEA in the culture supernatant fluid was 28 mg (70 µg/ml) (Table 1). The recovery of the SEA from the Red A gel column was 36%. The SEA was eluted in one large peak with 300 mM $KHPO_4$, pH 6.8 buffer (Fig. 1). The pooled SEA fractions was homogeneous by SDS-PAGE (Fig. 2). Several small peaks containing SEA were eluted also, but these contained impurities. They were pooled and stored separately.

DISCUSSION

The purification of the first SE (SEB) reported in 1959 using acid precipitation, alumina chromatography, starch gel electrophoresis and alcohol precipitation (3). Since that time many improvements have been made in the purification as newer materials became available, such as the ion exchange resins and gel filtration materials. Usually the procedures included three or four steps to obtain a high degree of purity, with relatively low recoveries, particularly the SEs that are produced in small amounts. The steps included two ion-exchange steps followed by one or two gel filtration steps (1).

The latest method developed utilized dye ligand chromatography requiring only one step after the culture supernatant fluids were concentrated by filtration and dialyzed (5, 13). The recovery of SEA, SEB and SE was > 50% of the SEs present in the culture supernatant fluids (5). Although the procedure is referred to as affinity chromatography, it is a form of ion-exchange chromatography. Although the developers of the method employed several liters of culture supernatant fluids, the high recovery rate makes it possible to obtain several milligrams of SE, for example SEA, from a relatively small volume of culture supernatant fluid. For example, we were able to obtain 10 mg of homogeneous SEA from 400 ml of culture supernatant fluid, which was 50% of that put on the col-

umn. This was less than the percent recovery reported for SEA for this method (5, 13), but it may have been due in part to the smaller amount of SEA that we were using. In adition more SEA was lost due to the extra procedures used in the preparation of the SE for the chromatography. However, this is an adequate amount for the preparation of good quantities of specific antibodies to SEA. Usually < 1 mg is required to imunize one rabbit from which more than 100 ml of specific antisera may be obtained (14).

The use of the sac culture method for production of the SEA made it possible to obtain a relatively large amount (28 mg) in a small volume (400 ml). Thus it is possible for small laboratories with minimal facilities to produce and purify sufficient quantities of SEA for preparation of specific antibodies. Emphasis is placed on SEA because all of the foods implicated in staphylococcal food poisoning in Brazil that have been examined for SE contained SEA (6, 7). A number of strains that have been isolated from such foods have produced SEB also, and some have produced SEC, but these SEs have not been detectable in the foods. Even if specific antibodies were needed for these SEs, the purified SEs would be relatively easy to purify because of the large amounts of these SEs that can be produced.

RESUMO

Purificação de enterotoxina estafilocócica A por cromatografia de afinidade com corante

A purificação de enterotoxina A foi obtida a partir de 400 ml de cultura de *Staphylococcus aureus* FRI-722. O método de cultura em saco de diálise foi utilizado para a produção da enterotoxina. Foram recuperados 10 mg de enterotoxina A purificada (36%) a partir de 28 mg presentes no sobrenadante da cultura. A enterotoxina purificada apresentou-se homogênea quando analisada por eletroforese em gel de poliacrilamida dodecilsulfato de sódio. A metodologia descrita neste trabalho mostrou-se adequada para purificar uma quantidade satisfatória de enterotoxina e pode ser utilizada em laboratórios onde não existem equipamentos necessários para manipular grandes volumes de sobrenadante de cultura.

Palavras-chaves: enterotoxina estafilocócica A; purificação; cromatografia.

REFERENCES

1. Bergdoll, M.S. - *Staphylococcus aureus. In*: M. P. Doyle (ed.), Foodborne bacterial pathogens. Marcel Dekker, Inc. N.Y. p. 463-523, 1989.
2. Bergdoll, M.S. - Analytical methods for *Staphylococcus aureus*. *Int. J. Food Microbiol.* **10**: 91-100, 1990.
3. Bergdoll, M.S.; Sugiyama, H. & Dack, G.M. - Staphylococcal enterotoxin. I. Purification. *Arch. Biochem. Biophys.* **85**: 62-69, 1959.
4. Bradford, M.M. - A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**: 248-254, 1976.
5. Brehm, R.D.; Tranter, H.S.; Hambleton, P. & Melling, J. - Large-scale purification of staphylococcal enterotoxins A, B and C_2 by dye ligand affinity chromatography. *Appl. Environ. Microbiol.*, **56**: 1067-1076, 1990.
6. Carmo, L.S. do; Dias, R.S.; Anunciação, L.L.C. & Bergdoll, M.S. - Staphylococcal food poisoning in Minas Gerais State (Brasil). *Rev. Microbiol.*, Submitted.
7. Cerqueira-Campos, M.L.; Furlanetto, S.M.P.; Iaria, S.T. & Bergdoll, M.S. - Staphylococcal food poisoning outbreaks in São Paulo (Brazil). *Rev. Microbiol.* Submitted.
8. Donnelly, C.B.; Leslie, J.E.; Black, L.A. & Lewis, K.H. - Serological identification of enterotoxigenic staphylococci from cheese. *Appl. Microbiol.*, **15**: 1382-1387, 1967.
9. Friedman, M.E. & Howard, M.B. - Induction of mutants of *Staphylococcus aureus* 100 with inccreased ability to produce enterotoxin A. *J. Bacteriol.*, **106**: 289-291, 1971.
10. Laemmli, U.K. - Cleavage of structural proteins during the assembly of the head bacteriophage T_4. *Nature*, **227**: 680-685, 1970.
11. Lopes, H.R.; Noleto, A.L.S. & Bergdoll, M.S. - Production of staphylococcal enterotoxins A, D and E by sac culture. *J. Food. Protect.*, **54**: 650-652, 1991.
12. Meyer, R.F. & Palmieri, M.J. - Single radial immunodiffusion method for screening staphylococcal isolates for enterotoxin. *Appl. Environ. Microbiol.*, **40**: 1080-1085, 1980.
13. Reynolds, D.; Tranter, H.S.; Sage, R. & Hambleton, P. - Novel method for purification of staphylococcal enterotoxin A. *Appl. Environ. Microbiol.*, **54**: 1761-1765, 1988.
14. Robbins, R.N. & Bergdoll, M.S. - Production of rabbit antisera to the staphylococcal enterotoxins. *J. Food Protect.*, **47**: 172-176, 1984.
15. Robbins, R.; Gould, S. & Bergdoll, M.S. - Detecting the enterotoxigenicity of *Staphylococcus aureus* strains. *Appl. Microbiol.*, **28**: 946-950, 1974.
16. Wieneke, A.A. - Enterotoxin production by strains of *Staphylococcus aureus* isolated from foods and human beings. *J. Hyg., Camb.* **73**: 255-262, 1974.

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INCIDENCE OF MYCORRHIZA IN NURSERY AND *EUCALYPTUS SPP.* FORESTS, IN VIÇOSA, MINAS GERAIS [1].

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SUMMARY

The incidence of mycorrhiza in the region of Viçosa, Minas Gerais State was verified slender roots and soil were collected from nursery and from two *Eucalyptus spp* forests. The ectomycorrhiza was indefied through the comparison of the mycelium and rhizomorphs surrounding the ectomycorrhiza with those found in the basidiocarp basis and the mycorrhiza interaction. The endotrophic colonization was observed by roots discoloration in KOH 10% coloration in cotton blue. The spores were extracted from the soil through centrifugation in saccharose. The yearly frequency of both mycorrhizal types was studied and related to environmental factors as air temperature, rain precipitation and kind of soil (pH). The findings were that the high air temperature and humidity besides the pH values of 4.5 to 7.0 favored the colonization by micorrhizae fungi the ectomycorrhizas were: 1. *Eucalyptus grandis* + *Scleroderma spp*, 2. *Eucalyptus citriodora* + *Pisolithus tinctorius*, 3. *Eucalyptus robusta* + *P. tinctorius* and the endotrophic fungi: *Acaulospora scrobiculata* and *Glomus clarum*.

Key Words: ectomycorrhiza, endomycorrhizal, *Eucalyptus*, *Pisolithus tinctorius*.

INTRODUCTION

Until the year 2000 comes, it is predicted that the reforested area with *Pinus* and *Eucalyptus* in Brazil will reach 16 million hectares. In 1980 the *Eucalyptus* covered the largest part of the cultivated area, occupying, approximately 3 million hectares (4). Therefore, it is possible to predict the spreading of its areas for the years yet to come.

Eucalyptus form symbiotic correlations with mycorrhizal fungi. The occurrence of ectomycorrhiza has been verified in different types of *Eucalyptus*, in several countries (21, 2, 5, 6), including Brazil (3, 22). There are, however, very little information about the infection by endomycorrhizal fungi, vesicular-arbuscular type, associated to some species of *Eucalyptus*.

In Brazil, it is still unknown the potential and occurrence conditions of the two mycorrhiza types in reforested regions with *Eucalyptus*. The possibility that the incidence of each type can be related to the species, plant age, and some ecological factors must be considered. Thus, this study had as principal aims: a) To characterize mycorrhiza in *Eucalyptus spp.* and identify the symbiotic fungus involved witch them and b) Evaluate the incidence of mycorrhiza in *Eucalyptus spp.* and relate this incidence in correlating factors to the plant of the environment.

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MATERIAL AND METHODS

Identification of ectomycorrhiza in nursery and *Eucalyptus* spp forests.

The mycorrhiza and soil collecting were made in nursery and forests in the surrounding of Viçosa, Minas Gerais.

In the nursery, a monthly collecting of the seedling samples was raised out of two *Eucalyptus grandis* lots, one sown on September/82 and another on June/83. Each sample consisted of three seedlings of each lot that were transported to the laboratory and immediately processed.

The soil of each seedling was separated from the roots and these ones were washed in current water to eliminate the exceeding of the adherent soil. Next, the roots were divided in sub-samples for macroscopic and microscopic characterization of the ectomycorrhiza and the endomycorrhizal study. The soil was stored at 4°C, to the extraction of VAM spores.

In the forest, 4 trees of *Eucalyptus citriodora* and four trees of *Eucalyptus robusta*, 3 and 14 years old respectively were shown trimestrially at random. For each of these species, 4 samples of 400 to 500g of soil and thin roots were collected.

The roots were separated from the soil by screening and washed in current water for a period of two hours approximately. Next, the roots were divided in sub-samples for characterization and determination of colonization level by ecto and endomycorrhizal fungi. The soil was divided in 4 sub-samples of 100g and stored at a 4°C, for the extraction of VAM spores.

For the macroscopic characterization of the ectomycorrhiza, Zak's technique was used (23, 24). Characteristics such as color, shape, presence or absence of rhizomorphs and the type of surrounding mycelium of mycorrhiza were observed.

Microscopically, ectomycorrhiza were observed and described based on the characteristics defined by Zak (23, 24) and Chilvers (5). The presence or absence of connection clamps at the surrounding mycelium hypha and the presence of rhizomorphs were observed after the setting of preparations. For Hartig net description and mantle thickness determination, permanent slides were set according to Johansen's technique (11).

For the identification of the symbiotic fungus two criteria were made, suggested by Zak (23, 24): the connection between root and basidiocarp and the comparison of the mycelium basidiocarp with the surrounding of the mycorrhiza.

The taxonomic identification of the symbionts was made based on the basidiocarp characteristics in key presented by Dring (7).

Incidence of ectomycorrhiza in nursery and *Eucalyptus* spp forests.

For the determination of the ectomycorrhizal colonization level, the roots collected in nursery and forests were washed, cut in pieces 1cm long and observed through the optic microscope (x 320).

The level of root colonization was determined after observation at random of 100 root fragments of the same size set in lactophenol between the slide and the little slide. On each slide 10 pieces of root were placed. The colonization level, expressed in percentuals (%), was based on the number of colonization roots and on the total number of roots observed and calculated by Nicholson's equation mentioned by Read et alii (18).

The roots which presented mantle were considered positively colonizated.

The level of colonization by ectomycorrhizal fungi was monthly evaluated for the nursery, trimestrially for the forest ones, and it was related to some ecological factors as air temperature, pluviosity, and soil pH.

Identification of the VAM endomycorrhizas in nursery and Eucalyptus forests.

The colonization by endomycorrhizal fungi in the nursery seedling and in forests was observed according Phillips and Hayman's technique (17).

For the extramatricial component study, as extraction of spores from the soil was performed by the centrifugation technique in saccharose solution described by Jenkins (10). The spores were observed in stereoscopic-microscope (x 40), being the ones of similar aspect transferred, with the help of Pasteur's pipette, to bottles with sterilized water. These bottles were stored at 4°C, for the spore identification of the symbiotic fungus.

For the symbiotic fungus identifications, fresh preparations were made between the slide and little slide, with spores of similar aspect. The taxonomic keys of Mosse & Bowen (14), Gerdemann & Trappe (9) and Nicholson & Schenck (16) were used.

Endomycorrhizal fungi incidence type V-A in nursery and *Eucalyptus* spp forests.

After root discoloration by Phillips and Hayman's technique (17) the colonization percentage

was determined by the same methodology followed by the colonization study by endomycorrhizal fungi. The roots which presented vesicles, arbusculos or mycelium were considered colonizated.

The colonization level by endomycorrhizal fungi was also related to the environmental factors as air temperature, pluviosity and soil pH.

For quantifying the inoculation of endomycorrhizal fungi, a sample of 100g of soil collected at the *E. citriodora* forest was used in two times of the year: June/83 and March/84. The spores extraction method used was described by Jenkins (10). After extraction, the spores were placed on Petri's plate, under water and counted through the stereoscopic microscope (x 40).

RESULTS

Macroscopic and microscopic characterization of ectomycorrhiza in nursery and forests of *Eucalyptus* spp.

Macroscopic and microscopic characterization of the collected ectomycorrhizas, each from a different forest as *E. grandis*, *E. robusta* and *E. citriodora*, led to the distinction of three types of ectomycorrhiza. The type 1, found in *E. grandis* was white, pyramidal, with surrounding downy white mycelium and white rhizomorphs, 28,0 to 30,0 µm wide interconnected to the mycelium. Type 2, found in *E. citriodora*, was mustard-yellow, pyramidal with smooth surrounding mycelium light yellow and mustard-yellow colored with rhizomorphs 52,5 67,5 µm wide. Type 3, found in *E. robusta*, presents the same characteristics of type 2, but with rhizomorphs 65,0 to 70,0 µm wide. In all the three types connection clamps were observed.

Mycorrhiza identification

Type 1 ectomycorrhizal symbiont was identified by the use of two selected criteria. For the mycorrhizas types 2 and 3, the symbionts were identified before the use of only one of these criteria.

In the nursery, the plants of lot 1 as well as the ones of lot 2, presented white ectomycorrhiza. However, in lot 1 only, the connection between the basidiocarp and ectomycorrhiza was proved. The connection is an evidence of ectomycorrhizal association (Zak, 24). Also, by the microscopic observations of both the slides set in water with surrounding mycelium of the ectomycorrhiza and basal mycelium of basidiocarp in connection, the presence of hyaline hyphas with identical thicknesses and connection clamps was observed. It was also verified that the ectomycorrhiza mycelium hyphas of lots 01 and 02 were alike, what leads us to suppose they were the mycorrhiza formed by the same fungus.

The symbiotic fungus, found in the connection, was identified by observations of some basidiocarp characteristics as *Scleroderma sp.*

In the *E. citriodora* and *E. robusta* forests *Pisolithus tinctorius* basidiocarp were observed with characteristics identical to the ones described by Dring (7). The connection of the *P. tinctorius* basidiocarp to the radicular system of eucalyptus was not proved. Therefore, through this criterion, it was not possible to identify the symbiotic fungus of the ectomycorrhizal types 2 and 3, present in the forests. However, similarity was observed between the ectomycorrhiza mycelium hyphas and the ones of the basidiocarp basal mycelium. It was verified, in both cases, the presence of light yellow hyphas, with the same thickness and connection clamps. Through this similarity, the symbiotic fungus with *P. tinctorius* was identified.

Based on the findings of the identification of symbiotic fungus, the types of mycorrhiza were named binomially, as it follows:

Type 1: *Eucalyptus grandis* + *Scleroderma sp.*
Type 2: *Eucalyptus citriodora* + *Pisolithus tinctorius.*
Type 3: *Eucalyptus robusta* + *Pisolithus tinctorius*.

TABLE 1 - Monthly levels of Ectomtcorrhiza in *E. grandis* in the Nursery in the region of Viçosa, MG.

Plant age (Months)	Levels of Ectomycorrhiza (%)	
	LOT 1 1/	LOT 2 2/
3	-	15 (Sept/83)
4	-	20
5	-	30
6	48 (March/83)	18
7	45	28
8	43	9
9	40	15
10	47	6 (April/84)
11	31	-
12	38 (Sept/83)	-

1/ Planting in Sept/82.
2/ Planting in June/83.
- Not observed.

Colonization level by the ectomycorrhizal fungi in nursery and forests of *Eucalyptus* spp.

The level of colonization by ectomycorrhizal fungi in *E. grandis* (lots 01 and 02), *E. citriodora* and *E. robusta* are presented on the Tables 1 and 2. This level was related to the values of air temperature, pluviosity and pH of the soil.

TABLE 2 - Trimestral levels of Ectomycorrhiza in *E. citriodora* (3 years) and *E. robusta* (14 years old) at the region of Viçosa, MG.

Collection date of Ectomycorrhiza	Levels of Ectomycorrhiza (%)	
	E. citriodora	*E. robusta*
March/83	33	30
June/83	15	20
September/83	23	23
December/83	43	32

Characterization of the VAM endomycorrhiza in nursery and forests of *Eucalyptus* spp.

In *E. grandis* (lots 01 and 02), in the nursery and in *E. citriodora* and *E. robusta* in the forest, vesicles and mycelium on the cortex region were observed, but not arbusculos. Zambolim and Barros (25), also rarely found arbusculo in forest of *E. saligna*, *E. citriodora* and *E. grandis*.

The extramatricial components of the endomycorrhizal funus have their characteristics presented on Table 3. It was verified that the same types of spores were presented in the 3 soils studied.

Colonization levels by endomycorrhizal fungus in nursery and Eucalyptus spp forests.

The colonization taxis by endomycorrhizal fungus in nursery and in *Eucalyptus spp* forests are presented in Table 4 and 5.

TABLE 4 - Monthly levels of Ectomtcorrhiza in *E. grandis* in the Nursery around in the region of Viçosa, MG.

Plant age (Months)	Levels of Ectomycorrhiza (%)	
	LOT 1 1/	LOT 2 2/
3	-	12 (Sept/83)
4	-	8
5	-	60
6	40 (March/83)	44
7	32	35
8	35	5
9	30	10
10	23	27 (April/84)
11	59	-
12	36 (Sept/83)	-

1/ Planting in Sept/82.
2/ Planting in June/83.
- Not observed.

TABLE 5 - Trimestral levels of Ectomycorrhiza in *E. citriodora* and *E. robusta* in the region of Viçosa, MG.

Collection date of Ectomycorrhiza	Levels of Ectomycorrhiza (%)	
	E. citriodora	*E. robusta*
March/83	3	12
June/83	6	3
September/83	8	5
December/83	25	272

TABLE 3 - Characteristics of spores of V-A fungus extracted from soils with *Eucaliptus* spp.

Type	Color	Shape	Fructification	Type of spore	Diameter (µm)	Wall characteristics
01	Hyaline to light yellow	Spherical or ellipsoid	Free spores without hypha connected	Azygouspore	90-110	Multiple
02	Hyaline	Spherical	Free spores or in sporocarps	Clamydospores	270-390	Double
03	Red	Spherical or oval	Free spores or in sporocarps	Clamydospores	408-520	Simple

Symbiotic fungus identification

From the characteristics observed in the spores, it was possible to identify the fungus species V-A with *Acaulospora scrobiculata* (Type 1) and *Glomus clarum* (Type 2). Type 3 was not identified because of the non total observation of the necessary taxonomic characteristics.

DISCUSSION

After analyzing the macroscopic and microscopic characteristics of the collected ectomycorrhizas, it was verified that the mycorrhiza *Eucalyptus grandis* + *Scleroderma sp* do not fit in any of the 8 types described by Chilvers (5). However, this classification does not involve all

the ectomycorrhiza types associated to *Eucalyptus* (23). Nevertheless, the characteristics of mycorrhizas *E. citriodora* + *Pisolithus tinctorius* and *E. robusta* + *P. tinctorius* fit in the described ones for the type 5 of Chilvers (5) classification, for being also the pyramidal type, mustard-yellow presenting rhizomorphs a little thick with connection clamps, although, the surrounding mycelium hyphas did not present themselves as containing pigments.

Associating the colonization level by ectomycorrhizal fungi, in the nursery with the decrease in the temperature, a decrease in the ectomycorrhizas percentage in *E. grandis* in lot 01 was verified. In August/83, the lowest percentage was noticed (31%), when besides the air temperature reached the least value (15,8°C), the precipitation was practically zero (0,5mm). In September/83, alough the temperature had not increased much, a good precipitation had occurred, and again the levels of mycorrhizal infection increased. In lot 02, it was verified that in the months of September to November/83, an increase in the mycorrhizal infection had occurred, which coincided with the increase of the temperature and precipitation. The reduction in the levels of mycorrhiza infection verified on Dec/83, when the temperature was high, can be probably explained by the high value of precipitation (315,2mm) that, in the nursery conditions (flat arca and clayish soil), might have kept a high water saturation in the soil as a consequent reduction of the airing. This condition seems to be unfavorable to the development and survival of mycorrhizas (1).

However, in February/84, when the highest value of the temperature (23,5°C) had occurred, it was verified a small percentage of mycorrhizas (9%). It is possible that the increase of the air temperature led to soil conditions, that somehow, caused a reduction in the contamination capacity of the ectomycorrhizal fungi. It is known that the percentage of infection depends on the sampled plant, plant age and ecological factors, besides the genera and species of the plant (12, 15).

Trimestrial levels data of ectomycorrhizas (Table 2) and the ones of air temperature and pluviosity showed that even in both *E. citriodora* and *E. robusta* the alterations in the ectomycorrhizas percentage occurred with the year season. In March and Dec/83, the highest percentages were observed, also corresponding to the highest values of air temperature and pluviosity. During the coldest and driest periods, June and September, there had been a low percentage of ectomycorrhizas. Few papers refer to the influence of air temperature and pluviosity in the ectomycorrhiza levels. However, it was already proved that external factors which affect photosynthesis and the quantity of photoassimilated substance produced by the hosting plant influence the development of the mycorrhizas (20).

The highest values of temperature and pluviosity, during March and December/83, may have promoted the plant growth causing, a bigger disponibility photoassimilated of substance for the roots, what can relate to the regulation of the mycorrhizal infection (8).

The soil humidity can affect the development of the mycorrhiza depending on the reached levels (15). In places of soaked soils, the colonization by ectomycorrhizal fungi is inhibited due to the lack of oxygenation, while constant drought conditions can reduce the ectomycorrhizas, approaching their occurring frequency to zero (1). Probably this fact contributed to the low levels of ectomycorrhizas (15 to 20%) in June/83, since the total pluviosity in the period was of 10mm. When this condition occurs, the rhizomorphs persists and constitutes inoculous for the colonization in more favorable periods (1, 15).

Another factor which may have influenced positively the level of ectomycorrhiza in nursery and forests was the low pH value in the soil. The ectomycorrhizal fungi, in their majority, are acidofilos with an excellent pH layer between 4,0 - 6,0 (13).

The pH values can select the symbiotic fungus for the mycorrhizal association, being the reason not known yet for these differences in the optimum pH and tolerance layers for colonization, once that, in pure culture these fungi can have different values of optimum pH for growth (15).

