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VIBRIO CHOLERAE NON-01 ISOLATED FROM SPORADIC CASES OF DIARRHEA IN RECIFE, BRAZIL

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SUMMARY

Among 3036 private patients with diarrhea, *Vibrio cholerae* non-01 was isolated from the stools of 0.7% (4 of 572) children less than two years of age. On the other hand, only one (0.04%) strain was recovered from patients after the second year of life.

Apart one infant, who also furnished *Campylobacter* sp., *V. cholerae* non-01, when present, was the sole enteric pathogen identified. The invasive character of the infection was suspected, because leukocytes were visualized in most fecal specimens positive for *V. cholerae* non-01. Cultures showed beta-hemolysis, but none was able to produce the cholera toxin. Despite the small number of isolates, this finding indicates that Recife has ecological conditions capable of supporting *V. cholerae*.

Key Words: *Vibrio*, Non-01 *Vibrio cholerae*, Vibrio linked diarrhea

INTRODUCTION

Vibrio cholerae non-01, formerly called non-agglutinable vibrio due to its failure to react in polyvalent 01 antisera, has long been recognized as an important cause of enteric and extraintestinal opportunistic human infections (3, 7, 14, 15, 16). Gastroenteritis linked to that microorganism are generally mild (14); however, a high fatality rate has been found at a hospital for diarrheal diseases in Bangladesh (8).

Vibrio cholerae non-01 is the most frequent vibrio species recovered at the Microbial Diseases Laboratory in California (9). In South America, nevertheless, only there are two references on human diarrhea associated to *V. cholerae* non-01; the first from Peru (10) and another from Brazil, when it was identified after an outbreak of gastroenteritis occurred in the south of Bahia (5). In Rio de Janeiro, the microorganism has been found in samples obtained from two sewage treatment stations (6).

Since cholera is now spreading on the west coast of South America and neighboring Amazon, we decided report our data on the isolation of *V. cholerae* non-01 from five patients with diarrhea in Recife.

MATERIAL AND METHODS

Clinical specimens – From April 1989 through March 1991, we selected 3036 diarrheal stools received for microbiologic diagnosis at a private clinical laboratory in Recife. Among them, 572 fecal samples came from children under 2 years old.

Laboratory methods – Standard methods were used to detect *Salmonella* spp., *Shigella* spp.,

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Campylobacter spp., enteropathogenic, enterotoxigenic, and enteroinvasive *Escherichia coli, Aeromonas* spp., *Plesiomonas shigelloides, Giardia lamblia, Entamoeba histolytica, Cryptosporidium parvum*, and rotavirus (11).

For recovering *Vibrio*, fecal samples were enriched in alkaline peptone water (pH 8.5) supplemented with 2% NaCl and subcultured to thiosulfate-citrate-bile salts-sucrose agar (TCBS; Difco Laboratories, Detroit, Mich.). Sucrose-positive (yellow) colonies were purified on sheep blood agar, biochemically characterized as *V. cholerae* by using accepted criteria (4), and tested for agglutination with polyvalent 01 *V. cholerae* antisera (Denka, Seiken Co., Tokyo).

Production of cholera toxin was evaluated by carrying out a reversed passive latex agglutination test (Denka, Seiken Co.). This test was done by following the manufacturer directions.

Leukocytes were searched on Gram stained fecal smears. A number equal or larger than five polymorphonuclear cells per high power field was recorded as a positive finding.

RESULTS

Vibrio cholerae non-01 was isolated from the stools of 0.7% (4 of 572) patients less than two years of age. On the other hand, only one (0.04%) strain was detected among patients after the second year of life. This strain of *V. cholerae* non-01 was isolated from a 54-year-old men, who developed his diarrhea 24 h after consuming fried fish. None child, with a positive culture for *V. cholerae* non-01, had a history of contact with salt water or had eaten seafood shortly before the onset of diarrhea.

Apart one infant, who also furnished *Campilobacter* spp., *V. cholerae* non-01, when present, was the sole enteric pathogen recovered. Two patients exhibited watery diarrhea without fecal leukocytes. The other three showed loose stools with a large number of leukocytes on smears. One of the infants showed bloody diarrhea. Vomiting was usually found among children but was absent in the adult patient.

On TCBS, *V. cholerae* non-01 colonies were large, mucoid, and deeply yellow. All strains were oxidase positive, failed to react in polyvalent 01 *V. cholerae* antisera, and gave the expected results in the identification tests, except strain 03 which was resistant to vibriostatic agent 0/129 (Table 1). Cultures were beta-hemolytic on sheep blood agar, but none was able to produce the cholera toxin.