The endomycorrhizal fungi V-A type, *Glomus clarum* and *Acaulospora scrobiculata*, presented different levels of colonization in lots 01 and 02 of *E. grandis* in nursery. It is probable that this difference was due in part lots sowing time. The plants of lot 01 were gown in September/82, when the temperature started to rise and the plants in lot 02, in June/83, when the cold period had started.

It the field, for *E. citriodora*, it was observed that the endomycorrhiza percentage was low, and, relatively, very little different in the coldest months (from March to September/83), increasing in Dec/83, when the air temperature and pluviosity were higher. The same was observed in

E. robusta, although the percentage of mycorrhizal colonization in March/83 was higher than in *E. citriodora*, this levels might have been a consequence of more favorable conditions occurred in the months of January and February/83. As it was mentioned for the ectomycorrhiza, it is possible that the periods of the most elevated air temperature and the highest pluviosity imply the plant growth with a higher number of short roots, which would result in higher number of sites for coloniazation.

It is possible that the type of vegetation present in the forests, predominately gramineous, influenced the levels of endomycorrhizal colonization, since these ones are hosts of fungus V-A. In the *E. robusta* forest, this type of vegetation used to be less abundant and probably, also due to this fact, the level of endomycorrhizal colonization in *E. robusta* was higher.

RESUMO

Incidência de micorrizas em viveiros de *Eucalyptus* spp em Viçosa, Minas Gerais

Na região de Viçosa - MG, foram coletadas raízes finas e solo, de viveiro e de duas florestas de *Eucaliptus spp.* verificando-se a ocorrência de ectomicorrizas. A identificação do fungo ectomicorrízico baseou-se na comparação do micélio e rizomorfas circundantes da ectomicorriza com aqueles encontrados na base do basidiocarpo e conexão entre micorrizas e basidiocarpos. Também foi observada colonização endotrófica por descoloração de raízes em KOH 10% e coloração em azul de algodão. O fungo simbionte foi identificado pela cor, forma e tamanho dos esporos, extraídos do solo através de centrifugação em sacarose. A freqüência anual de ambos tipos micorrízicos foram relacionados com fatores ecológicos como temperatura do ar, pluviosidade e tipo de solo (pH). Verificou-se que, de maneira geral, temperaturas do ar, umidade elevada e pH de 4,5-7,0, favoreceram a colonização.

Foram identificados as ectomicorrizas: 1. *Eucalyptus grandis* + *Scleroderma spp*, 2. *Eucalyptus citriodora* + *Pisolithus tinctorius*, 3. *Eucalyptus robusta* + *P. tinctorius* e os fungos endotróficos : *Acaulospora screobiculata* e *Glomus clarum.*

Palavras-chave: ectomicorriza, micorriza vesículo-arbuscular, *Eucalyptus*, *Pisolithus tinctorius.*

REFERENCES

1. Ashton, D.H. Studies on the mycorrhizae of *Eucalyptus regnan* F. Muell. *Aust. J. of Bot.*, **24**: 723-741, 1976.
2. Bakshi, B.K. Mycorrhiza in eucalyptus in India. *Indian Forester*, **92**: 19-20, 1966.
3. Barros, N.F.; Brandi, R.M. & Reis, M.S. Micorriza em eucalipto. *Rev. Árv.*, **2**: 130-140, 1978.
4. Carvalho, P.E.R. & Carpanezzi, A.A. Espécies florestais com associações simbióticas, promissoras ou indicadas para plantio no sul do Brasil. In: Seminário sobre atualidades e perspectivas florestais, 7, 1982. *Anais*. Curitiba, EMBRAPA, p. 7-17, 1982.
5. Chilvers, G.A. Some distinctive types of eucalypt mycorrhiza. *Aust. J. of Bot.*, **16**: 49-70, 1968.
6. Chu-Chou, M. & Grace, L.J. *Hymenogaster albus* - a mycorrhizal fungus of *Eucalyptus* in New Zeland. *New Zeland J. Fores. Scie.*, **11**: 186-190, 1981a.
7. Dring, D.M. Gasteromycetes. In: Ainsworth, G.C.; Sparrow, F.K. & Sossman, A.S (eds). *The fungi*. Academic Press, New Press, New York. p. 451-478, 1973.
8. Ferguson, J.J. & Menge, J.A. The influence of light intensity and artificially extended photoperiod upon infection and sporulation of *Glomus fasciculatus* on sudan grass and on root exudation of sudan grass. *The New Phytol.*, **92**: 183-191, 1982.
9. Gerdemann, J.W. & Trappe, J.M. The Endogonaceae in the Pacific Northwest. *Myc. Memoir.*, **5**: 1-76, 1974.
10. Jenkins, W.R. A rapid centrifugal-flotation technique for separating nematodes from soil. *The Plant Dis. Rep.*, **48**: 692, 1964.
11. Johansen, D.A. *Plant Microtech.* McGraw-Hill, New York, 523p., 1940.
12. Malloch, D. & Malloch, B. The mycorrhizal ecology of two Northern poplars. North American Conference on Mycorrhizae, Fifth. *Prog. and Abs.* Quebec, Canada, p. 36, 1981.
13. Mikola, P. Application of mycorrhizal symbiosis forestry practice. In: MARKS, C.G. & KOZLOWSKI,T.T. (eds) *Ectomycorrhizae: their ecology and physiology*. Academic Press, New York. p. 383-411, 1973.
14. Mosse, B. & Bowen, G.D. A key to the recognitions of some *Endogone* spores types. *Trans. Brit. Mycol. Soc.*, **51**: 469-483, 1968.
15. Mosse, B.; Stribley, D.P. & Le Tacon, F. Ecology of mycorrhizae and mycorrhizal fungi. *Adv. Microb. Ecol.*, **5**: 137-210, 1981.
16. Nicolson, T.H. & Schenck, N.C. Endogonaceous mycorrhizal endophytes in Florida. *Mycologia*, **71**: 178-198, 1979.
17. Phillips, J.M. & Hayman, D.S. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Brit. Mycol. Soc.*, **55**: 158-161, 1970.
18. Read, J.; Koucheki, H.K. & Hodgson, J. Vesicular-arbuscular mycorrhiza in natural vegetation systems. I. The occurrence of infection. *The New Phytol.*, **77**: 641-653, 1976.
19. Smith, S.S.E. Mycorrhizas of autotrophic higher plants. *The Biol. Rev.*, **5**: 475-510, 1980.
20. Trappe, J.M. Fungus associates of ectotrophic mycorrhizae. *The Bot. Rev.*, **38**: 538-606, 1962.
21. Trappe, J.M. & Guzmán, G. Notes on some hypogeous fungi from Mexico. *Mycologia*, **63**: 317-332, 1971.

22. Yokomizo, N.K.S. *Associação ectomicorrízica de Pisolithus tinctorius (Pers.) Coker e Couch com espécies de Eucalyptus L'Héritier*. Piracicaba, São Paulo, 54p., 1981. (Tese M.S.).
23. Zak, B. Characterization and classification of mycorrhizae of douglas fir. II. *Pseudotsuga menziesee + Rhizopogon vinicolor*. *Can. J. Bot.*, 49: 1079-84, 1971.
24. Zak, B. Classification of ectomycorrhizae. In: Marks, C.G. & Kozlowski, T.T. (eds.). *Ectomycorrhizae. their ecology and physiology*. Academic Press, New York. p. 43-78, 1973.
25. Zambolim, L. & Barros, N.F. Constatação de micorriza vesicular-arbuscular em *Eucaliptus spp* na região de Viçosa, MG. *Rev. Árv.*, 6: 95-97, 1982.

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SEROLOGICAL DISTRIBUTION OF BRADYRHIZOBIUM JAPONICUM FROM BRAZILIAN "CERRADOS" AREAS UNDER SOYBEAN CULTIVATION

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SUMMARY

Greenhouse experiments were carried out in order to study the serological distribution of *B. japonicum* in nodules formed in soybean cultivated in soils collected from major soybean cultivated areas in Cerrados. Serogroup 566 was the dominant one in 13 out of 20 areas that apparently had never been inoculated with strains from that serogroup. Similar results were observed at experimental areas of CPAC, with serogroup 566 occupying as much as 94% of nodules in sites where that strain had never been inoculated. In order to study the seasonal serological distribution in a field area, soil samples were collected, distributed in pots and sown with non-inoculated soybean Doko. Even though statistically significant fluctuations occurred within each serogroup, no shift of serological dominance of the strains was observed throughout the seasons.

Key Words: Savannas, nodulation, serogroup, nitrogen fixation.

INTRODUCTION

Soybean (*Glycine max* L. Merril) has been established as the most important grain crop in the Brazilian Cerrados. Although there is no native *Bradyrhizobium japonicum* in soils of this region (18, 19, 21, 22, 23) strains of this bacteria are now widely distributed throughout the soybean production areas, as a result of continuous cultivation of inoculated soybean. Inoculation of soybean with selected strains of *B. japonicum* in areas that already have an established population of that bacteria has sometimes failed in influencing nodulation or enhancing nitrogen fixation above values observed in non- inoculated plants (3, 11, 20). Among the factors reported as influencing inoculation response of soybean plants are the presence and quality of the soil *B. japonicum* population (2, 16, 17, 18).

The objective of this study was to determine the serological distribution of *B. japonicum* strains in nodules formed on soybean sown in soils collected from some soybean cultivated areas in Cerrados. The seasonal serological distribution in the field was also studied at the CPAC experimental station, as related to some environmental parameters.

MATERIAL AND METHODS

Experiment I

Soil samples were collected at several locations in the Cerrados, including twenty soybean

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growing farms and some experimental areas at Brazilian Cerrados Research Center (CPAC) where inoculation history was known. In the latter, sampling was taken from bare soils from October to November, just before the soybean planting date. At least twenty samples were taken at each site, from the 0-20 cm depth soil layer. After homogeneization, the soils were distributed in pots (2.5 kg/pot), irrigated, and sown with sterile seeds of Doko cultivar in the greenhouse. The experimental design was a complete randomized block with two replicates. At early flowering stage, nodules were taken from the plant roots, and identifyed serologically (50 nodules/pot) using the imuno-agglutination method (20). Nodules were crushed individually in test tubes, diluted in physiological solution and boiled at 100°C for one hour. Nodules suspensions were delivered into serological trays end tested against specific antibodies obtained from rabbits immunized with the *Bradyrhizobium japonicum* strains used in the experiment.

Experiment II

This experiment was carried out in 1990 and was aimed to follow the seasonal fluctuation of *B. japonicum* serogroups at an experimental area in CPAC. The area was divided into 14 plots with dimensions 10 x 20 m. Soil samples were collected monthly at the 0-20 cm layer (twenty sub-samples per plot) and had their moisture content determined gravimetrically. At early flowering stage, nodules were taken from the plant roots, counted, dried at 60° C for 72 h and identifyed serologically (50 nodules/pot) using the imuno-agglutination method (20). Enumeration of the soil rhizobial population was carried out using the Most Probable Number (plant dilution)Technique (26).

RESULTS AND DISCUSSION

Experiment I

The percentages of the serogroups found in the nodules of twenty sites in the year of 1990 are in Table 1. Except for four sites, more than 70% of the nodules were antigenically related to either one of three serogroups: 587, 29W or 566. Serogroup 566 was the dominant one in 13 sites and either serogroups 29W or 587 was the dominant one in seven of the sites. Strain 566 was isolated from an inoculant produced in the United States by Dixie Inoc., and was used by the brazilian inoculant industries until 1978. Since 1980, when soybean cultivated area started to increase in the Cerrados region, the commercial inoculants were prepared with solely strains 29W and 587, and strain 566 was never more used in the brazilian inoculants (24). The occurrence of strain 566 in soils where this bacteria has never been introduced may be attributed to its introduction in soil through agricultural tools and seeds originated from old soybean growing areas in southern Brazil, where strain 566 was used.

In some experimental areas at CPAC with careful control of inoculation procedures, strain 566 was present in more than 68% of the nodules in five out of seven sites (Table 2). In one site strain 566 was introduced through seed inoculation, and this strain occupied only 2% of nodules of non-inoculated soybeans two years after, but this level reached 35% in the second evaluation. In the other sites, even though strain 566 had never been inoculated, that strain was present in up to 96% of the nodules formed in non-inoculated plants. The factors affecting the dominance of one strain in soybean nodules are poorly understood (10,14). In major soybean growing areas of the USA Midwest, the most competitive population of

TABLE 1 - Serological distribuition of nodules in non-inoculated soybean grown in pots with soil from several soybean growing farms.[1]

Site	Serogroups						
	29W	587	566	532	CB 1809	965	Without reaction
Tabatinga-1 (DF)	28	11	38	9	0	0	14
Tabatinga-2 (DF)	6	8	65	11	0	1	9
Rio Preto-1 (DF)	27	15	39	3	0	0	16
Rio Preto-2 (DF)	34	12	17	9	0	0	28
Rio Preto-3 (DF)	32	9	55	2	-	-	2
Jardim-1 (DF)	30	35	14	10	0	0	11
Jardim-2 (DF)	23	1	54	12	0	0	10
PAD-DF-1	29	13	16	3	0	0	4
PAD-DF-2	16	16	69	-	-	-	-
PAD-DF-3	45	17	13	6	0	0	19
PAD-DF-4	26	17	40	3	0	0	13
PAD-DF-5	19	14	37	8	0	0	22
PAD-DF-6	22	22	17	23	0	0	16
Pipiripau (DF)	36	8	14	11	0	0	31
Taquara (DF)	11	2	62	10	0	0	15
Cristalina (GO)	33	23	17	11	0	0	16
Vilhena-1 (RO)	8	20	72	-	-	-	-
Vilhena-2 (RO)	19	16	65	-	-	-	-
São Gabriel do Oeste (MS)	23	13	63	-	-	-	2
Maracaju (MS)	21	25	51	3	-	-	-

Serogroups 566, CB 1809 and 965 are the same serogroups of strains USDA 123, 136 and 110, respectively.

TABLE 2 - Serological distribution of nodules from non-inoculated soybean grown in pots with soil collected at the CPAC experimental sites.

strains used	Inoculation date[a]	First evaluation				Second evaluation			
		29W	587	566	965	29W	587	566	965
29w, 587, 566[b]	1976	20	30	2	1	40	22	35	0
29w, 965[b]	1979	90	0	0	7	22	19	59	0
29w, 587[c]	1982-1	-	-	-	-	20	13	68	0
29w, 587[c]	1982-2	-	-	-	-	8	11	81	0
29w, 587[c]	1982-3	-	-	-	-	2	4	94	0
29w, 587[c]	1982-4	-	-	-	-	0	4	96	0
29w, 587[c]	1982-5	-	-	-	-	31	51	19	0

[a] Inoculation was carried out when soybean was planted for the first time in the field, but not in subsequent years.
[b] Evaluations in non-inoculated soybeans were in 1980 (first) and 1985 (second).
[c] Evaluation carried out in 1989.

B. japonicum is that of serogroup 123, which has the same serological reaction of strain 566 (5, 6, 7, 27) and some relatioship was found with the dominance of that strain in soybean nodules and some soil characteristics, as soil pH (5) and exchangeable Mg (1). In a toposequence with pH grading from 5.9 to 8.3, serogroup 123 tends to dominate in soils with low pH, whereas serogroup 135 was the dominant one in soils with high pH (5).

In an experiment carried out in 1978 in the Southern part of Minas Gerais State, strain 29W was found in all the nine sites sampled, reaching as much as 79% of the nodules formed in non- inoculated plants (21), although this strain had never before been used in the brazilian commercial inoculants. Strains 29W and 566 have quite different phenotypes, such as competitiveness and host specificity (13), N_2 -fixing efficiency (26) and intrinsic antibiotics resistance (15). A study of those two strains as related to their establishment in soils may help to understand the dynamics of the rhizobial ecology in Cerrados soils.

Experiment II

Seasonal fluctuations of nodulation and serological distribution in soybean at one site in CPAC/EMBRAPA is presented in Table 3. Plants were well nodulated in all the sampling dates, similar to other values found in the literature (18, 19, 21, 22, 23). Serogroup distribution had seasonal fluctuations: serogroup 29W shifted significantly throughout the months, reaching highest values in January and December. Serogroup 587 had significantly lower values from July through December and serogroups 566 and 532 had highest values on April and June-July, respectivelly. However, the dominance of the serogroups was kept throughout the year, in the following order: 29W > 587 > 566 > 532 > R54. Those results are in disagreement with a field experiment carried out in the USA, where planting date promoted the shift in the dominance of some serogroups (4).

The variables analyzed, aparently do not explain the fluctuations in the serogroup distribution. No statistical correlation was found between serogroup distribution and gravimetric soil water

TABLE 3 - Nodulation and serological distribution of nodules in non-inoculated soybean grown in pots containing soils collected at different dates from the same sampling sites[a].

Sampling date	Nodulation per plant		Serogroups (%)					Rhizobiol population[b]	Rainfall
	Number	Weight (mg)	29W	587	566	532	R54	(cells/g soil) x 1000	(mm)
January	124 b	371 bc	57 ab	31 abc	11 b	0 e	1 a	32,5 (22,7)	169,7
February	89 ef	360 c	47 de	33 ab	6 cd	10 bc	0 b	359,8 (160,7)	153,8
March	104 cd	299 d	54 abc	27 bcde	9 bc	3 de	0b	11,8 (3,3)	159,0
April	143 a	358 c	48 cde	29 abcd	15 a	3 de	1 a	51,8 (18,3)	70,0
May	124 b	293 d	47 de	35 a	9 bc	6 cd	0 b	11,8 (3,3)	148,4
June	84 f	238 e	44 ef	31 ab	5 cd	18 a	0 b	9,2 (7,3)	0,0
July	93 def	301 d	44 ef	24 cde	8 bc	16 a	0 b	5,0 (1,9)	67,7
August	72 g	242 e	39 f	24 cde	4 d	10 bc	0 b	9,7 (4,2)	12,6
September	99 cde	392 bc	45 def	27 bcde	3 d	11 b	0 b	6,7 (2,1)	98,4
October	103 cd	379 bc	51 bcd	23 de	6 cd	4 de	0 b	3,4 (0,9)	168,7
December	108 c	401 b	60 a	22 e	4 d	2 de	0 b	48,3 (19,9)	102,4
Coef. var.	13%	12%	15%	26%	67%	62%	37%		

[a] Values followed by the same letter in columns do not differ statistically by the Duncan test at 5% probability.
[b] Rhizobial populations: determined through the most probable number (MPN). numbers within brackets are the standard error of the mean.

TABLE 4 - Correlation coeflicients of gravimetric soil water and percentage of nodules occupied by each serogroups.

Month	Serogroup					
	29W	587	566	532	R54a	N/R[1]
January	0.31	0.10	0.40	-	-0.10	-0.09
February	-0.23	-0.05	0.11	0.44	-0.49	-0.15
March	0.20	-0.14	-0.34	0.20	-	0.01
April	-0.54	0.49	0.16	0.11	0.31	-0.03
May	-0.25	0.06	0.04	-0.20	0.19	0.70**
June	0.16	-0.33	-0.12	0.21	-	0.38
July	-0.15	-0.34	0.30	0.18	0.24	0.21
August	-0.20	-0.24	-0.41	0.25	-	0.06
September	0.09	-0.51	0.33	0.18	-	0.19
October	0.11	-0.28	-0.28	-0.16	-	0.31
November	-0.05	0.09	0.16	-0.08	-	-0.23

** Significant at the 0.01 level.
[1]N/R = no reaction.

content (Table 4) probably because this variable is not suitable for use in rhizobial ecology studies. According to Papendick and Campbell (12), soil water content is of little use in determining the effect of water on biological systems. On the other hand Vargas and Bezdiceck (25) reported a strong effect of soil water matrix potential on rhizobial survival in soil. Harris (8) recomend the use of water potential as the best variable to study microbial activity in soil. *B. japonicum* population in soil fluctuated throughout the year (Table 3), and tended to be higher in the months with high levels of rainfall. Seasonal fluctuation of *B. japonicum* in the field was reported by Mahler & Wollum (9) in soils of the United States, with lowest populations found in the spring, prior to soybean planting date.

RESUMO

Distribuição sorológica de *Bradyrhizobium japonicum* em áreas de "cerrados" no Brasil cultivadas com soja

Foi conduzido um estudo sobre ocorrência de sorogrupos de *Bradyrhizobium japonicum* em nódulos de soja cultivar Doko. O experimento foi conduzido em vasos com solos coletados em vários locais dos Cerrados, sendo a soja semeada sem inoculação O sorogrupo 566 foi o dominante em 13, dentre 20 áreas de cultivo de soja, que, aparentemente, nunca haviam sido inoculadas com estirpes desse serogrupo. Resultados semelhantes foram encontrados na área experimental do CPAC, onde essa estirpe nunca havia sido inoculada, mas que apresentou plantas de soja com até 94% dos nódulos ocupados por essa estirpe. Em outro estudo, objetivou-se acompanhar as flutuações sazonais da ocorrência dos serogrupos na soja em uma área no campo, através de amostragens mensais de solo que eram distribuídos em vasos e semeados com soja não inoculada. Apesar de ocorrerem flutuações significativas nas percentagens de ocorrência dos serogrupos, não houve alterações na ordem de dominância das estirpes durante os meses em que foi conduzido o experimento.

Palavras-chave: Savannas, nodulação, serogrupo, fixação de nitrogênio.

REFERENCES

1. Bezdicek, D.F. Effect of soil factor on the distribution of *Rhizobium japonicum* serogroups. *Soil Sci. Soc. Am. Proc.* **36:** 305-307, 1972.
2. Boonkerd, N.; Weber, D.F. & Bezdicek, D.F. Influence of Rhizobium japonicum strains and inoculation methods on soybean grown in rhizobia-populated soils. *Agron. J.* **70:** 547-549, 1978.
3. Caldwell, B.E. & Vest, G. Effects of *Rhizobium* strains on soybean yields. *Crop. Sci,* **10:** 19-21, 1970.
4. Caldwell, B.E. & Weber, D.F. Distribution of *Rhizobium japonicum* serogrups in soybean nodules as affected by planting dates. *Agron. J.* **62:** 12-14. 1970.
5. Damirgi, S.M.; Frederick, L.R. & Anderson, I.C. Serogroups of *Rhizobium japonicum* in soybean nodules as affected by soil types. *Agron. J.* **59:** 10-12, 1967.
6. Ellis, W.R.; Ham, G.E.; Schmidt, E.L. Persistence and recovery of *Rhizobium japonicum* inoculum in a field soil. *Agron. J.* **76:** 573-576, 1984.
7. Ham, G.E.; Caldwell, V.B. & Johnson, H.W. Evaluations of *Rhizobium japonicum* inoculants in soils containing naturalized populations of rhizobia. *Agron. J.* **63:** 301-303, 1971.
8. Harris, R.F. Effect of water potential on microbial growth and activity. in: Water Potential Relations in Soil Microbiology. *Am. Soc. Microb. Spec. Publ.* **9:** 1981.
9. Johnson, H.W.; Means, V.M. & Weber, C.R. Competition for nodule sites between strains of *Rhizobium japonicum. Agron. J.* **57:** 179-185, 1965.
10. Mahler, R.L. & Wollum, A.G. Seasonal fluctuations of *Rhizobium japonicum* under a variety of field conditions in North Carolina. *Soil Science* **134:** 317-324, 1982.
11. Moawad, H.A.; Ellis, W.R. & Schmidt, E.L. Rhizosphere response as a factor in competition among three serogroups of indigenous *Rhizobium japonicum* for nodulation of field-grown soybeans. *Appl. environm. microb.* **47:** 607-612, 1984.
12. Papendick, R.I. & Campbell, G.S. Theory and measurement of water potential. *Agron. Abstr.* **144:** 1978.
13. Peres, J.R.R. & Vidor, C. Seleção de estirpes de *Rhizobium japonicum* e competitividade por sítios de infecção nodular em cultivares de soja. *Agron. Sulriograndense.* **16:** 205-209, 1980.

14. Robert, F.M. & Schmidt, E.L. A comparision of lectin binding activity in two strains of *Rhizobium japonicum*. *FEMS Microbiol. Lett.* **27**:281-285, 1986.
15. Scotti, M.R.; Sá, N.M.; Vargas, M.A.T. & Dobereiner, J. Streptomycin resistance of *Rhizobium* isolates from Brazilian cerrados. *An. Acad. bras. Ciên.* **54**: 733-738, 1982
16. Singleton, P.W. & Tavares, J.W. Inoculation response of legumes in relation to the number and effectiveness of indigenous *Rhizobium* populations. *Appl. environm. microbiol.* **51**:1013-1018, 1986.
17. Thies, J.E.; Singleton, P.W. & Ben Bohllol, B. Influence of the size of indigenous rhizobial population on establishment and symbiotic performance of introduced rhizobia on field-grown legumes. *Appl. environm. microbiol.* **57**:19-28, 1991.
18. Vargas, M.A.T. & Suhet, A.R. Efeito de tipos e n níveis de inoculante na soja cultivada em um solo de Cerrados. *Pesq. agropec. bras.* **15**:343-347, 1980a.
19. Vargas, M.A.T. & Suhet, A.R. Efeito da inoculação e deficiência hídrica no desenvolvimento da soja em um solo de Cerrado. *Re. bras. Ci. Solo*, **4**:17-21, 1980b.
20. Vargas, M.A.T.; Peres, J.R.R. & Suhet, A.R. Reinoculação de soja em função de serogrupos de *Rhizobium japonicum* predominantes em solos de Cerrados. In: SEM. NAC. PESQ. SOJA, 2. Brasília, 1980. Anais... EMBRAPA-CPAC. p. 715-722, 1981.
21. Vargas, M.A.T. & Suhet, A.R. Adubação nitrogenada, inoculação e épocas de calagem para a soja em um solo sob Cerrado. *Pesq. agropec. bras.* **17**:1127-1132, 1982a.
22. Vargas, M.A.T.; Peres, J.R.R. & Suhet, A.R. Adubação nitrogenada e inoculação da soja em solos de Cerrado. Comunicado Técnico nº 13, Planaltina, EMBRAPA-CPAC, 11p. 1982b.
23. Vargas, M.A.T.; Peres, J.R.R. & Suhet, A.R. Fixação de nitrogênio atmosférico pela soja em solos de Cerrados. *Informe agropec.* **94**: 20-3, 1982c.
24. Vargas, M.A.T., Mendes, I.C. ; Suhet, A.R. & Peres, J.R.R. Fixação biológica do nitrogênio. In: CULTURA DA SOJA NOS CERRADOS, (Ed.) Potafos, Piracicaba, SP, pp. 159-182, 1993.
25. Vargas, M.A.T. & Bezdicek, D. Influence of water potential on the enumeration of *Bradyrhizobium* sp (Cicer). *Pesq. agropec. bras.* **26**:1081-1089, 1991.
26. Vincent, J.M. A manual for the practical study of root-nodules bacteria. International Biological Programme. London, Burguess, 1970, 164p.
27. Weber, D.F.; Keyson, H.H. & Uratsu, S.L. Serological distribuition of *Bradyrhizobium japonicum* from U.S. soybean production areas. *Agron. J.* **81**:786-789, 1989.

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EVALUATION OF THREE METHODS OF PRESERVATION FOR ANAEROBIC BACTERIA

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ABSTRACTS

Three methods of preservation of bacteria were studied observing the viability and stability of some morphological and biochemical characteristics of *Bacteroides fragilis, Clostridium perfringens, C. difficile, Fusobacterium nucleatum, Peptostreptococcus* sp *and Prevotella melaninogenica (B. melaninogenicus)* strains. After periods of storage, *B. fragilis* showed the best viability in lyophilization and freezing methods, whereas *Clostridium* strains were the most resistant bacteria, surviving up to one year after lyophilization, freezing or subculturing. *Peptostreptococcus and P. melaninogenica* lost the viability at the begining of storage, in all methods studied. The stability of colonial, morphological and physiologic-biochemical aspects of all strains seemed have not been altered throughout this study.

Key Words: Preservation, Viability, Stability, Anaerobic bacteria.

INTRODUCTION

The study of anaerobes has undergone transformations, in the last years, basically due to the development of simple methodology of isolation [1-3] and its applicability to clinical use [4, 5], rendering to them importance in human diseases.

Recently, the investigations have been regarded on the determination of virulence factors [6-8], and thus organisms entirely preserved are required for these researches. However, few comparable studies have been conducted on preservation of varied anaerobic bacteria strains. In an attempt to examine the short and long-term methods of conservation: subculture, freezing and freezedrying, bacterial strains isolated from clinical and normal flora were tested. In this respect the viability up to one year and possible variations on morphological and colonial aspects, and biochemical tests caused by conservation methodology and storages were observed in strains of *Bacteroides fragilis, Fusobacterium nucleatum, Peptostreptococcus* sp, *Clostridium perfrigens, C. difficile* and *Prevotella melaninogenica* (*B. melaninogenicus*) [11].

MATERIAL AND METHODS

Bacteria and culture conditions: The bacteria tested were isolated as follows: *C. difficile* (feces), *P. melaninogenica* and *F. nucleatum* (gingival crevice) and *B. fragilis* and *Peptostreptococcus* sp (clinical specimens). One strain of *C. perfringens*, from American Type Culture Collection - 10543, was also included in this investigation. All bacteria were cultivated in Blood Agar supplemented with haemin and vitamin K (BAS) (3) and

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incubated in anaerobiosis, using a gas mixture (N_2, 80%; H_2 10% and CO_2, 10%).

Characterization of strains: Before and after storage, all strains were identified by biochemical tests using prereduced sterilized anaerobically media (PRAS) (3).

Growth curve determination: The generation time of each strain was determined on Brain Heart Infusion (BHI -PRAS) (3). The knowledge of this time is considered essential to the following procedures, in order to work with strains in their late logaritmic phase of growth.

Preparation of bacterial suspension: *B. fragilis* strain was inoculated in BHI-PRAS, while *C. difficile*, *P. melaninogenica*, *F. nucleatum* and *Peptostreptococcus* were in BAS. After the incubation period, which was nearly on late logaritmic growth phase, bacterial cultures were harvested and their respective pellets and mixed with 10% skim milk, freshly prepared, in flasks containing glass beads and magnetic bar in order to obtain a good mixture under CO_2 flux.

Preservation methods and storage conditions: From the bacterial suspension, equal volumes were distributed, under CO_2 flux, in all methods used.

Subculture (SC): The bacterial suspension was inoculated in BHI-PRAS. The tubes were sealed, incubated 24 h at 37°C and stored, at room temperature.

Freezing (F): Aliquots, immediately after distribution in tubes were frozen, in dry ice alcohol bath which were maintained enveloped in aluminum foil for storage, in freezer at - 24°C.

Freezing drying (FD): The bacterial suspension was distributed in ampoules and frozen in dry ice alcohol bath, using the shell freezing technique (1). Then, the frozen filled were submitted to final lyophilization on freeze-dryer (The Virts Co) and sealed, under vacuum (13). After the process concluded they were wrapped with aluminum foil, and storage inside of another tube containing silica gel indicator, in freezer (-24°C).

Survival recoveries: The determination of viability was performed immediately before and after each conservation method (time zero) and also after time intervals of storage: 60, 180, 240 and 365 days. Lyophilized cells were reconstituted by adding BHI-PRAS and incubated at 37°C by a period smaller than its generation time, before viability determination. The viable count was determined by diluting the suspension and plating by drop counting technique of Miles & Misra (17), except for *C. perfringens*. For this one the pour-plate technique was employed to prevent its swarm.

Cultures stored at room temperature were only homogenized and inoculated into a new BHI-PRAS to determine the quantitative viability.

RESULTS

The results express the viable count of different anaerobes before and after the methodology of

TABLE 1 - Viability of different anaerobes before and after subculture (SC); Freezing (F) and Freezing-Drying (FD) by 12 months period of storage.

Species	Before each methold	Method	Periods of storage (months) 0	2	6	8	12
C. perfringes	8,7	SC	10,5	9,5	9,1	5,7	4,3
		F	9,3	5,2	5,6	4,7	4,5
		FD	8,5	8,1	7,8	5,5	4,5
C. difficile	6,9	SC	7,5	7,9	7,6	4,5	4,5
		F	6,4	4,5	3,5	-	2,5
		FD	5,5	5,5	4,9	4,5	4,5
B. fragilis	9,0	SC	11,9	6,5	5,0	4,6	0
		F	5,3	4,6	4,3	3,2	2,5
		FD	7,7	8,5	8,9	7,0	5,5
P.melaminoge nica	7,0	SC	6,0	"0"	-	-	-
		F	7,2	3,4	-	-	-
		FD	-	2,5	-	-	-
F. nucleatum	10,0	SC	13,0	"0"	-	-	-
		F	7,5	4,8	-	-	-
		FD	-	4,3	-	-	-
Pepstostrepto coccus sp	9,4	SC	12,6	7,6	-	-	-
		F	9,1	7,8	-	-	-
		FD	5,5	-	-	-	-

- The number indicates average of 3 determinations of units forming colony/ml (in Log_{10})
- "O": Recovered, but not detected after dilutions
- -: unviable

TABLE 2 - Percentage of recovery in maximum period of viability of anaerobes tested, in each method of preservation.

Microorganisms / Methods	Percentage/Maximum period of viability (months) Subculture	Freezing	Freezing-drying
C. perfringens	49.4/12	51.7/12	51.7/12
C. difficile	65.2/12	36.2/12	65.2/12
B. fragilis	51.1/8	27.7/12	61.1/12
P. melaninogenica	85.7/0	68.6/2	61.4/2
F. nucleatum	130.0/0	34.0/2	25.0/2
Peptostreptococcus	80.1/2	83.0/2	58.5/0

conservation (Table 1). They represent the average of three determinations. The percentage of recovery of each microorganism is listed in Table 2.

DISCUSSION

The different methods of conservation here evaluated showed a considerable variability in their performance for the anaerobic bacteria strains tested. By the method of subculture we found results that emphasize the remarkable dependence between microorganisms and methodology employed. It is possible that due to the characteristics of this methodology cell damage occurs by nutrients reduction or even by deleterious substances produced by the cells themselves. In addition, the bacteria can be or not recovered depending of nutrients offered by the medium (18) or by their resistance to starvation. Impey (19) showed that in cooked meat broth (CMB) the spore forming bacteria do not survive over twelve months of storage. Among our strains, spore forming anaerobic bacteria were the most resistant. The relative decrease of cellular concentration of our strains by freezing seems to be due to cryo-injury since freezing and thawing leads, invariably, to cellular damages. Nagel & Kunz (20) found *C. perfringens* strains surviving up to one year, using horse blood and glass beads at -70°C. The nature and function of the cryoprotector deserves, of course, attention, since it is recognized that the correlation between cellular damage and the loss of viability is directly related to ruptures of cell membranes by internal ice formation. The skim milk, here used, has the capacity to bind hydrogen and to regulate the cellular rehydration process through proteins and other macromoleculars (21).

Strains of *Clostridium* and *B. fragilis* kept viable for twelve months when they were freeze-dried. Surely, the methodology was extremely severe for *Peptostreptococcus* strain, since it was reduced over 50% in its original population. These results do not agree with those from Staab & Ely (22) that recovered *C. perfringens* in shorter time and preserved *Peptostreptococcus* strain stored one year, using the same cryoprotector, skim milk. Probably, this short period of viability of the *C. perfringens* strain found by (22) can be attributed to the low stimulus of sporulation in cellular mass preparation (23), although we did not take into consideration in the present investigation.

Now a days it has been the concern about stability of important components such as surface structures responsible for morphological and virulence aspects. In this regard, bacteria recently isolated from clinical specimens were also used, showing stable in all aspects studied throughout the investigation, probably due to the repair time that we took into account.

In conclusion, it was observed that freeze-drying was the best methodology for *B. fragilis* strain, while *C. perfringens* and also *C. difficile*, due their characteristics of sporulation, kept resistant in all methods tested. However, the most fastidious strains, such as *P. melaninogenica*, *F. nucleatum* and *Peptostreptococcus* lost the viability in all methodologies yet at the begining. This investigation confirms the necessity to attempt for others factors in order to improve the methods of preservation.

RESUMO

Avaliação de três métodos de preservação para bactérias anaeróbias

Três métodos de preservação para bactérias foram estudados observando-se a viabilidade e a estabilidade para algumas características morfológicas e bioquímicas de cepas de *Bacteroides fragilis*, *Clostridium perfringens*, *C. difficile*, *Fusobacterium nucleatum*, *Peptostreptococcus* sp e *Prevotella melaninogenicus* (*B. melaninogenicus*). Após períodos de estocagem, *B. fragilis* apresentou ótima viabilidade nos métodos de liofilização e congelamento, enquanto ambas as cepas de *Clostridium* foram as bactérias mais resistentes, sobrevivendo até um ano após terem sido liofilizadas, congeladas e sub-cultivadas. As cepas de *Peptostreptococcus* e *P. melaninogenica* perderam a viabilidade já no início da estocagem em todos os métodos estudados. Todas as cepas mostraram, entretanto, estabilidade nos seus padrões coloniais, morfológicos e fisiológicos-bioquímicos durante os seus tempos de estocagem.

Palavras chave: preservação, viabilidade, estabilidade, bactérias anaeróbias.

REFERENCES

1. ARANKI, A.; SYED, S.A.; KENNEY, E.B. & FRETER, R. Isolation of anaerobic bacteria from human gingiva and mouse cecum by means of a simplified glove box procedure. *Appl. Microbiol.* 17, 568-576, 1969.

2. SMITH, L.D.S. & HOLDEMAN, L.V. - The pathogenic Anaerobic Bacteria, pp 17-59, Charles C. Thomas, Springfield, Illinois, USA, 1968.
3. HOLDEMAN, L.V.; CATO, E.P. & MOORE, W.E.C. - Anaerobe Laboratory Manual, 4th ed. Virginia Polytechnic Institute Anaerobe Lab. Blacksburg, Virginia, USA, 1977.
4. KILLGORE, G.; STARR, S.E.; DEL BENE, V.E.; WHALEY, D.N. & DOWELL, V.R. - Comparison of three anaerobic systems for the isolation of anaerobic bacteria from clinical specimens. *Amer. J. Clin. Pathol.* **59**, 552-559, 1972.
5. ROSENBLATT, J.E.; FALLOU, A. & FINEGOLD, S.M. - Comparison of methods for isolation of anaerobic bacteria from clinical specimens. *Appl. Microbiol.* **25**, 77-85, 1973.
6. ONDERDONK, A.B.; MOON, N.E.; KASPER, D.L. & BARTLETT, J.G. - Adherence of *Bacteroides fragilis* "in vivo". *Infect. Immun.* **19**, 1083-1087, 1978.
7. ROTSTEIN, O.D.; KAO, J. & HOUSTON, K. - Reciprocal synergy between *E. coli* and *B. fragilis* in an intra-abdominal infection model. *J. Med. Microbiol.* **29**, 269-276, 1989.
8. SIMON, G.L.; KLEMPER, M.S.; KASPER, D.L. & GORBACH, S.L. - Alterations in opsonophagocytic killing neutrophils of *Bacteroides fragilis* associated with animal and laboratory passage: effect of capsular polysaccharide. *J. infect. Dis.* **145**, 72-77, 1982.
9. CUCHURAL, G.J.Jr.; TALLY, F.P.; JACOBUS, N.V.; GORBACH, S.L.; ALDRIDGE, K.E.; CLEARLY, T.J.; FINEGOLD, S.M.; HILL, G.B.; IANNINI, P.B.; O'KEEFE, J.P. & PIERSON, C.L. - Antimicrobial susceptibilities of 1,292 isolates of the *Bacteroides fragilis* group in the United States: comparison of 1981 with 1982. *Antimicrob. Agents Chemoter*, **26**, 145-148, 1984.
10. CUCHURAL, G.J.Jr.; TALLY, F.P.; JACOUNUS, N.V.; FINEGOLD, S.M.; HILL, G.; IANNINI, P.; O'KEEFE, J.P. & PIERSON, C. - Comparative activities of newer B-lactam agents against members of the *Bacteroides fragilis* group. *Antimicrob. Agents Chemoter.* **34**, 479-480, 1990.
11. SHAH, H.N. & COLLINS, D.M. - *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formely classified in the genus *Bacteroides. Int. J. Syst.* **40**, 205-208, 1990.
12. HECKLEY, R.J, - Preservation of bacteria by lyophilization. *Adv. in Appl. Microbiol.* **3**, 1-75, 1961.
13. SWATZ, H.M. - Effect of oxygen on freezing damage: III Modification by B-mercaptoethylamine. *Criobiology* **8**, 543-549, 1971.
14. CHOATE, R.V. & ALEXANDER, M.T. - The effect of the rehydration temperature and rehydration medium on the viability of freeze-dried *Spirillum atlanticum.* Criobiology **3**, 419-422, 1967.
15. RAY, B.; JEZESKI, J.J. & BUSTA, F.F. - Effect of rehydration on recovery repair, and growth of injured freeze-dried *Salmonella anatum. Appl. Microbiol.* **22**, 184-189, 1971.
16. VALDEZ, G.F.; GIORI, G.S.; HOLGADO, A.R. & OLIVER, G. - Effect of drying medium on residual moisture content and viability of freeze-dried lactic acid bacteria. *Appl. Environm. Microbiol.* **49**, 413-415, 1985.
17. MILES, A.A. & MISRA, S.S. - The estimation of the bactericidal power of the blood. *J. Hyg.* **38**, 732-749, 1938.
18. BRETZ, H.W. & AMBROSINI, R.A. - Survival of *E. coli* frozen in cell extracts. *Cryobiology* **3**, 40-46, 1966.
19. IMPEY, C.S. - Preservation of anaerobes. In: Anaerobic Bacteria in Habitats Others Than Man. *Soc. for Appl. Bacteriol. Symp. Series* **13**, 411-419, 1986.
20. NAGEL, J.G. & KUNZ, L.J. - Simplified storage and retrieval of stock cultures. *Appl Microbiol.* **23**, 837-838, 1972.
21. JANSEN, D.W. & BUSTA, F.F. - Influence of milk components in the injury, repair of injury and death of *Salmonella anatum* cells subjected to freezing and thawing. *Appl. Microbiol.* **26**, 725-732, 1973.
22. STAAB, A.J. & ELY, J.K. - Viability of lyophilized anaerobes in two media. *Cryobiology* **27**, 174-178, 1987.
23. STEEL, K.J. & ROSS, H.E. - Survival of freeze dried bacterial cultures. *J. Appl. Bact.* **26**, 370-375, 1963.

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ZEARALENONE PRODUCTION IN FUSARIUM GRAMINEARUM VARIANTS AFTER TREATMENT WITH NITROSOGUANIDINE

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SUMMARY

Zearalenone is a secondary metabolite produced by *Fusarium graminearum* and apparently involved in the control its sexual reproduction. When ingested by some animals it can induce estrogenic effects. The catalytic reduction of zearalenone produces zearalanol, which has anabolic effects in bulls and sheeps. In a *Fusarium graminearum* isolate, good producer of zearalenone, the possibility to obtain variants with greater zearalenone production through the action of a mutagenic agent (Nitrosoguanidine) was investigated. Forty samples, differing from the control on morphological aspects, growth rates and pigmentation, were selected. Through zearalenone quantification by HPLC, 10 variants that showed and increase in yields of 2 to 16 times, in comparison to the control, were obtained. The stability for zearalenone production in these variants was tested by 12 sucessive transfers in cultive medium. In the conditions tested, 2 stable variants which maintained zearalenone overproduction were obtained.

Key words: mycotoxins, zearalenone, nitrosoguanidine *Fusarium graminearum*

INTRODUCTION

The overproduction of metabolites in filamentous fungi is one of the most important and developed subject in the chemistry, physiology and genetics of microorganisms.

Due to the complexity in the biosynthesis of the majority of secondary metabolites produced by filamentous fungi, there are several factors involved, that may affect their production.

Zearalenone was first characterized by Urry et al. (24) as [6(10-hydroxy-6-oxo-trans-1-undecenyl)-B-resorcylic acid-lactone and is considered as a secondary metabolite or mycotoxin produced by several species of Fusarium that contamine corn, wheat, rye and other cereals. Zearalenone shows estrogenic and growth promoter activity in domestic and laboratory animals (20, 21). The study of zearalenone appeared as a consequence of intoxication in animals, caused by ingestion of mouldy cereals (14). The outbreak of zearalenone in food is in connection with favourable environment conditions. Temperatures between 20 and 25°C aid the fungal growth, but lower temperatures (6-12°C) are required to increase the zearalenone production (15).

Mainly in swines, the signs caused by zearalenone ingestion are: uterine enlargement, hemorrhage and death, embrionary development interference, stillborn increase, a "splay-leg" uncoordination of the hind limbs, decrease in the

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foetus number, uterine weight and others (1, 13, 17, 18 and 19).

A pair of diastereoisomers is obtained by catalytic reduction of zearalenone. One of these diastereoisomers, the α-zearalanol, has been used as an anabolic agent for sheep (14) and bovine (22), under the trademark RALGRO.®

The knowledge of the zearalenone production capacity of a Fusarium isolate is important to the study of secondary metabolites and in the control of food contamination by mycotoxins.

This study was undertaken to increase the zearalenone production of a *Fusarium graminearum* strain Fg 42 by means of a transformation induced with N-methyl-N'-nitro-N-nitrosoguanidine and also, to study along several generations, the stability of the genetic transformation introduced.

MATERIALS AND METHODS

Microorganisms - The strain used in this study (*Fusarium graminearum* - Fg 42), was collected in a wheat plantation in Maringá (State of Paraná). The strain was submited to monosporic isolation and has morphological and cultural growth characteristics similar with those described by Booth (6) to *Fusarium graminearum*. The samples submited to the stability tests (variants) were originated by treatment of Fg 42 macroconidia with the mutagenic agent nitrosoguanidine (NTG).

Culture media - Potato Dextrose Agar (PDA): 200 g potato (scrubbed and diced); 15 g dextrose; 20 g agar; 1 litre water (6).

Bilay's medium modified by Joffe (6): 1 g potassium dihydrogen phosphate; 1 g potassium nitrate; 0.5 g magnesium sulphate; 0.5 g potassium chloride; 0.2 g starch powder; 0.2 g glucose; 0.2 g sucrose; 15 g agar; 1 litre water. Fine strips of pure cellulose lens paper were added before the agar had set. This medium was utilized to enhance the yield of macroconidia.

Rice medium - 14 g of dry and clean rice in 8.5 ml of distilled water were distributed horizontally in 70 ml screw capped tubes and sterilized twice with 24 hours interval each. This medium was utilized to zearalenone production.

Treatment with Nitrosoguanidine (NTG) - 10^5 to 10^6 macroconidia . ml-1 obtained from plates cultivated with Bilay's medium, were allowed to react with 0.5 to 2.0 mg-1 of Nitrosoguadinine for 1 hour, at 28oC, with agitation each 10 minutes. This procedure was based in the methodology used by Avalos et al. (2). After incubation, the macroconidia were washed with sterile distilled water and centrifuged for 10 minutes at 1200 g. This process was repeated three times. The pellet obtained was resuspended in sterile distilled water (1 ml) and used as inocula for the selection of variants. The manipulation and disposal of NTG material and glassware was according the procedure described by Ehrenberg & Wachtmaister (12).

Selection of Variants - The macroconidial suspension obtained after the NTG treatment was diluted to contain 1000 macroconidia per milliliter. Inocula were made, dispensing 0.1 ml of this dilution in Petri dishes containing PDA medium. Growth was allowed for 4 days at 25°C, in the absence of light. The selection work started after 4 days of growth, comparing the colonies obtained with those of the control (colonies arising from macroconidia without treatment). Colonies which had distinct morphological aspects, slower growth rates and pigmentation, were selected (9). These colonies were transferred to Petri dishes and tubes with PDA medium, and allowed to grown during seven days, at 25°C, without light. Selected Petri dishes were photographed to accompaniment.

Production, extraction and purification of zearalenone - The control (Fg 42) and the isolates obtained after treatment with NTG, were cultivated in rice media with cultived agar fragments (5 mm in diameter) as inoculum. Incubations were done at 25°C, during 12 days and, in sequence, at 12°C, for 6 weeks , without light. The mycelial mass and cultivated media of each tube was triturated with spatula until small fragments and then extracted during 24 hours with 40 ml of acetonitrile. The material was filtered under vacuum (filter paper) through a Büchner filter. The residue obtained was re-extracted again and the filtrates of each sample were combinated. To reduce the volume, a flux of air produced by a compressor was passed over the samples, in a water bath, at 60°C, in a fume hood. The resulting material was partitioned twice, with n-Hexane (1:1). The hexane fractions were discarted, and the acetonitrile fractions were evaporated to dryness as described above. The final extracts were suspended in a minimal volume of chloroform (0.5 to 1.0 ml) and purified by column adsortion chromatography (silica gel, Sigma 60 A). The columns were filled with silica suspended in chloroform and eluted with chloroform: methanol (97:3). The separation of zearalenone was fol-

lowed by its characteristic fluorescence under short-wave ultraviolet light. The eluates were collected, concentrated to dryness and stored in dark flasks at 4°C to posterior analysis.

Qualitative analysis - A previous qualitative analysis by TLC was executed in order to select interesting variants, those that maintained or inclusive, increased the zearalenone production. 3 µl of each extract obtained was applied on TLC plates and developed with chloroform: methanol (97:3). Zearalenone at 1 mg.ml-1 was used as standard. Revelation was performed using fast violet B 0.7%, sodium borate buffer at pH 9.0 and 50% sulphuric acid (23).

Quantitative analysis - Quantitative determination of zearalenone, in the selected variants, was obtained with HPLC on a CG-480C chromatograph equipped with UV detector (CG-435) and a 10 µm Lichrosorb RP 18 column. The solvent system was methanol: water (65:35) with 0.5 mM NaOH with a flux of 1 ml.min-1. Zearalenone standard (Sigma) was 0.1 mg.ml-1. Extracts of samples were dissolved in 1 ml HPLC grade methanol. When necessary, dilutions were done . The concentration values were obtained by area calculation through CG-300 integralization equipment.

Stability tests - Nine variants with quantitative analysis upper to the control value, were selected to a zearalenone production stability test.. From the stock isolates of these variants, sucessive transfers, in tubes containing PDA medium, were done until a total of twelve, with 7 days interval to each one. The morphological and pigmentation characteristics were documented in each stage. The subcultive number 4 was designed R-4 generation in relation to the initial isolation after the NTG treatment. The same criteria was used to R-8 and R-12. These generations were cultivated in rice medium and submited to the same procedures of extraction, purification and quantification already described.

RESULTS AND DISCUSSION

Among the techniques utilized to genetic improvement, mutation and recombination with selection through conditional lethal protocol are generally utilized (4). Nitrosoguanidine is usually utilized as chemical indutor of mutations in several fungi species (3, 9, 11, 12).

Secondary metabolites, by definition (5) are not essential to the life of the producing organism (although they may serve some selective advantage in the life cycle). Thus, mutant screening cannot utilize a conditional lethal protocol, which allows recovery of the desired phenotypes against a background of unrecovered lethals. Since all the products of a mutant hunt must be screened when studying secondary metabolites, isolation an event that occurs with a frequency of 1 on 10 6 requires a prodigious amount of labor. Needless to say, this makes analysis of genetic fine structure virtually impossible. In this work the selection was made by a sifting, many colonies were grown and some of them were chosen due to their desirables characteristics as differences in morphology, pigmentation and growth rates, comparing with the initial isolate (Figure 1A and 1B). This choice was made because these characteristics are related with natural isolates of high levels of zearalenone production (9). According to these characteristics, 40 variants were selected. Qualitative analysis by TLC revealed that among these variants 34 produced zearalenone and were therefore submited to quantitative determination by HPLC. Typical chromatograms for Fg 42 and Fg-NTG19 are shown in Fig. 2. Results obtained by HPLC are presented in TABLE 1. Increase, decrease and in some cases, values near to the control average of zearalenone production (0.671 mg.g-1 of rice) were obtained. Differences among the average of zearalenone production in the control were significative in 10 samples (Fg-NTG6, Fg-NTG9, Fg-NTG10, Fg-NTG11,Fg-NTG-14, Fg-NTG19,Fg-NTG23, Fg-NTG27, Fg-NTG28 and Fg-NTG35). That is, 25% of the population produced more zearalenone than the control ($p<0.05$), 60% of the variants had production average less than the control and only 15% had no significative difference. Fg-NTG35, which had the greater zearalenone

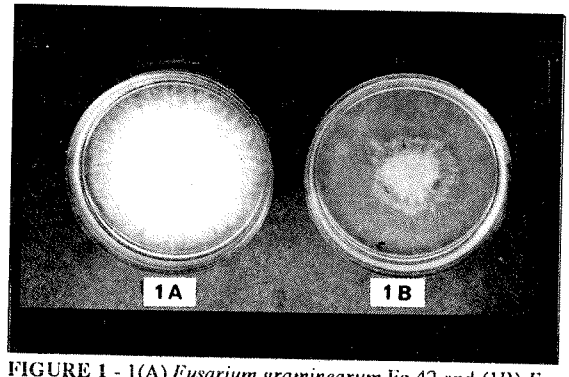

FIGURE 1 - 1(A) *Fusarium graminearum* Fg 42 and (1B) *Fusarium graminearum* variant NTG 35, grown in PDA during 7 days at 25°C.

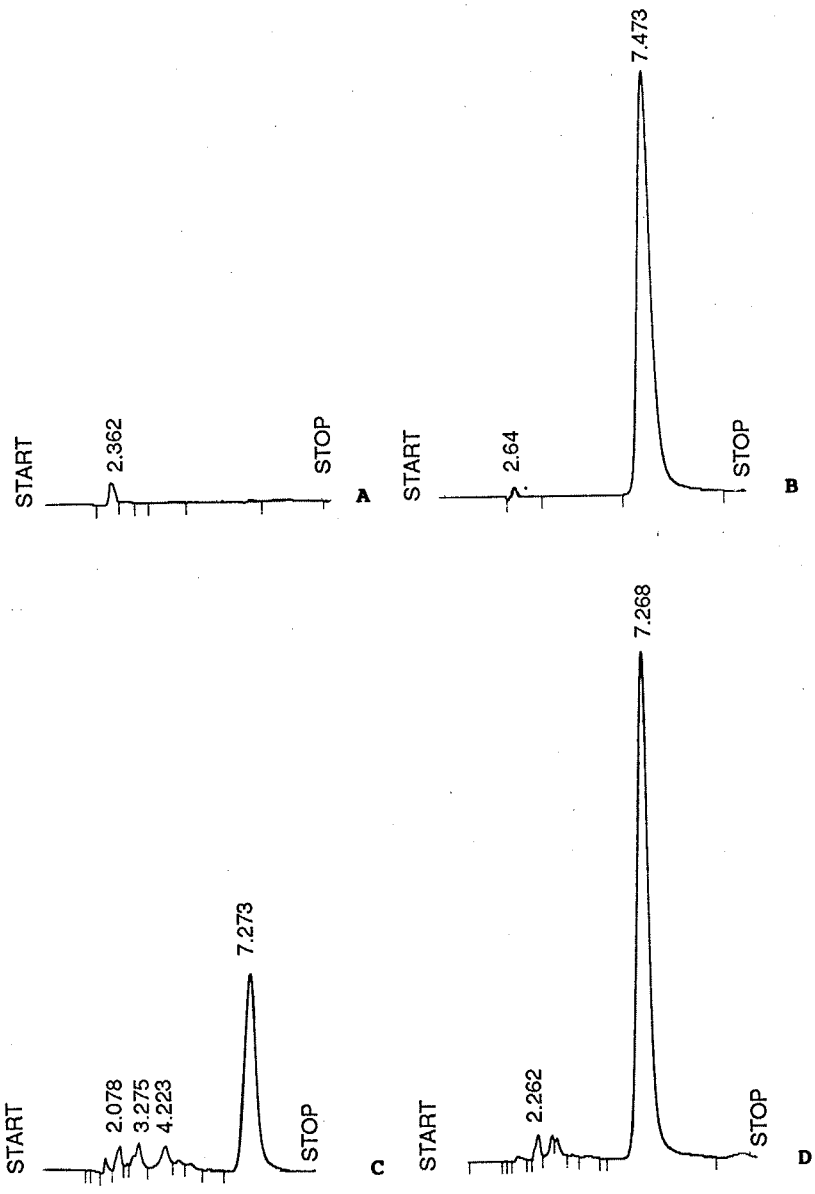

FIGURE 2 - Quantitative analysis of Zearalenone by HPLC. (A) methanol; (B) Zearalenone standard (Sigma) 0.1 mg.ml-1; (C) Fg 42 diluted 1:100; (D) NTG19 diluted 1:400. Conditions as described in materials and methods.

production (11.300 mg.g-1 of rice) also had the more distinct morphological growth characteristics when compared with the control, and even, with other variants. During the experiments, it was possible to observe that pigment production was exacerbated in the majority of cases. Among the 40 variants obtained, 10 (25%) may be considered as overproducing strains (9), in which zearalenone was 2 to 16 times greater than in the initial Fg 42 isolate (TABLE 1).

A decrease of zearalenone production in 60% of variants was not surprise, Duncan & Bu'Lock (11), also obtained variants from a *Fusarium graminearum* strain. It would appear that toxin production is apparently more easily lost from, than induced in. Yabe (28) also obtained mutants of *Aspergillus par-*

TABLE 1 - Zearalenone production by *Fusarium graminearum* variants.

Sample	Production (mg zearalenone . g rice^{-1})*
Fg-NTG5	0.433
Fg-NTG6	1.654
Fg-NTG9	1.732
Fg-NTG10	1.252
Fg-NTG11	1.152
Fg-NTG13	0.718
Fg-NTG14	1.122
Fg-NTG17	0.997
Fg-NTG19	6.003
Fg-NTG21	0.077
Fg-NTG23	7.189
Fg-NTG24	0.103
Fg-NTG25	0.735
Fg-NTG26	0.292
Fg-NTG27	4.937
Fg-NTG28	6.174
Fg-NTG30	0.016
Fg-NTG31	0.011
Fg-NTG32	0.050
Fg-NTG33	0.040
Fg-NTG34	0.052
Fg-NTG35	11.300
Fg-NTG36	0.029
Fg-NTG36	0.047
Fg-NTG39	0.039
Fg-NTG40	0.239
Fg-NTG41	0.435
Fg-NTG42	0.019
Fg-NTG43	0.033
Fg-NTG44	0.074
Fg-NTG45	0.045
Fg-NTG46	0.690
Fg-NTG47	0.024
Fg-NTG48	0.073
Fg 42	0.671

* Determined by HPLC analysis. Average of three experiments.

asiticus, by UV radiation, in which the ability to produce aflatoxins was reduced or lost.

For the stability tests, 8 variants with production of zearalenone greater than the control and also a variant Fg-NTG13 with lower zearalenone production were used. The results are presented in TABLE 2, and as can be seen, the behaviour was very heterogeneous. Samples Fg-NTG9, NTG10, NTG23, NTG27, NTG28 and NTG35 presented a decrease of production in R-4, a increase in R-8 and again, decrease in R-12 generations. The NTG6 variant had sucessive increase in R-4 and R-8 generations, with a subsequent drop of production in R-12. The variants NTG13 and NTG19 presented decrease in R-4, increase in R-8 and a slight increment in R-12, that was less significant

TABLE 2 - Zearalenone production by *Fusarium graminearum* after twelve transfers.

Variants	Initial	R-4	R-8	R-12
	(mg zearalenone . g rice^{-1})			
NTG 6	1.654	1.968	4.931	1.490
NTG 9	1.732	0.024	0.221	0.022
NTG 10	1.252	0.297	1.201	0.261
NTG 13	0.718	0.082	1.848	2.741
NTG 19	6.003	0.926	12.057	12.645
NTG 23	7.189	6.139	8.185	2.590
NTG 27	4.937	0.093	4.403	2.048
NTG 28	6.174	5.255	14.944	12.539
NTG 35	11.300	7.253	4.261	4.261
Fg 42	0.671	0.104	0.059	0.004

Zearalenone production for nine variants and for the original strain (Fg 42 control), after initial isolation and after sucessive transfers (R-4, R-8 and R-12).

** Determined by HPLC analysis. Average of three experiments.

that the second. In fact, all of them had a zearalenone production decrease in R-4, except the NTG6. At R-12 generation, the majority presented a decrease in production, being equal or superior to the initial value, in few cases. In spite of the fact that the experiments were realized in triplicate, a expressive variability in production was observed. It is important to mention that the subcultures were made from mycelia grown on solid media, having therefore two aspects to consider: First, the difficulty in standardize the inoculum. Second, the aleatory choice of cultived agar pieces as inocula can conduce to a non representative sample, with distinct characteristics. Despite of this, it was possible to observe that the variant NTG28 e NTG35 demonstrated a stability during the sucessives subcultives, still maintaining a good zeara–lenone production. NTG19 variant, although presented good zearalenone production had greater variability and consequent instability.

When following the development of the colonies during sucessive generations, changes in morphology, pigmentation and growth rates were observed mainly to NTG6, NTG9, NTG13, NTG23 and NTG29. Little variation was observed in NTG28 and NTG35 when compared with the initial condition, and thus, they were considered genetically stable.

On the decrease in the production of the control, it is known that culture instability was a serious problem in the early days of industry, when the microorganisms required the transfer on culture medium for its restoration (7). To overcome

this problem, original isolates are stored on earth and by freeze drying.

It is interesting to mention that, in this work, only colonies which had distinct pigmentation and slower growth rates relative to the original isolate were selected. However, Kuiper-Goodman (16) reported that mutants of *Gibberela zeae* with high levels of zearalenone were obtained without the concomitant formation of pigments. Thus, it could be possible to have isolates with high zearalenone production that were rejected according the systematic used in the selection process.

RESUMO

Produção de Zearalenona em variantes de *Fusarium graminearum* após tratamento com nitrosoguanidína

A Zearalenona é um metabólito secundário produzido por *Fusarium graminearum* e, aparentemente, envolvido no controle de sua reprodução sexuada. Em alguns animais, quando ingerida, provoca efeitos estrogênicos. A redução química da zearalenona produz o derivado zearalenol, que possui efeito anabolizante em bois e ovelhas, predominando sobre o efeito estrogênico. A partir de um isolado de *Fusarium graminearum*, considerado bom produtor de zearalenona, investigou-se, através da ação do mutagênico nitrosoquanidína sobre seus conidios, a possibilidade de obtenção de variantes com maior produção de zearalenona. Foram selecionadas 40 amostras em função de seus aspectos morfológicos, pigmentação e tempo de crescimento compatíveis com exemplares mais produtores de zearalenona em isolamento natural. Através de quantificação de zearalenona em HPLC, obtiveram-se 10 variantes com produção entre 2 a 16 vezes superior ao controle quando do seu isolamento. A estabilidade destes variantes foi testada através de 12 transferências sucessivas em meio de cultivo. Nas condições testadas obtiveram-se 2 variantes considerados estáveis e que mantiveram a superprodução de zearalenona.

Palavras-chave: micotoxina, Zearalenona, nitrosoguadína, *Fusarium graminearum*.

REFERENCES

1. Abbas, H.K.; Mirocha, C.J.; Kommedahl, T.; Burnes, P.M.; Meronuk, R.A. & Gunter, R. - Toxigenicity of *Fusarium proliferatum* and other *Fusarium* species isolated from corns ears in Minnesota. *Phytopatology*, **78**: 1258-1260, 1988.
2. Avalos, J.; Casadesús, J. & Cerdá-Omedo, E. - *Gibberella fujikuroi* mutants obtained with U.V. radiation and N-methyl-N-Nitro-N-Nitrosoguanidine. *Applied and environmental Microbiology*, **49**(1): 187-191, 1985.
3. Avalos, J.; Cerdá-Omedo, E - Carotenoid mutants of *Gibberella fujikuroi*. *Curr. Genet.*, **11**: 505-511, 1987.
4. Azevedo, J.L., et al. - Genética de microorganismos em Biotecnologia e Engenharia Genética. Piracicaba, Ed. FEALQ. 1985.
5. Bennett, J.W. - Overproduction of Microbial Products - FEMS Symposium nº 13. London, Academic Press Inc. Ltd, 1982.
6. Booth, C. - *Fusarium* - Laboratory guide to the identification of the major species - Kew, Surrey, England. Ed. by Commonwealth Agricultural Bureaux. 1977.
7. Calan, C.T. - The long-term storage of microbial cultures in industrial practice - In: Kirsop, B.E., ed. The Stability of Industrial Organisms. Kew, Surrey, England. Commonwealth Agricultural Bureaux, p. 32-38, 1980.
8. Chaudhary, K.; Lakshminarayana, K.; Dev, I.K. & Uyas, S.R. - Nitrosoguanidine induced mutation of *Aspergillus niger* for obtaining high citric acid production mutants. *Indian Journal of Microbiology*. **14**: 42-43, 1974.
9. Cullen, D.; Caldwell, R.W. & Smalley, E.B. - Cultural characteristics, pathogenicity and zearalenone production by strains of *Gibberella zeae* isolated from corn. *Phytopathology* 72(11): 1415-1418, 1982.
10. Detroy, R.W.; Freer, S. & Ciegler, A. - Aflatoxin and anthraquinone biosynthesis by nitrosoguanidine-derived mutants of *Aspergillus parasiticus*. *Can. J. Microbiol.*, **19**: 1373-1378, 1973.
11. Duncan, J.S. & Bu'Lock, J. - Degeneration of zearalenone production in *Fusarium graminearum*. *Experimental Mycology*. 9: 133-140, 1985.
12. Ehrenberg, L. & Wachtmaister, C.A. - Safety precautions in work with mutagenic and carcinogenic chemicals. - In: Kilbey, B.J.; Legator, M.S.; Nichols, W. and Ramel, C., ed. - Handbook of mutagenicity test procedures. Amsterdam, Elsevier/North-Holland Biomedical Press, p. 401-410, 1977.
13. Etienne, M. & Jemmali, M. - Effects of zearalenone (F2) on estrous activity and reproduction in gilts. *Journal of Animal Science* 55(1): 1-10, 1982.
14. Hidy, P.H.; Baldwin, R.S.; Greasham, R.L.; Keith, C.L. & McMullen, J.R. - Zearalenone and some derivatives: production and biological activities. *Advances in Applied Microbiology*. **22**: 59-82, 1978.
15. James, L.J. & Smith, T.K. - Effect of dietary alfafa on zearalenone toxicity and metabolism in rats and swine. *Journal of Animal Science*. 55(1): 110-118, 1982.
16. Kuiper-Goodman, T.; Scott, P.M. & Watanabe, H. - Risk assessment of the mycotoxin zearalenone. *Regulatory Toxicology and Pharmacology*. 7: 253-306, 1987.
17. Long, G.G. & Diekman, M.A. - Characterization of effects of zearalenone in swine during early pregnancy. *Am. J. Vet. Res.*, 47(1):184-187, 1986.
18. Long, G.G. & Diekman, M.A. - Effect of purified zearalenone on early gestation in gilts. *Journal of Animal Science*. 59(6): 1662-1670, 1984.
19. Miller, J.K.; Hacking, A. & Gross, V.J. - Stillbirths, neonatal mortality and small litters in pig associated with the ingestion of *Fusarium* toxin by pregnant sows. *Vet. Rec.*, **93**: 555-559, 1973.

20. Mirocha, C.J.; Christensen, C.M. & Nelson, G.H. - Estrogenic metabolite produced by *Fusarium graminearum* in stored corn. *Appl. Microbiol.*, **15**: 497-503, 1967.
21. Mirocha, C.J.; Pathre, S.V. & Christensen, C.M. - Zearalenone. - In: Mycotoxins in Human and Animal Health. Pathotox Publishers. p. 345-364, 1977.
22. Ralston, A.T. - Effect of zearalanol on weaning weight of male calves. *J. Anim. Sci.*, **47**(6): 1203-1206, 1978.
23. Swanson, S.P.; Corley, R.A.; White, D.G. & Buck, W.B. - Rapid Thin Layer Chromatographic Method for Determination of Zearalenone and Zearalenol in Grains and Animal Feeds. *J. Assoc. Off. Anal. Chem.*, **67**(3): 580-582, 1984.
24. Urry, W.H.; Wehrmeister, H.L.; Hodge, E.B. & Hidy, P.H. - The structure of zearalenone. *Tetrahedron Letters*, **27**: 3109-3114, 1966.
25. Yabe, K et al. - Isolation and characterization of *Aspergillus parasiticus* mutants with impaired aflatoxin production by a novel tip culture method. *Applied and Environmental Microbiology*. **54**(8): 2096-2100, 1988.

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ECOLOGY OF SEDIMENT MOLDS FROM A POLLUTED PALEO-CARSTIC LAKE IN SOUTHEASTERN BRAZIL

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SUMMARY

Sediment samples were taken monthly during one year at five different stations at Lake Olhos d'Agua - MG. The diversity of substrates among the sampling stations was reflected by the mold community. The similarity index confirmed the observed differences between the types of substrate from each station, and was a good parameter based on the molds community structure of comparison between different stations. The prevalence genera were the common molds: *Penicillium spp.*, *Aspergillus spp.*, *Cladosporium spp.* and non sporulating fungi. Seasonal variations were not observed. There were high mold densities down to 6cm in the sediments and progressive reduction to 20cm depth. The prevalence of non sporulating fungi at more polluted stations suggested this group of microrganisms as a possible indicator of organic poluttion. The indices of ricchness and diversity responded to changes in the fungi community mainly at the more polluted stations.

Key words: mold, pollution indicators, tropical fresh water.

INTRODUCTION

Artificial eutrophication by human use of the aquatic ecossistems has been changing the natural balance of these environments and lead to a reduction in the availability of high quality water (5). Two main approachs to the study of pollution effects have been the use of measurements based on community structure and on measurements based on indicator microrganisms (20). Some works have characterized the water mycoflora and its use to establish reliable parameters for the assessment of water quality and monitoring purposes (1,7,11,16,18,19). Futhermore, molds and yeasts are inclued among the probable microbiologic indicators of pollution (7,11,16,18). The Standard Methods for the Examination of Water and Wastewater (1) suggest that there is an increased number of molds and yeasts in highly polluted aquatic environments. High counts of molds are considered to be a good indicator of the presence of concentrated organic material, and highly diversified microbiota is indicative of populational adaptation to that improved environment (1).

The specificity of some groups of moulds to a substrate can be helpful in revealing the presence of these substances in the water (14). Another important advantage in using molds in ecological

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studies is the ease in their isolation and identification. Futhermore, the mycological methods are simple, rapid and cheaper than the majority of the microbiological assessment techniques commonly in use, exept recent fecal contamination (8).

Purchio et al (16) pointed out a parallel between coliform counts and relative frequencies of the non-sporulating fungi in marine waters, suggesting the use of frequencies of this group as a reliable microbial pollution indicator of coastal waters. Our objective was to determine if sediment mold data can be applied to determine levels of eutrophication in tropical fresh water and to verify the applicability of biotic indices in assessing impacts on the molds community.

MATERIAL AND METHODS

Lake Olhos d'Agua is located at the city of Lagoa Santa, approximately 38Km north of Belo Horizonte, in the Lagoa Santa Karst Plateau (19 44' and 19 33' S; 44 05' and 43 50' W (2, 9). This lake was described as a paleo-carstic one since it is not exclusively based on a calcareous rock matrix (9). The pluviometric regime ranges from 1000 to 1200mm and the mean anual temperature varies between 19 C and 21 C (17). Lake Olhos d'Agua is a shallow lake (3 to 3,70m) showing characteristics of eutrophication due to intense human occupation of its vicinities. As a consequence, there are several macrophytes species, including *Nymphaea sp.*, *Eleocharis sp.*, *Salvinia sp.*, *Typha sp.* and *Chara sp.*

Sampling sites were described by Rosa et al (19). The sediment samples were taken monthly between June 1988 and July 1989. Samples were taken with a modified Eckman-Birge core sampler (6) disinfected with 70% ethanol. Interface water was sampled assepticaly from the core sampler by sterile 0.5cm diameter tubes and seringes into 200ml sterile wide mouthed bottles. The sediment was pushed out of the sampler with a wood embolus. As it emerged the upper portion of the sediment sample was fractioned with a sterile spatula in two subsamples, corresponding to the layers of 0-2 and 2-4cm depth in the sediment. Vertical distribution of molds in 2cm intervals in the sediment was estimated to a depth of 20cm at a central site in August and November 1988 and February 1989. Petri dishes were filled with these subsamples and transported to the lab on ice within 8 hours.

Fungi were isolated on Sabouraud's dextrose agar (Biobrás), suplemented with chloranphenicol (100 mg/l), penicillin (100 mg/l) and yeast extract (5 g/l) (12), Spread plates were made with 0,2ml of an asseptically prepared suspension of 1g sediment in 10ml of sterile destilled water shaken for 15 minutes at low velocity. Incubation was at 25 C (average temperature of the lake water) for 3 to 10 days. Colonies were described, enumerated and transferred to Sabouraud's dextrose agar slants. Molds were identified according to techniques described in Barnett (3) and McGinnis (12). The molds which did not produce spores on potato agar, cornmeal agar, and Czapeck agar were included under the designation of non sporulating fungi - NSF.

The Sorensen's similarity index between each station was measured monthly and the similarity

TABLE 1 - Mold taxa isolated from the sediment of Lake Olhos d'Água at stations I, II, III, IV and V, from June/88 to May/89.

	Percent of total population				
TAXA	I	II	III	IV	V
Penicillium spp.	22.4	16	15.8	38.2	28.4
Aspergillus spp.	29.2	14.1	16.3	5.5	9.5
Paecilomyces spp.	11.4	4.6	5.2	9.3	12.2
Byssochlamys spp.	11.7	4.3	4	10	18.2
Fusarium spp.	3.3	2	3.5	0.3	0.7
Cladosporium spp.	2.1	6	18.2	1.7	11.5
Eurotium spp.	1.3	1.5	1.5	0.1	1
Trichoderma spp.	2	3.1	5.1	0.5	3.3
Phoma spp.	1.5	2.6	18.7	-	0.2
Rhizopus spp.	1.4	0.3	0.8	-	0.2
Mucor spp.	1.1	-	0.2	-	0.1
Absidia spp.	0.1	0.2	0.4	-	0.1
Gliocladium sp.	-	0.2	0.1	-	-
Gliomastix sp.	0.4	0.1	-	-	0.2
Sagrahamala sp.	-	-	-	-	-
Hamigera sp.	-	0.1	0.2	6.2	0.4
Eupenicillium sp.	-	-	-	-	-
Hyalodendron sp.	-	-	-	0.1	-
Helmintosporium sp.	-	-	0.8	-	-
Memnoniella sp.	-	0.2	0.1	6.4	0.4
Curvularia spp.	-	0.1	0.2	-	0.2
Chaetomium spp.	0.5	0.8	0.4	2.6	1
Circinella spp.	-	-	0.2	-	-
Endocochlus sp.	0.5	-	0.1	-	-
Phialophora sp.	0.1	0.1	0.1	0.1	-
Alternaria spp.	-	0.2	0.2	-	0.1
Botrytis sp.	0.2	-	0.1	-	-
Scopulariopsis sp	-	-	0.1	-	-
Dactylium sp.	0.1	-	0.2	-	0.1
Deuteromycetes	0.6	0.1	0.2	-	-
Ascomycetes	0.5	0.1	0.2	-	-
Coelomycetes	-	0.1	0.1	-	0.1
Mucorales	-	0.1	0.1	-	-
NSF	3.8	42.9	17.5	19	12.3
Mean counts*	199.3	258.1	153.6	565.4	220.4

* - monthly mean counts as colony-forming unity per gram (CFU/g).

degree estimated by the anual mean, expressed as percent (10, 13, 20). It was calculated the Shannon-Weaver's diversity index and the Simpson's richness index for each station (13, 20).

RESULTS

A list of the fungi isolated, their mean densities and occurrences is presented in Table 1. The 16760 colonies identified included 29 genera and other taxa, totalizing 34 groups. From stations I to V, were counted respectively: 2391, 3097, 1843, 6785 and 2644 C.F.U. (colony forming units). The dominant genera were *Penicillium*, *Aspergillus*, *Cladosporium* and the non sporulating fungi. Figure 1 shows the monthly mean mold counts in the sediment of each station and the mean variation of the five stations. The highest densities were found between August 1988 to January 1989 and April and May 1989. However, seasonal variations were not identified, except for

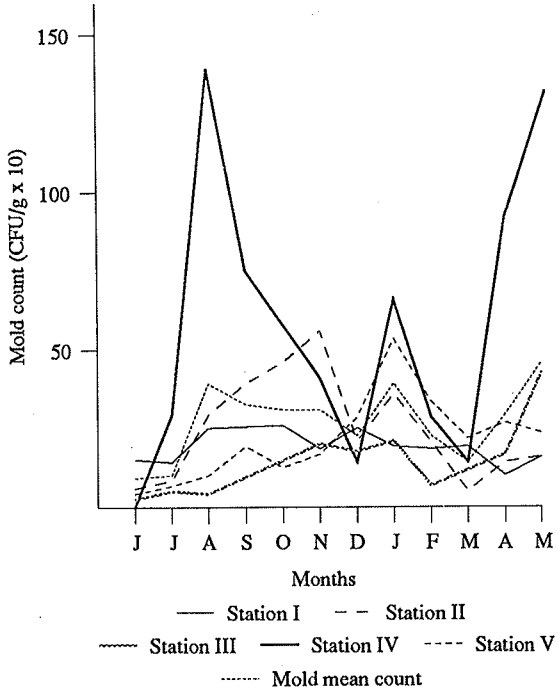

FIGURE 1- Mold counts in the sediment of the Lake Olhos d'Água between June/88 and May/89.

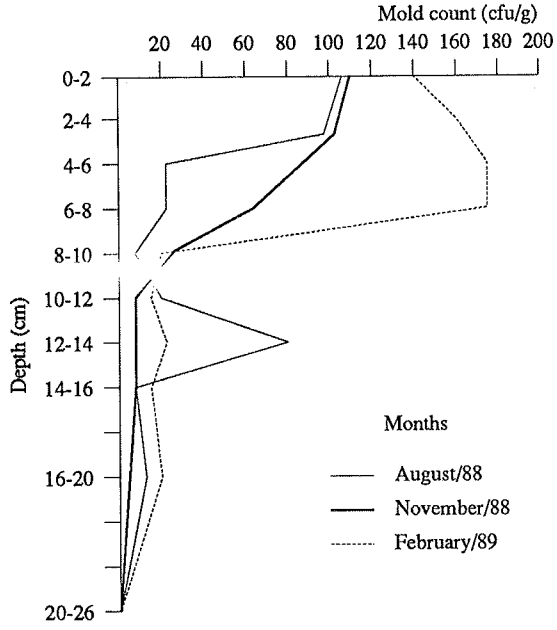

FIGURE 2 - Vertical distribution of molds in Station I, Lake Olhos d'Água.

TABLE 2 - Sorensen's indices of similarity for five sampling stations, at Lake Olhos d'Água, Lagoa Santa, MG.

		Month											
ST.	X (Cs)	J	J	A	S	O	N	D	J	F	M	A	M
I x II	0.44	0.21	0.34	0.44	0.3	0.24	0.42	0.56	0.46	0.49	0.39	0.83	0.59
I x III	0.45	0.23	0.56	0.21	0.25	0.14	0.6	0.65	0.74	0.49	0.51	0.6	0.38
I x IV	0.32	0.06	0.49	0.16	0.2	0.1	0.41	0.6	0.38	0.48	0.7	0.16	0.16
I x V	0.42	0.31	0.43	0.32	0.35	0.14	0.7	0.43	0.47	0.31	0.51	0.47	0.55
II x III	0.47	0.33	0.57	0.2	0.3	0.48	0.5	0.5	0.54	0.5	0.57	0.7	0.51
II x IV	0.28	0.19	0.16	0.29	0.18	0.68	0.25	0.43	0.27	0.22	0.41	0.16	0.16
II x V	0.45	0.44	0.53	0.4	0.54	0.33	0.43	0.32	0.57	0.24	0.35	0.47	0.73
III x IV	0.27	0.26	0.22	0.04	0.14	0.42	0.27	0.58	0.34	0.2	0.47	0.21	0.14
III x V	0.49	0.59	0.65	0.38	0.48	0.5	0.68	0.5	0.44	0.18	0.41	0.5	0.59
IV x V	0.29	0.21	0.24	0.11	0.26	0.35	0.4	0.47	0.3	0.36	0.31	0.31	0.18

X(Cs) = average indices of similarity.

Cladosporium spp. and *Aspergillus spp.* at station I. The first showed an increased density between October and March while the second had the lowest densities during the summer, between December 1988 and March 1989. There was not a significant variation of mold count data in the 0-2 and 2-4 cm layers (figure 2).

The similarity indices are presented in Table 2. The diversity indices and the richness indices are showed in Table 3. Lake Olhos d'Agua had during the time of this present study a richness index of 1.65 and diversity index of 2.02.

TABLE 3 - Shannon-Weaver's indices of diversity and Simpson's indices of richness for five sampling stations, at Lake Olhos d'Água, Lagoa Santa, MG.

Stations	Diversity	Richness
I	2.038	1.86
II	1.89	1.89
III	2.274	1.95
IV	1.877	1.02
V	2.021	1.55

DISCUSSION

There are few studies concerning the isolation of molds from aquatic environments in Brazil and none dealing with molds from the sediment or analyzed by statistical indices. In spite of the difficulties in estimating the mold density in nature, the relation between the environmental changes and the variations in mold densities can be a good indicator of their activity, as suggested by several authors (4, 18), and corroborated our results. Some reports suggest an over estimation of the molds due to the recovery of dormant spores which could limit the use of mold densities in assessments of environmental problems (4, 18).

As shown in Table 1, the mold counts were higher in the more polluted stations (II and IV) and lower in the station I, limnetic zone, free of effluents. Although the absence of seasonal variation it was found a direct relation between the mold community structure and the type of substrate that enter in the water. Progressive europhication could have provided high amounts of organic matter in decomposition to support the larger mold community. Allochtonous materials from the sewage and pluvial discharges could be another source of incoming fungic spores to the sediment.

The lake showed a heterogeneous mold community structure along the 5 collection stations (Table 1). Certainly, the different substrates discharged into each station may have modified the water and sediment conditions, which was reflected in the mold community structure. In stations II and III which received sewage NSF were prevalent representing 50% of the isolates. In station IV, with sediment having high levels of decomposing plant materials, Penicillium spp predominated, followed by the NSF. In stations I and V the NSF were isolated in low density predominating Penicillium spp. These data were consistant with those of Purchio et al (16) for marine waters. In both situations the NSF have high isolation frequencies in polluted waters.

A possible hypothesis is that the increased concentration of organic matter could lead to an unbalance in the mold reproduction favoring vegetative reproduction rather than sporulation. A similar phenomenon occur in laboratory conditions due to the low stress situation of a mold in enriched culture media (10). However, this approach must be tested in order to obtain more detailed information and to standardize specific techniques.

The prevalene of NSF at more polluted stations suggested this group as a possible indicator of organic pollution.

Some authors reported a relation between organic pollution, mold densities (11) and NSF frequency (16) when relating mold densities with organic matter and NSF frequencies in each station.

The results of diversity index shown in table 3 were in accord with diversity of substrates in each station. The richness index follow the same trend (Table 3). Since molds act more on larger particles during plant decomposition (4, 14), a high density was expected in station IV. This station has a principal substrate resource of macrophytes that permits a lower number of ecologic niches to be occupied, and the lower richness of "taxa" (1.02) reflects this substrate prevalence. In contrast, station III has a high substrate diversity because it receives domestic sewage and pluvial waters. This probably explains the high richness of "taxa" (1.95) and mold diversity (2.274) in spite of the lower mold density in this station. The lower monthly range in mold densities at station I reflects the absence of shore influence and its low susceptibility to domestic effluent effects (Figure 1). Rosa et ali (19) notes a higher yeast density at station II during the rain period, that is in accord with our data and was probably due to the increased sewage discharge during this period.

Our data on the vertical distribution (Figure 3) confirmed the profile shown in Quinn (18).

The calculation of similarity is based on the presence and absence of "taxa" and its relation to the total count (Table 2). In this study the similarity index reflected the kind of substrate at each station. High similarities were found for station IV and low ones for the other stations. The stations with low macrophyte density (I and V) and those receiving sewage discharges (II and III) showed higher similarity index. These data suggested the use of this index to compare sites in relation to the type of substrate.

Some studies have pointed out that more diversified substrates leads to higher mold diversity (4), so the mold diversity index could be applied to set a value on substrates diversity. Analysis of the diversity associated with the similarity reflected the different modifications at each station. The use of such indies based on fungi and substrate presence to compare sites could be an useful tool for environmental impact assessment.

RESUMO

Ecologia de bolores do sedimento de uma lagoa paleo-cárstica poluída do sudeste do Brasil

Foram feitas amostragens mensais de sedimento na lagoa Olhos d'Água - MG, durante um ano, em cinco diferentes estações de coleta. Os efluentes de diferentes origens em cada estação provocam uma diversidade de substratos entre elas, a qual se reflete na microbiota fúngica. O coeficiente de similaridade dos bolores estudados foi uma ótima ferramenta para a comparação das regiões,refletindo o grau de semelhança entre os substratos de cada estação. Verificou-se a predominância de bolores comuns à maioria dos ambientes: *Penicillium spp*, *Aspergillus spp.*, *Cladosporium spp.* e fungos não esporulados. Não foi observada sazonalidade. Os bolores apresentaram grande densidade até 6cm de profundidade reduzindo, progressivamente, até o estrato de 20cm. Foi observada a dominância de fungos não esporulados nas estações mais poluídas, sugerindo a possibilidade de se usar este grupo como indicador microbiológico de poluição. Os índices de riqueza de "taxa" e diversidade indicam alterações na comunidade fúngica, principalmente nas estações que apresentavam ações antrópicas mais acentuadas.

Palavras chaves: bolores, indicadores de poluição, água doce tropical.

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REFERENCES

1. American Public Health Association (APHA) - *Standard methods for the examination of water and waste water.* 14 ed. Washington, APHA, 1980.
2. Barbosa, F.A.R.; Coutinho, M.E.; Araújo, L.M.; Menendez, R.M.; Barbosa, P.M.M.; Figueira, J.E.C. and Esteves F.A. - Estudos limnológicos na região cárstica central de Minas Gerais. 1. Caracterização preliminar da Lagoa Santa. *An. Sem. Reg. Ecol.*, **4**: 399-437, 1984.
3. Barnett, H.L. and Hunter, B.B. - *Ilustrated genera of Imperfect fungi.* 3th ed. Minneapolis, Burgess Pub. Co., 1972.
4. Christensen, M. - A view of fungal ecology. *Mycologia,* **81**(1): 1-19, 1989.
5. Esteves, F.A. and Barbosa, F.A.R - Eutrofização artificial: a doença dos lagos. *Ciência Hoje,* 27(5): 56-61, 1986.
6. Fukuhara, H. and Sakamoto, M. - An improved Ekman-Birge Grab for sampling an undisturbed bottom sediment core sampler. *Jpn. J. Limnol.*, **48**: 127-32, 1987.
7. Hagler, A.N.; Santos, S.S. and Mendonça-Hagler, L.C. - Yeasts of a polluted Brazilian Estuary. *Rev. Microbiol.*, 10(1): 36-41, 1979.
8. Hagler, A.N.; Mendonça-Hagler, L.C.; Santos, E.A.; Farage, S.; Filho, J.B.S. and Schrank, A. - Microbial pollution indicators in Brazilian tropical and subtropical marine surface waters. *The Sccience of Total Environment.*, **58**: 151-160, 1986.
9. Kohler, H.C.; Coutard, P. and Queiroz, J.P. - *Excursão na região kárstica ao norte de Belo Horizonte, M.G.* São Paulo, USP. Col. Interdisc. Franco-Brasil 2, 1978.
10. Magurran, A.E. - *Ecological diversity and its measurement.* New Jersey. Princeton University Press, 1988.
11. Martins, M.T.; Gambale, W.; Paula, C.R.; Pellizari, V.H.; Matsumoto, E.F.; Ribeiro, G.; Malateaux, S. and Mayer, M.H. - Utilização de bactérias e fungos como indicadores na avaliação de fatores fisiográficos que interferem nos processos de auto-depuração de um córrego subtropical. *Rev. Microbiol.*, 20(3): 278-91, 1989.
12. McGinnis, M.R. - *Laboratory Handbook of Medical Mycology.* 2 ed. New York, Academic Press, 1980.
13. Odum, E.P. - *Ecologia.* 1 ed. Rio de Janeiro, Ed. Interamericana, 1985.
14. Park, D. - On the ecology of heterotrophic microorganisms in freshwater. *Trans. Br. Mycol. Soc.*, 58(2): 291-9, 1972.
15. Pugh, G.J.F. - Strategies in fungal ecology. *Trans. Br. Mycol. Soc.*, 75(1): 1-14, 1980.

16. Purchio, A.; Gambale, W. and Paula, C.R. - Molds from some beaches in the southern area of São Paulo State (Baixada Santista), Brazil. *Rev. Microbiol.*, **19**(2): 166-71, 1988.
17. Queiroz, R. - *Zoneamento agroclimático do estado de Minas Gerais*. 1 ed. Belo Horizonte, Secretaria Estado Agricul., 1980.
18. Quinn, J.P. - Seasonal occurrence of yeasts and other fungi in a freshwater lake. *Trans. Br. mycol. Soc.*, **83**(1): 53-8, 1984.
19. Rosa, C.A.; Resende, M.A.; Franzot, S.P.; Morais, P.B. and Barbosa, F.A.R - Distribuição de leveduras e coliformes em um lago do karst do planalto de Lagoa Santa - MG, Brasil. *Rev. Microbiol.*, **21**(1): 19-24, 1990.
20. Washington, H.G. - Diversity, biotic and similarity indices. *Water Res.*, 18(6): 653-94, 1984.

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STAPHYLOCOCCAL FOOD POISONING OUTBREAKS IN SÃO PAULO (BRAZIL)

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ABSTRACT

Six staphylococcal food poisoning outbreaks in São Paulo were investigated, the implicated foods being cream-filed cake (4 outbreaks), gnocchi (1 outbreak) and ice-cream (1 outbreak). The foods were examined for the presence of *Staphylococcus aureus* and staphylococcal enterotoxin. Staphylococcal counts of 107 to 109 cfu/g were recorded for all foods except the ice-cream from which no staphylococci were isolated. The staphylococci isolated from the foods produced enterotoxin A, and all foods samples contained enterotoxin A, the most common cause of staphylococcal food poisoning.

Key words: Food poisoning, *Staphylococcus*, Enterotoxin.

INTRODUCTION

Staphylococcal food poisoning occur world wide, but most cases are never reported. The major symptons, vomiting and diarrhea, are usually of short duration and most individuals affected never seek medical attention (1). In recent years several cases have been reported to public health officials in Brazil. Investigations of the outbreaks revealed the presence of staphylococci in the incriminated food, the number of organisms present in the food, and the production of enterotoxin by the staphylococci isolated from the food (4).

There have been a number of staphylococcal food poisoning outbreaks in the São Paulo area in the last two or three years in which a number of individuals were made ill. The food most often implicated was cake with cream filling, which was one of the major foods involved in the outbreaks recorded in the Belo Horizonte area (4). Usually staphylococci were present in large number, up to 10^8 cfu/g of food.

This report includes the results of testing implicated foods from six outbreaks in São Paulo in which the ill persons demonstrated the symptoms of staphylococcal food poisoning.

DESCRIPTION OF THE OUTBREAKS

Outbreak 1: This outbreak occured on February 25, 1991, among 40 persons attending a birthday party. Of 23 people interviewed, 16 (69.4%) became ill will symptoms of vomiting (100%), diarrhea (87.5%), abdominal cramps (6.3%) and nausea (6.3%). One individual required hospitalization. The mean incubations period was 4 hours

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(a range of 3.5% to 6 hours). Most of the food items served at the party were home-made: the attack rate implicated the birthday cake with whipped cream filling, which was acquired at a bakery, as the vehicle of intoxication. The cake was held at room temperature for several hours because it was too large for the refrigeration facilities available.

Outbreak 2: This outbreak occured on March 10, 1991, among approximately 200 persons at a residence during a wedding party. Of 56 people interviewed, 33 became ill and 12 required hospitalization. The mean incubation period was 4.5 hours and the symptoms were vomiting (72.7%), nausea (57.6%), abdominal cramps (57.6%), diarrhea (46.4%) and headache (33.3%). The food histories implicated the cake with cream filling as the vehicle of intoxication. The cake was prepared by a neighbor, with the cream filling being placed in the cake on day before it was delivered to the party residence. The cake sat in the sun after delivery and was not consumed until late in the evening.

Outbreak 3: On October 17, 1991, 9 people became ill with symptoms of vomiting, diarrhea, and abdominal cramps 3 hours after eating a cake filled with condensed sweet milk. All of the exposed persons required hospitalization.

Outbreak 4: Two persons became ill with vomiting, colic, and diarrhea after consuming a cake with cream filling. This cake was one of twelve prepared at a supermarket bakery; the filling of the cakes with cream filling was done at night. The cakes were stored without refrigeration and sold the following day. The cake was eaten shortly after its purchase.

Outbreak 5: Seven persons became ill with nausea, vomiting, colic, diarrhea and headache 2.5 hours after ingestion of gnocchi. An 82-year old man required hospitalization and died because of the other complications. The gnocchi was prepared by a commercial concern, was cooled at room temperature, and held at room temperature during sale.

Outbreak 6: On January 1, 1992, three members of a family experienced vomiting, diarrhea, and headache 3.5 hours following the ingestion of ice-cream which the family had mixed with yogurt. The ice-cream had been purchased from a commercial outlet and eaten shortly after purchase.

MATERIALS AND METHODS

Food samples - The samples of foods implicated in these outbreaks were obtained from the Secretaria Municipal de Abastecimento do Município de São Paulo, Brazil.

Isolation of staphylococci - Twenty-five or 50 grams of the implicated food were suspended in 225 or 450 ml, of buffered-peptone water; 0.1 ml of 10-1 to 10-6 dilutions was spread on the surface of Baird-Parker agar plates (9). The plates were incubated for 48 hours at 37°C. Ten typical colonies were tested for coagulase and thermonuclease (TNase) production. Coagulase-positive colonies were used for enterotoxin testing.

Growth of staphylococci for enterotoxin detection - Inocula were prepared by incubating the staphylococci isolated from the foods in brain heart infusion (BHI) broth overnight at 37°C. Sac culture flasks were prepared by placing 50 ml of BHI in a dialysis tube and placing it in the bottom of a 250 ml erlenmeyer flasks (5). Two ml of the inoculum was mixed with 20 ml of PBS and placed in the erlenmeyer flasks containing the sac. The flasks were incubated with shaking at 37°C for 18-20 hours. The cultures were removed from the flasks, centrifuged, and the culture supernatant fluid was used for enterotoxin testing.

Enterotoxin testing of strains - The optimum-sensitivity-plate (OSP) method was used (9). In this method 3 ml of agar (1.2% were placed in a 50 mm plastic petri plate with a tight lid: wells were cut according to the original specifications. Specific antisera was placed in the center well, enterotoxin (4 ug/ml) was placed in two smaller wells, and culture supernatant fluids were placed in the four larger outer wells. Different plates were required for each enterotoxin (SEA, SEB, SEC, SED). The plates were placed in a humidified closed container and incubated overnight at 37°C. Positive reactions were determined from precipitin lines formed by the culture supernatant fluids that coalesced with the reference precipitin line.

Enterotoxin testing of foods - Up to 100 g of the implicated food was homogenized in a Waring blender with 1-1.5 ml of fluid/g of food (7). The homogenized food was adjusted to pH 4.5 and centrifuged. The pH of the supernatant fluid was readjusted to pH 7.4 and recentrifuged if any precipitate occured. The extract was tested for the presence of enterotoxin by use of the ELISA ball kit obtained from Dr. Bornelli AG, Stationstrasse 12, CH-3097 Libbefeld-Bern, Switzeland (6). This method has a sensitivity of 0.5 ng/ml and is the reliable most of the sensitive methods

available for checking foods for enterotoxin. One antibody-coated ball for enterotoxins A-D plus two balls coated with normal rabbit sera were placed in 20 ml of food extract and mildly shaken overnight. Each ball was removed from the extract as washed with the wash solution and each placed in a color coded tube for treatment with the conjugate. After 6 hours the conjugate was removed and the balls washed with thw wash solution. One ml of the substrate was added and the color allowed to develop for 60 minutes. If any color developed, the extract was judged to contain enterotoxin.

RESULTS AND DISCUSSION

The staphylococcal counts in all foods examined varied from 3.0 x 10^7 to 9.0 x 10^8 cfu/g (Table 1), and the isolated strains examined by the OSP method were all SEA produces. One strain was also positive for SEB (Table 1). The fact that enterotoxigenic staphylococci were isolated in large numbers from five of the outbreaks was circunstantial evidence of staphylococcal food poisoning. However, by using the ELISA method, all examined foods were shown to be positive for the presence of SEA (Table 1), which confirmed the outbreaks as staphylococcal. Strong positive reactions by the ELISA method indicated relatively large amounts of enterotoxin in the foods. SEA is the leading cause of staphylococcal food poisoning (10) and agrees with the results of strains testing from food poisoning outbreaks in Belo Horizonte in which 12 of 13 outbreaks were due to SEA (4); only one was caused by SEB which is rarely involved in food poisoning.

Four of the outbreaks were due to cream-filled cakes which was one of the leading causes of the outbreaks in Belo Horizonte (4). In the outbreak due to ice cream, no staphylococci were isolated from the ice-cream, but SEA was detected in the ice-cream. The ice-cream was prepared commercially, but no information was available about how it was made. Most likely enterotoxin was produced in the milk or cream used in its making before they were pasteurized. This would have destroyed the staphylococci, but would not have inactivated the enterotoxin (2).

The outbreak from gnocchi was of interest because of its relative severity. All seven persons consuming the gnocchi were made ill; one required hospitalization and subsequently died. This individual was over 80 years of age and suffered from other complications. Although the death was not atributed to the food poisoning; severe vomiting and diarrhea can weaken the individual and result in death from other ailments. It is quite unusual for staphylococcal food poisoning to be fatal, however, it does occur occasionaly in very young children and older individuals suffering from other ailments (3).

The relative large number of staphylococcal food poisoning outbreaks attributed to cream-filled cakes indicates the lack of awareness that this type of food should be refrigerated at all times except when it is being prepared and consumed. Cream-filled bakery goods at one time were a leading cause of this type of food poisoning in the United States (1). Which very seldom occurs now because of the care used in handling of this type of food. It is difficult to avoid the contamination of foods by food handlers, particularly in the home, hence, care should be taken to refrigerate vulnerable foods. Refrigeration is the only satisfactory method for prevention of this type of food poisoning.

TABLE 1 - Staphylococcal food poisoning in São Paulo.

Outbreak	Food Involved	Persons involved (Nº)				*S. aureus*	Enterotoxin	
		At risk	Interwed	Iii	Hosp/death	cfu/g	Strains	Food
1	Cream-filled cake	40[a]	23	16	1/0	$5.3x10^8$	SEA	SEA
2		200[a]	56	33	12/0	$3.0x10^7$	SEA	SEA
3		9	9	9	9/0	$9.0x10^9$	SEA	SEA
4		2	2	2	0/0	$3.0x10^8$	SEA,SEB	SEA
5	Gnocchi	7	7	7	1/1[b]	$3.0x10^8$	SEA	SEA
6	Ice cream	3	3	3	0/0	neg	-	SEA

a- aproximate number.
b- The person who died was over 80 years of age and suffered from other complications.

RESUMO

Surto de intoxicação alimentar por *Staphylococcus* em São Paulo (Brasil)

Foram investigados seis surtos de doenças de origem alimentar ocorridos em São Paulo, Brasil. Os alimentos implicados foram: bolo recheado com creme(4 surtos), nhoque (1 surto) e sorvete (1 surto). Os alimentos foram analisados, quanto à presença de *Staphylococcus aureus* e enterotoxinas estafilocócicas. As contagens de *S. aureus*, nos alimentos, variaram de 10^7 a 10^9 UFC/g, exceto para o sorvete, do qual não se obteve o isolamento da bactéria. Os estafilococos isolados a partir dos alimentos mostraram-se produtores de enterotoxina A e todas as amostras, dos alimentos analisados, continham enterotoxina A, a mais comumente associada à intoxição alimentar estafilocócica.

Palavras chaves: intoxicação alimentar, *Staphylococcus*, enterotoxina.

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REFERENCES

1. Bergdoll, M.S. Staphylococcal intoxications, p. 443-494. In H. Reiman and F.L.Bryan (ed), Foodborne infections and intoxications, Academic Press, Inc., N.Y. 1979.
2. Bergdoll, M.S. *Staphylococcal aureus*. p. 463-523. In. M.P.Doyle (ed), Foodborne bacterial pathogens. Marcel Dekker, Inc., New York, 1989.
3. Bergdoll, M.S. Staphylococcal intoxication in mass feeding. p. 25-47. In A.T.Tu (ed). Food Poisoning, Handbook of Natural Toxins, Vol. 7. Marcel Dekker, Inc., New York, 1992.
4. Carmo, L.S. do, and Bergdoll, M.S. Staphylococcal food poisoning in Belo Horizonte (Brazil). *Rev. Microbiol.*, **21**: 320-323, 1990.
5. Donnelly, C.B., Leslie, J.E., Clack, L.A. and Lewis, K.H. Serological identification of enterotoxigenic staphylococci from cheese. *Appl. Microbiol.*, **15**: 1382-1387, 1967.
6. Fey, H and Pfister, H. A Diagnostic kit for the detection of staphylococcal enterotoxins (SET) A, B, C and D (SEA, SEB, SEC, SED), pg 345-348. In S. Avrameas (ed), Immunoenzymatic techniques. Elsevier/North-Holland Publishing Co., Amsterdam. 1983.
7. Freed, R.C., Everson, M.L., Reiser, R.F. and Bergdoll, M.S. Enzyme-linked immunosorbent assay for detection of staphylococcal enterotoxins in foods. *Appl Environ. Microbiol.*, **44**: 1349-1355, 1982.
8. Robbins, R.; Gould S. and Bergdoll, M.S. Detecting the enterotoxigenicity of *Staphylococcus aureus* strains. *Appl. Microbiol.*, **28**: 946-950, 1974.
9. Tatini, S.R.; Hoover, D.G. and Lachica, R.V.F. Methods for the isolation and enumeration of *Staphylococcus aureus*, p. 411-427. In Compendium of methods for the microbiological examination of foods. 2nd. ed. M.L.Speck (ed). American Public Health Association. Inc., Washington, DC. 1984.

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OCCURRENCE OF THERMOTOLERANT SPECIES OF CAMPYLOBACTER IN THREE GROUPS OF HENS MAINTAINED UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

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SUMMARY

The isolation rate of thermotolerant *Campylobacter* sp. in free-ranging domestic hens, laying hens and specific pathogen-free (SPF) hens was established. *Campylobacter* sp. were isolated in 66.7% of the first type of hens being less frequent (29.4%) in the second group. The lowest isolation rate (7.3%) was observed in SPF hens. All the biovars described for *C. jejuni* and *C. coli* were found in free-ranging hens. In SPF hens only biovars II of both species were isolated. The results show that as the environmental sanitary conditions and management of the hens improved, a significative reduction of the intestinal carriage and biovars distribution was observed.

Key words: *Campylobacter* sp., hens, environmental conditions, isolation rates.

INTRODUCTION

Thermotolerant campylobacters, especially *Campylobacter jejuni* and *C. coli*, are zoonotic bacteria recognized as common causes of diarrhea in humans throughout the world (2,8).

Several mammalian and avian species have been identified as natural reservoirs (2,6) and some foods of animal origin, as well as water and sewage, have been reported as potential vehicles of *Campylobacter* sp. (2,3,20).

Among avian species, hens and chickens are important reservoirs of *Campylobacter* sp. in developed and developing countries, and commercial poultry meat and viscera have been found to be contamined with these bacteria (8,18). In developed countries, chicken meat has been reported as source of various food-related outbreaks (11,18). Despite the isolation of *Campylobacter* sp. from chicken carcasses and retail raw meat (12,17), outbreaks due to its consumption are not well documented in developing countries. However, live birds seem to be important links in the transmission of thermotolerant campylobacters (10,14).

Environmental sanitary conditions have been suggested to be associated to the intestinal carriage in dogs (7,19). This is an epidemiological factor that needs further studies in relation to these and other animals.

In this work, the prevalence of *C. jejuni* and *C. coli* in three groups of hens maintained under different environmental conditions is reported.

MATERIAL AND METHODS

Four hundred forty three hens divided in three groups depending upon their origin and environmental sanitary conditions were studied.

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Group A: consisting of 150 free-ranging domestic hens obtained from families of low socioeconomical level, living in the periurban zone of Valdivia city (140,000 habitants, 39°48' southern latitude, Chile).

Group B: consisting of 143 laying hens, individually caged and fed with commercial prepared food and potable water. All the hens were from the same poultry-farm.

Group C: consisting of 150 specific pathogen-free (SPF) hens (5) maintained and managed under strictly controlled sanitary conditions at the Veterinary Medicine Faculty. A fecal sample from each bird was obtained introducing a sterile cotton swab into the cloaca. All samples were seeded directly onto modified Skirrow agar plates and incubated at 43°C for 48 hours under microaerobic conditions in a GasPak system without catalyst (7).

Suspected colonies were identified morphologically (Gram stain) and biochemically, using the differential tests proposed by Lior (13).

Statistical analysis (z test) was made using computer-assisted EPIINFO program.

RESULTS

In group A, *Campylobacter* sp. was isolated from 100 (66.7%) of the hens studied being *C. jejuni* more frequent (58.8%) than *C. coli* (8.0%).

TABLE 1 - Thermotolerant *campylobacter* species isolated from three groups of hens.

		C. jejuni		*C. coli*		Total	
HENS	(n)	nº	%	nº	%	nº	%
Group A	150	88	58.8	12	8.0	100	66.7*
Group B	143	32	22.4	10	7.0	42	29.4*
Group C	150	9	6.2	2	1.3	11	7.3*

Group A: Free ranging hens; Group B: Laying hens; Group C: SPF hens; (*) $p<0.05$

In group B, the overall isolation rate was lower (29.4%) than that observed in the first group. *C. jejuni* and *C. coli* were isolated in 32 (22.4%) and 10 (7.0%) of the hens respectively.

The lowest isolation rate (7.3%) was observed in group C, where 9 (6.2%) and 2 (1.3%) of the hens harbored *C. jejuni* and *C. coli* respectively.

The differences observed among the isolation rates obtained from the three groups of hens studied are statistically significant ($p<0.05$; Table 1).

All the biovars described for *C. jejuni* and *C. coli* were found in group A hens, being biovar I (34.0%) and II of *C. jejuni* the most frequent (Table 2). Biovar of *C. coli* was the most frequent (9.0%) in this group of hens.

In group B, three of the four biovars described for *C. jejuni* were found. Biovar I and II of *C. jejuni* and biovar I of *C. coli* were also the most frequent. Only biovar II from both, *C. jejuni* and *C. coli*, were isolated from hens of group C.

DISCUSSION

Thermotolerant species of *Campylobacter* can live a wide range of animals, being especially common in wild and domestic birds (2,6,8). From the latter group, hens and chickens have been frequently associated as infection sources for human beings (3,18).

We have studied three groups of hens mantained under different environmental conditions for the presence of thermotolerant species of *Campylobacter*. In group A, consisting of free-ranging domestic hens, we found that 66.7% of the birds harbored *Campylobacter*. This isolation rate agrees with that reported previously by us (60.0%) and by Grados *et alii*. (61.4%) for hens mantained under similar conditions (6,10). In group A we isolated all the biovars described by Lior (16) for *C. jejuni* and *C. coli*, being biovars I and II of both species the most frequent.

TABLE 2 - Distribution of *Campylobacter jejuni* and *C. coli* biovars isolated from three groups of hens

Strains Isolated From (n)	Biovar Distribution.					
	C. jejuni				*C. Coli*	
	I	II	III	IV	I	II
Group A (100)	34 (33.4)*	37 (37.0)	8 (8.0)	9 (9.0)*	9 (9.0)	3 (3.0)
Group B (42)	19 (45.2)	12 (28.6)	1 (2.4)	0 (0)	6 (14.3)	6 (14.3)
Group C (11)	0 (0)	9 (81.8)	0 (0)	0 (0)	0 (0)	2 (18.2)

* (%)

In group B, composed by laying hens, the frequency of *Campylobacter* was 29.4% and very near to the reported by Doyle (25.2%) for the same type of hens (4).

The lowest isolation rate was found among SPF hens (group C), where only biovars II of *C. jejuni* and *C. coli* were isolated.

The results obtained in the three groups of hens show that there is a strong realtionship between the sanitary environmental conditions where the birds are maintained and the isolation rate and biovars distribution found in each group under study. As sanitary conditions and management of the hens improved, a significative reduction of the intestinal carriage and biovars distribution was observed.

Free-ranging hens without veterinary control or sanitary management of their environment (group A), could have more opportunities to become infected with *Campylobater* sp. than the other two types of hens. The high prevalence of birds which are intestinal carriers, together with the wide distribution of *C. jejuni* and *C. coli* biovars among them, as well as our previous findings in surface water (8), in dogs feces deposited in the streets (9) and in wild and domestic animals (6) suggest the existence of a highly environment. Grados *et alii* (10) and Marquis *et alii* (14) demonstrated that hens and chickens mantained in close association with humans represent a significant potential source of *Campylobacter* infection. The frequency of human diarrhea due to *Campylobacter* in our city is 9.2% (8) and most of the cases treated at the County Hospital come from the peri-urban region where the hens fecal samples (group A) were taken. Biovars I and II from *C. jejuni* and *C. coli* were found most frequently among hens and they were, also, the most prevalent among diarrheic children.

Groups B and C, maintained under veterinary control, were individually caged with wire floor, conditions that prevent coprophagy and cross infection. On the other hand, they were fed with commercial poultry rations and potable water. Both conditions are low risk factors in *Campylobacter* dissemination (16,18).

The overall results obtained in this work suggest the contributory role of environmental sanitary conditions in the transmission of *Campylobacter* sp. to hens. Similar findings have been observed in developing countries among children living in urban slums (1,15) and among stray and pet dogs (7).

RESUMO

Ocorrência de espécies termotolerantes de campylobacter em três grupos de galinhas mantidas sob diferentes condições ambientais

Foi estabelecida a freqüência de isolamento de *Campylobacter* sp. em galinhas domésticas, galinhas poedeiras e galinhas livres de patógenos específicos. (LPE). *Campylobacter* sp. foi isolado em 66,7% e 29,4% das aves do primeiro e do segundo grupo, respectivamente. A menor freqüência (7,3%) foi observada nas galinhas LPE. Todos os biovar descritos para *C. jejuni* e *C. coli* foram encontrados nas galinhas domésticas. Nas galinhas LPE foi isolado o biovar II de ambas as espécies. Os resultados demonstram que, na medida que as condições higiênicas e sanitárias melhoram, observa-se significativa redução da freqüência de isolamento e da distribuição dos diferentes biovars de Campylobacter sp.

Palavras-chave: *Campylobacter* sp., galinhas, condições ambientais, freqüência de isolamento.

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REFERENCES

1. Araya, M.; Figueroa, G.; Espinoza, J.; Zaror, X. & Brunser, O. Acute diarrhoea and asymptomatic infection in Chilean preschoolers of low and high socioeconomic strata. *Acta Paediatr. Scand.*, **75**: 645-651, 1986.
2. Blaser, M.J.; Taylor, D.N. & Feldman, R.A. Epidemiology of *Campylobacter jejuni* infections. *Epidemiol. Rev.*, **5**: 157-156, 1983.
3. Butzler, J.P. & Oosterom, J. *Campylobacter*: pathogenicity and significance in foods. *Int. J. Food Microbiol.*, **12**: 1-8, 1991.
4. Doyle, M.P. Association of *Campylobacter jejuni* with laying hens and eggs. *Appl. Environ. Mirobiol.*, **47**: 533-536, 1984.
5. Dufty, J.H. Specific pathogen-free and gnotobiotic farm animals. *Austr. Vet. J.*, **52**: 355-361, 1976.
6. Fernández, H. Species and biotypes distribution of thermotolerant campylobacter in southern Chile. *Rev. Inst. Med. Trop.* (São Paulo), **30**: 357-360, 1988.
7. Fernández, H. *Campylobacter* intestinal carriage among stray and pet dogs. *Rev. Saúde Publ.* (São Paulo), **25**: 473-475, 1991.

8. Fernández, H. Thermotolerant *Campylobacter* species associated with human diarrhea in Latin America. *J. Braz. Ass. Adv. Sci.*, **44**: 39-43, 1992.
9. Fernández, H. & Arnés, V. Occurence of *Campylobacter jejuni* in dog feces from the streets of a southern Chilean city. *Rev. Inst. Med. Trop.* (São Paulo), **28**: 410-412, 1986.
10. Grados, O.; Bravo, N.; Black, R.E. & Butzler, J.P. Paediatric *Campylobacter* diarrhea from household exposure to live chicken in Lima, Peru. *Bull. WHO.*, **66**: 369-374, 1988.
11. Harris, N.V.; Weiss, N.S. & Nolan, C.N. The role of poultry and meats in the etiology of *Campylobacter jejuni/coli* enteritis. *Am. J. Publ. Hlth.*, **76**: 407-411, 1986.
12. Levi, A. & Ricciardi, I.D. *Campylobacter fetus* subsp. *jejuni (C. jejuni)*: identification of strains isolated from chickens in Rio de Janeiro. *Rev. Microbiol.* (São Paulo), **13**: 332-334, 1982.
13. Lior, H. New, extended byotiping scheme for *Campylobacter jejuni, Campylobacter coli* and *Campylobacter laridis*. *J. Clin. Microbiol.*, **20**: 636-640, 1984.
14. Marquis, G.S.; Ventura, G.; Gilman, R.H.; Porras, E.; Miranda, E.; Carbajal, L. & Pentafiel, M. Fecal contamination of shanty town toddlers in households with non-corraled poultry, Lima, Peru. *Am. J. Publ. Hlth.*, **80**: 146-149, 1990.
15. Molbak, K.; Hojlyng, N. & Gaarslev, K. High prevalence of *Campylobacter* excretors among Liberian children related to environmental conditions. *Epidemiol. Infect.*, **100**: 227-237, 1988.
16. Montrose, M.S.; Shane, S.M. & Harrington, K.S. Role of litter in the transmission of *Campylobacter jejuni*. *Av. Dis.*, **29**: 392-399, 1984.
17. Sakuma, H.; Franco, B.D.G.M. & Fernández, H. Occurrence of *Campylobacter jejuni* and *Campylobacter coli* in retail raw chicken meat and giblets in São Paulo, Brazil. *Rev. Microbiol.* (São Paulo), **23**: 13-16, 1992.
18. Shane, S.M. The significance of *Campylobacter jejuni* infection in poultry: a review. *Av. Pathol.*, **21**: 189-213, 1992.
19. Simpson, J.W.; Burnie, A.G.; Ferguson, S. & Telfer, W.A.B. Isolation of thermophilic *campylobacters* from two populations of dogs. *Vet. Res. Comm.*, **5**: 63-66, 1981.
20. Weber, G.; Manafi, M. & Reisinger, H. Importance of *Yersinia enterocolitica* and thermophilic campylobacters in water hygiene. *Zbl. Bakt. Hyg. B.*, **184**: 501-514, 1987.

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PRODUCTION OF MICROBIAL ALKALINE CELLULASE AND STUDIES OF THEIR CHARACTERISTICS

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SUMMARY

In order to obtain cellulases that improve the detergency of laundry detergent products, two alkalophilic microorganims, *Bacillus sp* B38-2 and *Streptomyces sp* S36-2, were isolated from soil and compost by incubating samples in enrichment culture medium containing CMC and Na_2CO_3 at pH9.6. It was found that they secrete a constitutive extracellular alkaline carboxymethyl cellulase (CMCase) in high quantity. The maximum enzyme activity was observed between 48hr to 72 hr at 30°C for the *Streptomyces* and between 72hr to 96hr at 35°C for the *Bacillus*. The optimum pH and temperature of the crude enzyme activities ranged from 6.0 to 7.0 at 55°C for the *Streptomyces* and 7.0 to 8.0 at 60°C for the *Bacillus*. Two crude CMCases activities were termostable at 45°C for 1hr and the both crude enzyme activities of the *Bacillus* as of the *Streptomyces* were stable at pH 5.0 to 9.0 after pH treatments in various buffer solutions at 30°C for 24hr.

Key words: alkaline cellulases, alkalophilic microorganisms, laundry detergent, alkalophilic *Bacillus*, alkalophilic *Streptomyces*.

INTRODUCTION

The cellulolytic complex enzymes are widely distributed in nature by microorganisms which decompose lignocellulosic materials. Most of the cellulases studied so far, as well as those commercially available, are of mold origin and are related to the exploitation of renewable biomass or more recently to the use in the pulp and cellulose industry. Nevertheless, these enzymes indicate pH optima in the acidic or neutral range.

Recently, a new application of cellulolytic enzymes has been developing firmly due to its use to improve household detergent efficacy (3). The use of proteolytic and amylolytic enzymes in the formulation of detergents began about 60 years ago (6, 10), however it regained importance because of environmental considerations, which led to a reduction or total elimination of phosphate in the conventional formulation of detergents.

To be used in laundry detergents the cellulases must fulfill essential requirements which make

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them compatible with the new medium: a) they have to be in the alkaline pH range; b) they have to prove alkali-stable; c) they have to prove termostable at 50°C; d) they have to prove non-dependent activity to calcium and magnesium, and, e) they must not be inhibited by chemical surfactant, chelating, or structural agents (10).

Thus, we have analysed microorganisms from soils, composts and dejets and selected two isolated microorganisms, the alkalophilic *Bacillus* sp called B38-2 and the alkalophilic *Streptomyces* sp called S36-2, both producing carboxymethyl cellulase (CMCases; endo-β-1,4-glucanase, EC 3.2.1.4) that fulfills the essential requirements for use in laundry detergents.

In this paper, we described the isolation of these microorganisms and some properties of the CMCases enzymes, produced by them.

MATERIALS AND METHODS

Culture media: The screening medium, M-1, was composed by the following reagents given in g per 100ml of distilled H_2O: CMC (7 H35F; Hercules Co), 1.0; meat extract (Difco), 1.0; Bacto-peptone (Difco), 1.0; NaCl, 1.0; KH_2PO_4, 0.1; Na_2CO_3 (Merck) 0.5 and agar-agar (Difco), 1.5. Sodium carbonate was sterilized separately (121°C/15 min) and added to the medium. The initial pH was 9.6.

The production medium, M-2, was composed by the following reagents given in g per 100ml of distilled water:CMC (7 H35F, Hercules Co), 1.0; Bacto yeast extract (Difco), 0.5; meat extract (Difco), 1.5; KH_2PO_4, 0.1 and Na_2CO_3 (Merck), 0.5. Sodium carbonate was sterilized separately (121°C/15 min) and added to the medium. The initial pH was 9.6.

Pre-selection of alkaline cellulase-producing microorganisms: The soil samples (about 0.5g) were suspended in 10 ml of saline solution (NaCL 0.85%) an then treated at 80°C for 30 min. The thermally treated suspensions were suitably diluted and inoculated on the M-1 medium surfaces in dishes. Then, the dishes were incubated at 30C for 3 days. The isolated colonies, developed in the dishes, were transferred to tubes with slant medium M-1 and then stored for extracellular enzyme production test.

Selection of extracellular alkaline CMCase-producing strains: One hook of each strain isolated from the M-1 medium was inoculated in 20ml of the M-2 production medium into 125ml Erlenmeyer flasks and incubated at 30°C under 150 rpm during 48hr shaking. After the fermentation period, the media were centrifugated at 6000g per 10 min and supernatants were used to determine the alkaline CMCase activity.

Crude enzyme preparation: The selected strains were inoculated in several 250ml Erlenmeyer flasks containing 50 ml of the production medium. The *Streptomyes sp* S36-2 was incubated at 30°C for 48hr, while the *Bacillus sp* B38-2 was incubated at 35°C for 72hr; both were shaked at 150rpm.

At the end of the respectivc incubation times, the supernatants of each fermented broth, obtained by centrifugation, were pooled and used to study CMCase enzyme properties referring to optima pH and temperature, pH stability and thermal stability, besides resistency to the structural agent of detergents (sodium tripolyphosphate). The crude enzyme storage at 5°C for 2 weeks or its freezing did not reduce the CMCase activity.

Determination of the CMCase activity: The reaction mixture was o.1ml of crude enzyme extract and 0.9 ml of CMC 1% solution (7 h35F, Hercules) at pH 6.5 (*Streptomyces sp* S36-2) and at Ph 8.0 (*Bacillus* sp S38-2), both with 0.3M McIlvaine Buffer.

After the incubation at 50°C for 30 min, the reducing sugar was determined by the 3,5-dinitrosalicylic acid method as Miller (7).

One unit (U) of each enzyme activity was defined as the amount of enzyme which produced 1.0 μ mol of reducing sugar, as glucose, per min under the above conditions.

Time courses for cell growth and production of CMCase enzymes: Cultivations were carried out at 30°C (S36-2) and at 35°C (B38-2) for 4 days with shaking. Samples were withdrawn at time intervals and enzyme activities were determined in supernatants of the culture medium under standard assay described above. For the cellular growth, the cells were harvested by centrifugation at 6000g for 10 min, washed twice with water and suspended in 20 ml of distilled water. The degree of growth was estimated by measuring the absorbance of the cell suspension in water at 660 nm employing a Coleman-290 colorimeter. The pH determination of the fermented medium was estimated potentiometrically by use of pHmeter (Digimed).

Effect of pH on the activity CMCase: The enzyme activities were determined in supernatants of the culture medium (100 μL) incubated for 10 minutes with 0.9 ml of buffer solutions of various

pH containing CMC (1%). The different buffers were McIlvaine's 0.3 M, pH 2.5 - 8.0 and Gly-NaOH 0.3 M, ph 8.0 - 10.5.

Effect of temperature on the activity CMCases: Enzyme activities were determined in supernatants of the culture medium (100 µL) mixed with 0.9 ml buffer solutions containing CMC (1%) and incubated at various temperatures by using McIlvaine buffer 0.3 pH 6.5 (S36-2) and McIlvaine's buffer 0.3 M pH 8.0 (B38-2).

pH stability CMCases: 50 µL of the supernatants of the culture medium (0.6 U/ml) were mixed with 200 L of the buffer solutions at various pH, incubated for 24hr at 30°C. The remainig activities were assayed at pH 6.5 (S36-2) and pH 8.0 (B38-2).

Thermal stability CMCases: The supernatants of the culture medium (0.6 U/ml) were incubated (without substrate) at various temperatures for 1 hr. The residual activities were assayed at pH 6.5 and 55°C (S36-2) and pH 8.0 and 60°C (B38-2).

RESULTS

Selection of microorganisms: From 447 soil samples colected in the field, two alkalophilic microorganisms produced a remarkable quantity of alkaline CMCase. They were classified until the genus according to the Bergey's Manual of Determinative Bacteriology (9) as *Bacillus* sp and *Streptomyces* sp and called, respectively *Bacillus* sp B38-2 and *Streptomyces* sp S36-2.

Time courses for cellular growth and production of alkaline CMCase activity: Both strains of the *Bacillus* sp B38-2 and of *Streptomyces* sp S36-2 were grown by continuous shaking in the M-2 medium in s shaker box at 30°C and 35°C for 96hr. Both grew at these temperatures but the strain of *Streptomyces* sp S36-2 was better suited at 30°C, regarding both cellular growth and enzyme production level. The strain of *Bacillus* sp B38-2 was more adapted to a 35°C temperature. The results are shown in Fig. 1.

The two strains did not register any medium pH change during the fermentation process. Frequently *Bacillus* sp increases medium pH when sporules; This did not occur probably due to the fact that initial pH was already highly alkaline (pH 9.6). Both microorganisms showed an enzyme production associated with cellular growth. *Streptomyces* sp S36-2 reached its maximum cellular growth as well as maximum enzyme activity after 48 hours of growth. *Bacillus* sp B38-2 reached its maximum cellular growth and enzyme activity with 72 hours of growth.

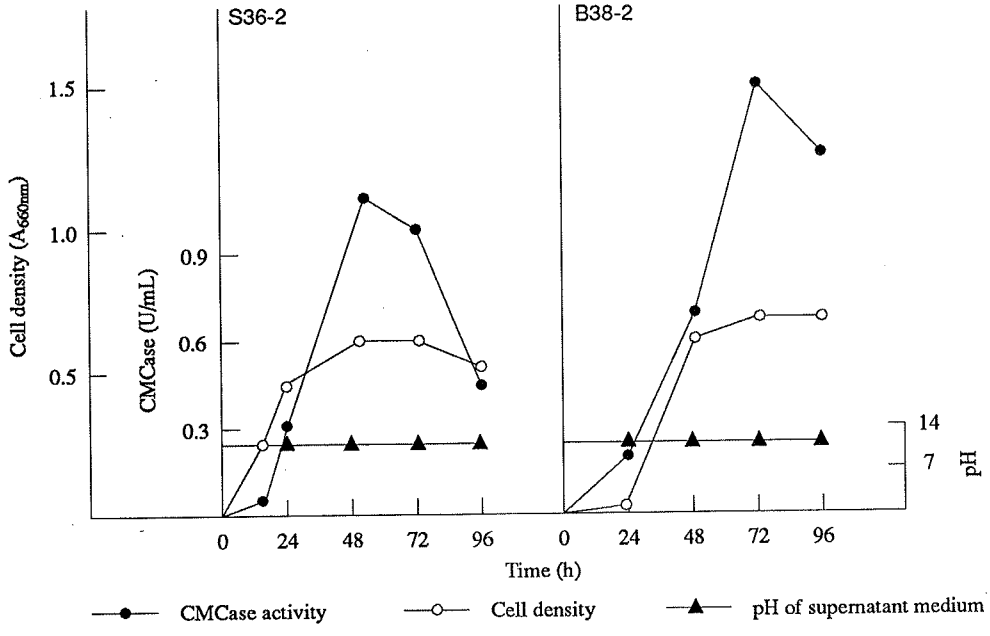
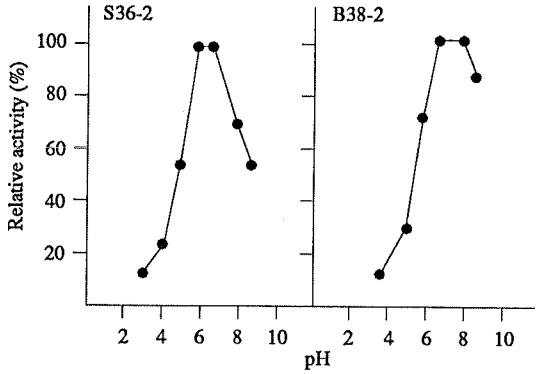


FIGURE 1 - Time course production of CMCase enzymes by *Streptomyces* sp S36-2 and *Bacillus* sp B38-2 grown in M-2 medium.

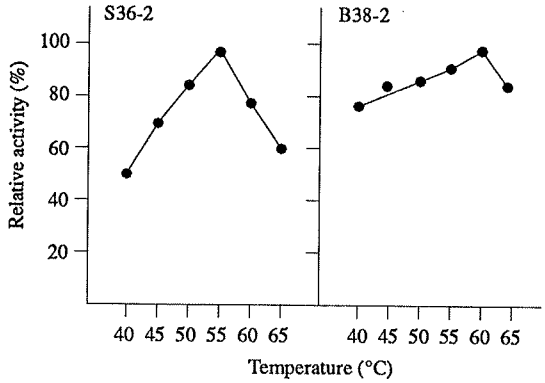
Effect of pH on the CMCases activities: The effect of pH on the CMCases activities of the crude enzyme extracts, S36-2 and B38-2, was determined in buffer solutions of various pH containing CMC substrate, as shown in Fig. 2. The maximum activity for the S36-2 was observed at pH 6.0 to 7.0, a typical CMCase neutral activity; at pH 9.0 the enzyme showed about 60% of the maximum activity. It was observed that the B38-2 strain registered maximum activity between pH 7.0 and 8.0, typical of alkaline enzyme. At pH 9.0 the enzyme showed about 90% of the maximum activity.



The different buffers were McIlvaine's 0.3M pH 2.5-8.0; Gly-NaOH buffer 0.3M, pH 8.0-10.5.

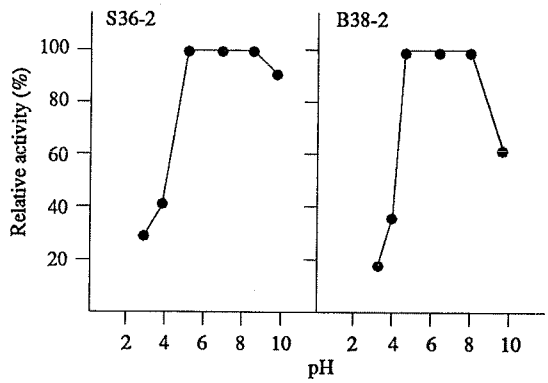
FIGURE 2 - Effect of pH on the activity of *Streptomyces* sp S36-2 and *Bacillus* sp B38-2 CMCases.

Effect of temperature on the crude CMCase activity: The optimum temperature of the CMCase activity for both isolated microorganisms was determined by varying incubation temperature of the enzyme-substrate reaction. As shown in Fig. 3, the optimum temperature of the CMCase activity for the S36-2 was at 55°C; the enzyme showed about 50% to 60% of maximum activity at 40°C and 65°C, respectively. The CMCase enzyme of the B38-2 was very active in a wide temperature range, being the optimum temperature at 60°C; it still showed about 75% to 90% of maximum activity at 40°C and 65°C, respetcively. These results are higher than those observed for CMCases of other alkalophilic *Bacillus* strains (8).



Both activities were assayed in McIlvaine's Bufffer 0.3M, pH 6.5 (S36-2) and pH 8.0 (B38-2).

FIGURE 3 - Effect of temperature on the activity of *Streptomyces* sp S36-2 and *Bacillus* sp B38-2 CMCases.



pH 2.5-8.0, McIlvaine's Buffer 0.3M; pH 8.0-10.5, Gly-NaOH 0.3M.

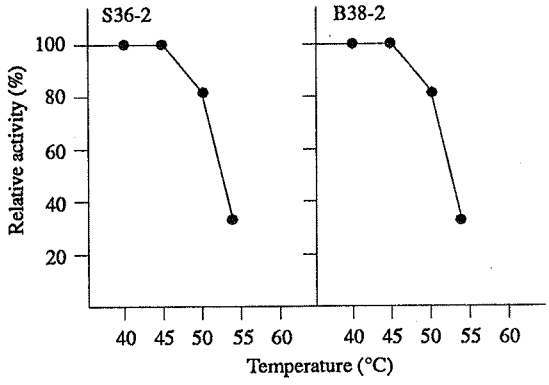
FIGURE 4 - pH stability of *Streptomyces* sp S36-2 and *Bacillus* sp N38-2 CMCases.

Effect of pH on the crude enzyme stability: The CMCase residual activities were measured at pH 6.5 and 8.0, respectively for the S36-2 and B38-2 strains, after the incubation of enzymes at 30°C for 24hr in buffer solutions at various pH without substrate. As shown in Fig. 4, the enzyme activities of both strains were totally unchangeable over broad pH ranges between 5.0 and 9.0.

Thermal stability of the CMCase activity: The thermal stability of the two enzymes were examined by incubating them without substrate at various temperatures for 1hr. The residual activity was measured under standard assay conditions.

As shown in Fig. 5, both enzymes showed practically the same characteristics in respect to thermal stability, maintaining 100% stable until 45°C; 30% of the original activity was retained after heat treatment at 55°C.

Effect of sodium tripolyphosphate on the enzyme activities: One of the most important characteristics of enzymes used in laundry detergents is their compatibility with surfactant agents



The residual activities were assayed at pH 6.5 and 55°C (S36-2) and pH 8.0 and 60°C (B38-2).

FIGURE 5 - Thermal stability of *Streptomyces* sp S36-2 and *Bacillus* sp B38-2 CMCases.

and sequestrant builders; among these, sodium tripolyphosphate is the most largely used one. Thus, the CMCase activity was analysed in the presence of 1% of sodium tripolyphosphate in the enzyme reaction mixture. The results obtained (not shown) indicated that the enzyme activity was not affected at all by the reagent.

DISCUSSION

There are, basically, two reasons for the application of alkaline enzymes, like proteinases, amylases, lipases (6, 10) and cellulases (3, 8) in the formulation of detergents. The first one is their capacity to improve the efficacy of releasing organic substances like proteins, starch, grease, and celluloses associated to dirty and stains of washable products.

On the other hand, the second reason is related to a change on the detergent composition. In some developed countries (USA, Japan, Germany,...) preoccupation towards environmental pollution has taken to a systematic reduction of phosphate level on the formulation of laundry detergents. This is occurring as much due to the producer's efforts as to specific legislation (6). In refer to this case, the presence of enzymes is important for compensating phosphate reduction, and keeping unchangeable the performance of detergents (6).

Alkaline cellulases have been reported by other researchers. HORIKOSHI et al (2) isolated a *Bacillus* sp strain N.4, similar to *Bacillus pasteurii*, and which grew in a high pH range. The crude CMCase strongly hydrolysed CMC and after purification it showed two components with alkaline CMCase activity and optimum pH at 10.0. FUKUMORI et al (1) isolated a strain of alkalophilic *Bacillus* sp N.1139 (similar to *B. firmus*) which produced inducible alkaline CMCase. KITADA et al (5) isolated a strain of alkalophilic and thermophilic *Bacillus* (IC), which grew well in alkaline medium at 55°C and was identified as *B. licherniforms*; the authors did not assert whether there was alkaline cellulase activity or not. KAWAI et al (4) reported an alkaline CMCase from a neutrophilic *Bacillus* sp KSM-522 strain, which is similar to *B. pumillus*. The partially purified enzyme was active between pH 7.0-10.0, being the optimum temperature at 50°C. ITO et al (3) described the isolation of alkalophilic *Bacillus* sp KSM-635, which produced CMCase considerably. The optimum activity was at pH 9.5 and optimum temperature at 40°C. The authors are the first to use this enzyme in the formulation of household detergents. The same group, SHIKATA (8), reports, in a further paper, the isolation of three strains of alkalophilic *Bacillus*, KSM-19, KSM-69 and KSM-520, which produced stable and suitable alkaline cellulases used as components to improve efficacy of laundry detergents. YOSHIMATSU (11) described the complete purification and characterization processes of two alkaline CMCases (E-H and E-L), produced by a strain of alkalophilic *Bacillus* sp KSM-635.

In the present paper, there was a successful isolation of two strains of alkalophilic microorganisms which produced a remarkable quantity of CMCases, S36-2 and B38-2. The microorganisms were temporarily classified as *Streptomyces* sp S36-2 and *Bacillus* sp B38-2. The CMCase S36-2 showed maximum neutral activity at range of pH 6.5 to 7.0 and optimum temperature at 55°C. The enzyme kept stable at pH 9.5 for 24hr; it also resisted to thermal treatment at 45°C for 1hr. The CMCase B38-2 showed maximum alkaline activity at optimum pH 8.0 and optimum temperature at 60°C. The enzyme kept stable at pH 9.5 for 24hr; it still maintained active for 1hr at 45°C. Both enzymes were active at pH range usually found in detergents (pH 9.0); at this range the enzymes showed 55% and 87% for maximum activity, respectively, for S36-2 and B38-2.

These preliminary tests of crude enzyme characterization assume that the enzymes are suitable to be used as components in detergents. B38-2 was considered the most suitable to be used for this purpose.

ACKNOWLEDGEMENTS

We wish to thank Alvaro Hattnher and Maurílio José Netto for language assistance.

RESUMO

Produção de celulase alcalina microbiana e estudos de suas características

Com o objetivo de obter celulases que melhorassem a eficiência de produtos detergentes, isolou-se , a partir de solos e compostagens, dois microorganismos, *Bacillus* sp B38-2 e *Streptomyces* sp S36-2, através de incubação de amostras em meio de cultura enriquecido, contendo CMC e Na_2CO_3 com pH 9.6. Verificou-se que eles secre–tavam uma carboximetil celulase (CMCase) alcalina constitutiva, extracelular, em quantidade. A atividade enzimática máxima foi observada entre 48h e 72h a 30°C para o *Streptomyces* e entre 72h e 96h a 35°C para o *Bacillus*. O pH ótimo e a temperatura ótima das atividades enzimáticas brutas estiveram situadas entre os pH 6.0 e 7.0 a 55°C para o *Streptomyces* e entre os pH 7.0 e 8.0 a 60°C para o *Bacillus*. As duas atividades CMCases brutas permaneceram termoestáveis a 45°C durante 1hr e ambas as atividades enzimáticas brutas, tanto do *Baccillus* como do *Streptomyces*, ficaram estáveis na faixa de pH 5.0-9.0 após tratamentos em várias soluções-tampão de diferentes valores de pH a 30°C durante 24h.

Palavras-chave: celulases alcalinas, microorganismos alcalofílicos, detergentes, *Bacillus* alcalofílico, *Streptomyces* alcalofílico.

REFERENCES

1. FUKUMORI, F.; KUDO, T. & HORIKOSHI, K. - Molecular cloning and nucleotide sequence of the alkaline cellulase gene from the alkalophilic *Bacillus* sp strain 1139. *J. Gen. Microbiol.* **132**: 2329-2335, 1986.
2. HORIKOSKI, K.; NAKAD, M.; KURONO, Y. & SASHIHARA, N. - Cellulases of an alkalophilic *Bacillus* strain isolated from soil. *Can. J. Microbiol.* **30**: 774-779, 1984.
3. ITO, S.; SHIKATA, S.; OZAKI, K.; KAWAI, S.; OKAMOTO, K. INOUE, S.; TAKEI, A.; OHTA, Y. & SATOH, T. - Alkaline cellulase for laundry detergents: Production by *Bacillus* sp KSM-635 and enzymatic properties. *Agric. Biol. Chem.* **53**: 1257-1281, 1989.
4. KAWAI, S.; OKOSHI, H.; OZAKI, K.; SHIKATA, S.; AKA, K. & ITO, S. - Neutrophilic *Bacillus* strain, KSM-522, that produces an alkaline carboxymetil cellulase. *Agric. Biol. Chem.* **52**: 1425-1431, 1988.
5. KITADA, M.; WIJAYANTI, L. & HORIKOSHI, K. - Biochemical properties of a thermophilic alkalophile. *Agric. Biol. Chem.* **51**: 2429-2435, 1987.
6. MAASE, F.W.J.L. & TILBURG, R. Van - The benefit of detergent enzymes under changing washing conditions. *J. Am. Oil Chem.* **60**: 1672-1675, 1983.
7. MILLER, G.L. - Use of Dinitrossalicylic acid reagent for determination of reducing sugars. *Anal. Chem.* **31**: 426-428, 1959.
8. SHIKATA, S.; SAEKI, K.; OKOSHI, H.; YOSHIMATSU, T.; OZAKI, K.; KAWAI, S. & ITO, S. - Alkaline cellulases for laundry detergents: Production by alkalophilic strains of *Bacillus* and some properties of the crude enzymes. *Agric. Biol. Chem.* **54**: 91-96, 1990.
9. SNEATH, P.H.A., in "Bergey's Manual of Systematic Bacteriology", vol. 2, ed. by SNEATH, P.H.A.; MAIN, N.S.; SHARPE, M.E. & HOLT, J.G. - The Williams and Wilkins Company, Baltimore, USA, 1986.
10. STARACE, C.A. - Detergent enzymes - Past, Present and Future. *J. Am. Oil Chem. Soc.* **60**: 1025-1027, 1983.
11. YOSHIMATSU, T.; OSAKI, K.; SHIIKATA, S.; OHTA, Y.; KOIKE, K.; KAWAI, S. & ITO, S. - Purification and characterization of alkaline endo-1,4-beta-glucanase from alkalophilic *Bacillus* sp KSM-635. *J. Gen. Microbiol.* **136**: 1973-1979, 1990.

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A NEW MEDIUM FOR GROUP B STREPTOCOCCAL ENRICHMENT THAT CAN DETECT THE BACTERIA IN HEAVILY COLONIZED WOMEN

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SUMMARY

We modified a recently described medium, LAL-1 (14) by the addition of 15 µg/ml of nalidixic acid. The LAL-2 allowed the detection of GBS isolated from heavily colonized women. The medium was very efficient for GBS enrichment since the detection of GBS increased in approximately 58%.

Key Words: Streptococci, *Streptococcus agalactiae*, GBS

INTRODUCTION

Group B streptococci (GBS) were initially known as an important cause of bovine mastitis (10). However, since the '60s, GBS have been recognized as a major cause of meningitis and septicemia among neonates (12, 7). Epidemiological data showed that between 5% and 30% of women carried GBS at the time of delivery, and their newborns were frequently contaminated during the birth (1) or even during their intrauterine lives (6). The contamination can lead to a precocious disease, where septicemia or pneumonia is a primary symptom (2). Although all GBS serotypes can produce the disease, type III is the foremost, common agent (6).

The aim of this study was [1] to verify the utility of the LAL-1 medium (14) modified by the addition of 15 µg/ml of nalidixic acid (LAL-2) for detection of GBS, directly from clinical materials, in heavily colonized women and, [2] to assess the GBS serotypes obtained from pregnant and non-pregnant women belonging to different groups of age, in Rio de Janeiro, Brazil.

Duplicate vaginal swabs were obtained from 184 patients. Ninety four out of these clinical materials were collected from non-pregnant women who attended gynecologic clinics of the Hospital Universitário Clementino Fraga Filho. Ninety out of 184 swabs were obtained from pregnant women from the Instituto de Pediatria e Puericultura Martagão Gesteira. All clinical materials were obtained during the period of June/1988 to December/1990. One swab was streaked by a semiquantitative method on Tryptose Blood agar plate with 5% of defibrinated sheep blood (TBA; Difco Laboratories), to assess the density of GBS

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colonization (15). The material that presented GBS colonies limited on primary zones of inoculation was classified as 1+; and 2+, 3+ and 4+ for the ones that presented GBS colonies on second, third and fourth streaks of the plates, respectively. The plates were incubated for 18 h at 37°C (15).

Beta-hemolytic colonies were inoculated in 50 ml of Tood Hewitt broth. After 18 h of incubation at 37°C, the streptococci isolated were grouped and typed by serological methods (3,5,8). The other swab was cut and introduced into 1ml of LAL-1 (14) with 15 µg/ml of nalidixic acid (LAL-2). The nalidixic acid was added to LAL-1 to inhibit the growth of Gram negative bacteria, that frequently colonize the vagina. After vigorous agitation, the tubes were incubated at 37°C. The readings were carried out each hour during 5 h. The cultures that showed a shift in the medium color (from purple to yellowish green), within 5-hour incubation, and cointained Gram positive cocci, were recorded as positive for presumptive GBS identification. After 5 h, aliquots from each tubes (positive or negative for GBS) were subcultured on TBA and the plates were incubated for 18 h at 37°C. Beta-hemolytic colonies were serogrouped and serotyped.

After enrichment in LAL-2, 27 β-hemolytic streptococci were isolated from 184 clinical materials analyzed. Twenty six of 27 (96.3%) streptococci were grouped as GBS and 1 as group G streptococcus. Only 15 (57.7%) GBS were isolated when the enrichment in LAL-2 was not used. Using LAL-2 in association with the Gram staining we presumptively identified 5GBS (19.2%) within 5 h of incubation. Among the total GBS detected by LAL-2, 4 (15.4%) were obtained from heavily colonized patients (nore than 2+) and the majority (84.6%) were isolated from women slightly colonized (1+; table 1). GBS carrying different capsular serotype were isolated, exception for serotype IV. Most of the strains carried antigen II (34.6%), although, among pregnant women, GBS type III (30%) were also prevalent. GBS were isolated from all groups of age studied (Table 2). Similar results were obtained previously in our laboratory using a different medium for GBS enrichment (3).

LAL-1 medium was described previously as a medium for rapid presumptive detection of GBS. The sensitivity and specificity of LAL-1 were respectively 96% and 99% (14). The addition of nalidixic acid (15 µg/ml) did not cause any alteration in these values and prevented the growth of Gram negative bacteria (data not shown). The parallel use of Gram staining and LAL-2 was necessary to sort out false-positive results. Indeed, it was imperative that the incubation of the cultures did not extend over 5 h due to the increase of false-positive reactions (14). The medium was very efficient for GBS enrichment since the detection of GBS increased in approximately 58% (table 1).

Even though the LAL-2 medium did not allow for presumptive identification of all GBS isolated, directly from clinical materials, its use is

TABLE 1 - Comparison between the density of GBS colonization and detection of GBS under different conditions.

Group of women Studied	Density of GBS (*)	Total of GBS isolates detected		
		LA -2 (**)	TBA (***)	Enrichment (****)
Non-pregnant	1+	1	6	13
	3+	2	2	2
Total		3	8	15
Pregnant	1+		5	9
	2+	1	1	1
	4+	1	1	1
Total		2	7	11

(*) Density of colonization was defined in text.
(**) The swabs were inoculated in LAL-2. Within 5h of incubation a shift in the color of the broth, from purple to yellowish green, and the presence of Gram positive cocci after were recorded as positive for presumpitive identification of GBS. See text for details.
(***) The clinical material was streaked onto Tryptose Blood agar plates with 5% of defibrinated sheep blood and incubated during 18h at 37°C; ß-hemolitic streptococci were then isolated, grouped and typed.
(****) After 5h of incubation in the LAL-2 broth at 37°C the culture was inoculated onto a TBA plate during 18h. See text for details.

TABLE 2- distribution of GBS serotypes isolated from women in Rio de Janeiro.

Age group	Pregnancy	Total of women studied(*)	Serotypes						
			Ia	Ib	II	III	IV	V	NT (**)
0 - 10	No	4							
11 - 20	No	18				1			1
	Yes	19			2	1			
21 - 23	No	10							
	Yes	57		1	2	1		2	
31 - 40	No	21	1		2				3
	Yes	11	1						
41 - 50	No	22			1			1	
	Yes	3				1			
51 - 60	No	12	1		1				
61 - 70	No	7			1			1	1

(*) Swabs from vagina of pregnant and non-pregnant women.
(**) NT, not typable.

recommended since all heavily colonized women studied was detected. Using the latex method, Wald e col (15) were able to detect only 66.7% of the GBS isolated from heavily colonized women.

A prompt identification of GBS is desirable as it can significantly reduce the mortality among infected newborn. Thus, rapid tests for GBS identification have been developed (13,9,14). Mothers with high-level GBS colonization were pointed out as a factor that increases the risk of newborn infection by these bacteria (4,11). Therefore, we recommended the use of the LAL-2 broth as an alternative for the enrichment of GBS isolated from vagina, and for rapid detection of GBS in heavily colonized women.

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RESUMO

Novo meio de enriquecimento para o isolamento de estreptococos do grupo B em material altamente contaminado

O meio LAL-1, descrito recentemente (14), foi modificado pela adição de 15 µg/ml de ácido nalidíxico (meio LAL-2). O LAL-2 permitiu a detecção de todos os GBS isolados de mulheres altamente colonizadas pelo microrganismo. O meio modificado foi bastante eficiente para o enriquecimento de GBS visto que a detecção do microrganismo aumentou em aproximadamente 57%.

Palavras-chave: estreptorocos, *Streptococus agalactiae*, GBS.

REFERENCES

1. Baker, C.J. - Group B streptococcal infections in neonates. *Pediatr. Rev.*, **1**: 5-15, 1979.
2. Baker, C.J. & Barrett, F.F - Transmission of group B streptococci among parturient women and their neonates. *Pediatr.*, **83**: 919-925, 1973.
3. Benchetrit, L.C., Fracalanza, S.E.L., Peregrino, H., Camelo, A.A. & Sanches, L.A.L.R. - Carriage of *Streptococcus agalactiae* in women and neonates and distribution of serological types: a study in Brazil. *J. Clin. Microbiol.*, **15**: 787-790, 1982.
4. Boyer, K.M., Gadzala, C.A., Kelley, P.G., Bund, L.I. & Gotoff, S.P. - Selective intrapartum chemoprophylaxis of neonatal group B streptococcal early-onset disease. II. Predictive value of prenatal culture. *J. Infec. Dis.*, **148**: 802-809, 1983.
5. El Kholy, A., Wannamaker, L.W. & Krause, R.M. - Simplified extraction procedure for serological grouping of β-hemolytic streptococci. *Appl. Microbiol.*, **28**: 836-837, 1974.
6. Franciosi, R.A., Knostman, J.D. & Zimmerman, R.A. - Group B streptococcal neonatal and infant infections. *J. Pediatr.*, **82**: 707-718, 1973.
7. Gilbert, G.L. & Garland, S.M. - Perinatal group B streptococcal infections. *Med. J. Aust.*, **1**: 566-571, 1983.
8. Jelinková, J. & Motlová, J. - Worldwide distribution of two new serotypes of group B streptococci: Type IV and provisional Type V. *J. Clin. Microbiol.*, **21**: 361-362, 1974.
9. Kotnick, C.M. & Edberg, S.C. - Direct detection of group B streptococci from vaginal specimens compared with quantitative culture. *J. Clin. Microbiol.*, **28**: 336-339, 1990.
10. Lancefield, R.C. - A serological differentiation of specific types of bovine hemolytic streptococci (group B). *J. Exp. Med.*, **59**: 441-458, 1934.
11. Lotz-Nolan, L., Amato, T., Iltis, J., Wallen, W. & Packer, B. - Evaluation of a rapid latex agglutination test for detection of group B streptococci in vaginal specimens. *Eur. J. Clin. Microbiol. Infect. Dis.*, **8**: 289-293, 1989.
12. McCracken, Jr., G.H. - Group B streptococci: The new challenge in neonatal infections. *J. Pediatr.*, **82**: 703-706, 1973.
13. Sánchez, P.J., Siegel, J.D., Cushion, N.B. & Threlkeld, N. - Significance of a positive urine group B streptococcal latex agglutination test in neonates. *J. Pediatr.*, **116**: 601-606, 1990.
14. Teixeira, L.A., Figueiredo, A.M.S. & Benchetrit, L.C. - Liquid medium for rapid presumptive identification of group B streptococci. *J. Clin. Microbiol.*, **30**: 506-508, 1992.
15. Wald, E.R., Dashefisky, B., Green, M., Harger, J., Parise, M., Korey, C. & Byers, C. - Rapid detection of group B streptococci directly from vaginal swabs. *J. Clin. Microbiol.*, **25**: 573-574, 1987.

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TECHNICAL NOTES

OBJECTIVE CRITERIA TO CONFIRM THE EXISTENCE OF THE EXPONENTIAL GROWTH PHASE IN A BATCH MICROBIAL PROCESS

Walter Borzani

SUMMARY

Three criteria are proposed with the aim to reduce the subjectiveness of the method used to verify the existence of the exponential growth phase.

Key words: Exponential growth phase. Batch process.

INTRODUCTION

The existence of an exponential growth phase in a batch microbial process is usually confirmed on the basis of a graphical representation of the function ln X = f(t). If, within a time interval Δt, ln X is linearly correlated to t, we say that un exponential growth phase exists during Δt, and the correspondent constant specific growth rate may be calculated. The goodness of the linear correlation is represented by its correlation coefficient.

The above criterion, however, when applied by different researchers to the same set of experimental points, may lead to significantly different results.

The main purpose of this paper is to propose three criteria in order to reduce the subjectiveness of the described method.

First criterion: minimum number of experimental points during the exponential phase

If a well designed test was carried out, the number and the distribution of the experimental points must assure a good graphical representation of the growth curve. In this respect, if t_f is the total duration of the experiment, it seems advisable, as a first tentative, to measure X at almost constant time intervals not larger than $t_f/10$.

Obviously, the above recommendation must be carefully applied. If, for instance, a diauxic phenomenon appears during the test, the number and distribution of the experimental points must be ajusted in order to have a good definition of the growth curve.

Assuming that a consistent growth curve was obtained, we propose, as the first criterion, a minimum of four consecutive points to verify the existence of the exponential phase.

Second criterion: To be applied when the error that affects x is known

In this case, if α is the relative error that effects X, a graphical representation of ln X $(1+-\alpha)$ = f(t), similar to that presented in Figure 1, may be obtained. Applying the FIRST CRITERION proposed above, we tentatively choose two arbitrary moments t_1 and $t_2>t_1$ and draw two straight lines, the first one defined by the points ln X_1 $(1-\alpha_1)$ and ln X_2 $(1+\alpha_2)$, and the second defined by ln X_1 $(1+\alpha_1)$ and ln X_2 $(1-\alpha_2)$. If the above straight lines intersect all the segments representing the experimental errors of X within the choosed time interval, we will say that an exponential growth phase probably exists from t_1 to t_2. Several tentatives must be carried out in order to have a difference t_1-

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t_2 as large as possible. A simple mathematical approach, based on the equations of the cited straight lines, may be used instead of the graphical method.

In the particular case represented in Figure 1, this SECOND CRITERION shows that an exponential growth phase probably exists from t=1.5h to t=4.0h; the correspondent straight lines permit to calculate (1) the specific growth rate: $(0.27 \pm 0.02)h^{-1}$.

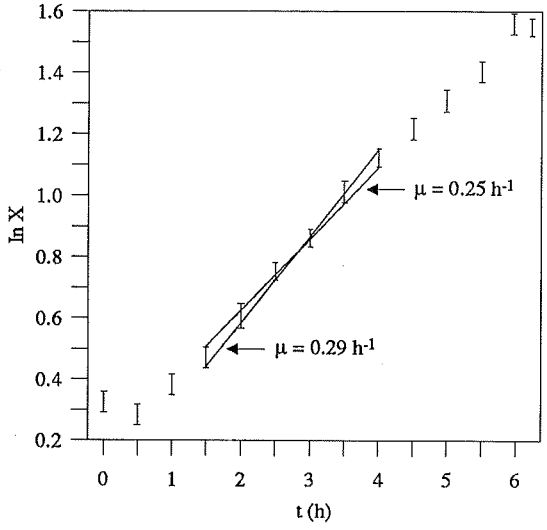

FIGURE 1 - Growth curve representing the errors that affect the values of X.

Third criterion: to be applied when the error that affects x is not known

A linear equation correlating ln X and t, within a time interval Δt, even if the correspondent correlation coefficient is relatively high, is not sufficient to assure that the exponential growth phase during Δt probably exists.

It is necessary to examine how the differences between the experimental values of X and the correspondent values calculated by the linear correlation distribute within Δt. If the above differences are randomly distributed within Δt, we may say that probably exists an exponential phase. If not, we may affirm that there is no exponential phase, whatever will be the practical applications of the obtained correlation.

Let us consider, for instance, the numerical examples presented in Table 1. The following correlations may be obtained from the values of Table 1:

Example nº 1: $\ln X = 1.41031 + 0.143t$ (r=0.993) (1)

Example nº 2: $\ln X = 0.49242 + 0.244t$ (r = 0.99990) (2)

TABLE 1 - Numerical examples.

t (h)	X (g/L) Example nº 1	X (g/L) Example nº 2
0	4.22	1.65
1	4.50	2.08
2	5.66	2.65
3	6.16	3.38
4	7.45	4.33
5	8.29	5.57

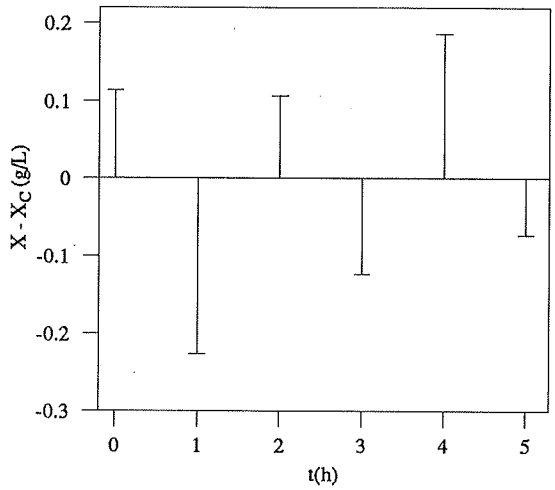

FIGURE 2 - Differences between the experimental and the calculated values of X (Example nº 1).

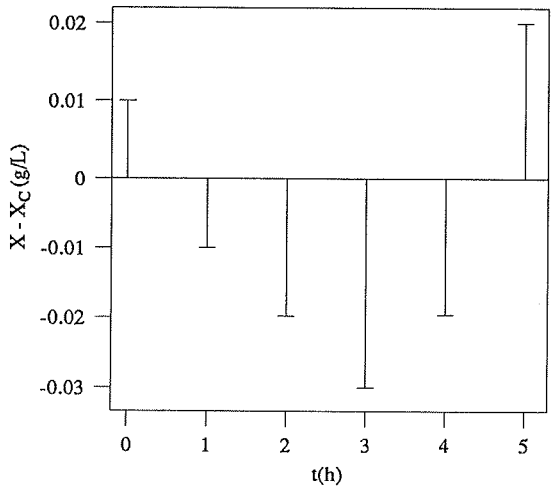

FIGURE 3 - Differences between the experimental and the calculated values of X (Example nº 2).

We may then calculate the differences between the experimental values of X (see Table 1) and the correspondent values calculated by equations (1) and (2). Figures 2 and 3 clearly show that in Example nº 1 we may affirm that the exponential phase probably exists, while in Example nº 2, in spite of the relatively high value of the respective correlation coefficient, we may conclude that the exponential model is not obeyed.

Nomenclature

r: correlation coefficient
t: time
t_f: duration of the experiment
X: cells concentration (dry matter)
X_1: value of X when $t=t_1$
X_2: value of X when $t=t_2$
X_c: calculated value of X
α: relative error that affects X
$\alpha 1$: relative error that affects X_1
$\alpha 2$: relative error that affects X_2
Δt: time interval

RESUMO

Critérios objetivos para confirmar a existência da fase exponencial de crescimento em processos de fermentação microbiana

Três critérios são propostos com a finalidade de tornar menos subjetivo o método utilizado para verificar a existência de fase exponencial de crescimento.

Palavras chave: Fase exponencial de crescimento. Processo em batelada.

REFERENCES

1. Borzani, W., Evaluation of the error that affects the value of the maximum specific growth rate. *J. Ferment. Technol.*, **58**: 299-300, 1980.

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ALTERNATIVE CULTURE MEDIUM FOR ISOLATION AND GROWTH OF BORRELIA BURGDORFERI

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Borrelia burgdorferi (Burgdofer, W. et al, 1982) is a spirochete that is the causative agent of an infectious disease: Lyme Disease (LD). Several isolates have been reported from a variety of ticks, animals and LD patients (see review by Barbour, A.G., 1988 for references).

The culture medium routinely used to grow Lyme spirochetes "in vitro" was described initially by Kelly (Kelly, R., 1971) subsequently modified by Stoenner (Stoenner, H.G., 1974) and Barbour (Barbour, A.G., 1984), resulting in the BSK medium (Barbour, Stoenner & Kelly). Two years latter, Preac-Mursic (Preac-Mursic, V., et al, 1986) proposed another modified Kelly's medium (MKP) to improve the sensitivity of isolation of spirochetes from clinical specimens and cultivation.

However, these media require expensive reagents and substancial time to produce. We describe here a relatively, rapid, simple and inexpensive alternative medium: Modified Spirolate Broth (MSB) for isolation and laboratory culture of Lyme disease spirochetes.

TABLE 1 - Formulation of Modified Spirolate Broth (MSB)

1. Spirolate Broth (BBL)	29.00 g
2. Brain Heart Infuison (DIFCO)	9.25 g
3. Sodium Thioglycollate (GIBCO)	1.00 g
4. Phenol Red	0.002 g
5. Water (qs)	911.00 ml

Adjust to pH: 7.6

Dissolve above in water qs 911 ml and autoclave at 121C for 15 minutes. Store in the dark at room temperature, and if not used within 7 days, must be heated to drive out the oxygen before use.

Add immediately before using:

6. 1M HEPES Buffer (GIBCO)	25.00 ml
7. 10% N-acetyl-glucosamine (SIGMA)	4.00 ml
8. Rabbit Serum	60.00 ml

The Spirolate Broth (BBL) was modified by addition of: brain heart infusion, sodium thioglycollate, HEPES buffer, N-acetyl-glucosamine and phenol red, as described in TABLE

For the comparative studies MSB was prepared as described above and used just after autoclaving (121C/15min) and BSK and MKP media as described previously. Five different LD spirochete isolates were utilized in these experiments (TABLE 2).

TABLE 2 - Isolates of Lyme Disease Spirochetes

Strain	Origin	Location	Donor
B-31	I. dammini	(NY-USA)	A. Barbour
JD-1	I. dammini	(MA-USA)	J. Donahue
Guilford	I. dammini	(CT-USA)	A. Steere
20047	I. ricinus	(France)	J. Anderson
CH90-2223	I. persulcatus	(China)	M. Zang/B. Cao

The stock cultures of spirochetes were grown in BSK medium and stored frozen at -85C in BSK medium with 30% glycerol. Spirochetes were grown in each of the media at 33C for 6 days (approximately 108 spirochetes/ml). The inoculum density was adjusted spectrophotometrically to an optical density of 0.400 at 500nm (Beckman DU-64 Spectrophotometer) and 0.01 ml from each culture was used to inoculate 10 ml of homologous medium.

The bacterial growth curve was determined by daily spectrophotometer readings and by direct darkfield microscopy counting each day for 10 days. The plasmid and protein profiles were done as described previously (Barbour, A.G., et al, 1988 and Hansen, K., et al, 1988).

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2. The Division of Vector-Borne Infections Diseases, National Center for Infectious Diseases, Centers for Disease Control, CO-USA

The five LD spirochetes strains showed no differences in grow rate, spirochete motility, cell yield, plasmid profiles or SDS-PAGE protein patterns when either of the 3 media were used.

The data from this study support the conclusion that the Modified Spirolate Broth is a suitable medium for cultivation of laboratory strains of Lyme spirochetes for DNA, plasmid, or antigen isolation at cost about 1/3 of others media.

REFERENCES

1. BARBOUR, A.G. Isolation and cultivation of Lyme disease spirochetes. *Yale J. Biol. Med.* **57**: 521-525, 1984.
2. BARBOUR, A.G. Plasmid analysis of *Borrelia burgdorferi lyme* disease agent. *J. Clin. Microbiol.* **26**: 475-478, 1988.
3. BURGDORFER, W., BARBOUR, A.G., HAYES, S.F., BENACH, J., GRUNWALT, E. & DAVIS J.P. Lyme disease-a tick-borne spirochetosis. *Science* **216**: 1317-1319, 1982.
4. HANSEN, K., BANGSBORG, J.M., FJORDVANG, H., STRANDBERG - PEDERSEN, N. & HINDERSSON, P. Immunochemical characterization of and isolation of the gene for a *Borrelia burgdorferi* immunodominant 60-kilodalton antigen common to a wide range of bacteria. *Infect. Immun.* **56**: 2047-2053, 1988.
5. KELLY, R. Cultivation of *Borrelia hermisii. Science* **173**: 443, 1971.
6. PREAC-MURSIC, V., WILSKE, B. & SCHIERZ, G. European *Borrelia burgdorferi* isolated from humans and ticks culture conditions and antibiotic susceptibility. *Zbl. Backt. Hyg. A263*: 112-118, 1986.
7. STEERE, A.C., GRODZICKI, R.L., KORNBLATT, A.N., CRAFT, J.E., BARBOUR, A.G., BURGDORFER, W., SCHMIDT, G.P., JOHNSON, E. & MALAWISTA, S.E. The spirochetal etiology of Lyme disease. *N. Engl. J. Med.* **308**: 733-740, 1983.
8. STOENNER, H.G. Biology of *Borrelia hermisii* in Kelly medium. *Appl. Microbiol.* **28**: 540-543, 1974.

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