TABLE 1 – Behavior of 5 *V. cholerae* non-01 strains, recovered from diarrheal stools, in the identification tests.

Test*	No. isolates tested				
	01	02	03	04	05
Growth in:					
0% NaCl	+	+	+	+	+
8% NaCl	-	-	-	-	-
Lysine decarboxilase	+	+	+	+	+
Arginine dehydrolase	-	-	-	-	-
Ornithine decarboxilase	+	+	+	+	+
ONPG	+	+	+	+	+
Voges-Proskauer	+	+	+	+	+
Gas in TSI	-	-	-	-	-
Acid from:					
Sucrose	+	+	+	+	+
Arabinose	-	-	-	-	-
Lactose	-	-	-	-	-
Inositol	-	-	-	-	-
Manitol	+	+	+	+	+
Salicin	-	-	-	-	-
Esculin	-	-	-	-	-
0/129 (150 ug/disc)	s	s	r	s	s

* Done in culture media amended with 1% NaCl. Except ONPG, tests were evaluated at 48 h. Symbols: + = positive; - = negative; s = susceptible; r = resistant.

DISCUSSION

Present results confirm previous report (5) on the occurrence of *V. cholerae* non-01 associated with human diarrhea in Brazil Northeast. This is a cause of public health concern because shows the existence of ecological niches for *V. cholerae* on the region. In fact, both *V. cholerae* 01 and non-01 are indistinguishable from the biochemical and genetical viewpoints, though most non-01 strains lack the gene which governs the cholera toxin synthesis (9).

In South America, *V. cholerae* non-01 is more frequently associated with infantile diarrhea. All five cases found in Peru occurred in patients less than three years of age (10). In Bahia (5) and Recife it was more prevalent among children. Such predilection of *V. cholerae* non-01 for infants is in contrast to that observed in the United States, where only adults have been infected (7). It differs of that noticed in Recife concerning *V. parahaemolyticus*, which has only been isolated from adults presenting watery diarrhea (13). Indeed, during the present survey we recovered from adult stools 24 strains of *V. parahaemolyticus*. This is an epidemiologic challenge since both microorganisms have the same habitat, and seafood repre-

sents a common source for human contamination. In the United States outbreaks, *V. parahaemolyticus* did not show any apparent preference for age or sex (1). Is still unclear why in Recife infants are more receptive to *V. cholerae* non-01 but adults to *V. parahaemolyticus.*

Concerning the child, who also furnished *Campylobacter* sp., it is hard to decide which microorganism was responsible for the diarrheal episode. However, if we consider that campylobacters are frequently found among ill and healthy infants in Recife (12), vibrio would have been the provable agent.

Since the infection was predominant among infants, they probably acquired the microorganism through cross-contamination of their milk with contaminated food handled in the same environments.

One of our children developed his gastroenteritis after drinking milk prepared at the kitchen, where shrimp had simultaneously been cooked. Another puzzling question is that the patients now examined were private, living in households with good sanitary-facility, and belonging to a higher economic class than those enrolled in a previous study, when the isolation rate of *V. cholerae* non-01 was practically the same (12). These intriguing data might suggest a paradoxical absence of correlation between socioeconomic class conditions, and the probability of infants develop in Recife *V. cholerae* non-01 associated gastroenteritis. Additional survey including a larger number of infected patients might elucidate the question.

Since the strains were non toxigenic and most elicited fecal leukocytes, invasiveness apparently might have played some part on the gastroenteritis of our patients. Furthermore, as the strains were strongly hemolytic, the role of non-01 *V. cholerae* hemolysin in disease should be better evaluated, mainly because it shares antigens and has biological activities similar to the hemolysin produced by the biotype E1 Tor of *V. cholerae* (16). Whereas strain 03 was resistant to 0/129, it was easily distinguished from *Aeromonas* because grew on TCBS and decarboxilated the ornithine; in addition, it did not attack arginine, arabinose, salicin, or esculin.

Despite the existence in Brazil of several ecological niches able to support *V. cholerae,* the inability of non-01 strains to transform into 01 epidemic ones, either in laboratory or in environment (2), indicates that cholera control policy remains in avoid importing the microorganism from abroad.

RESUMO

Vibrio cholerae não-01 isolado de casos esporádicos de diarréia no Recife, Brasil.

Entre 3036 pacientes de clínica privada com diarréia, *Vibrio cholerae* não-01 foi isolado das fezes de 0,7% (4 de 572) crianças menores de dois anos de idade. Por outra parte, apenas uma linhagem (0,04%) foi recuperada dos pacientes maiores de dois anos. Exceto uma criança, que também forneceu *Campylobacter* sp., *V. cholerae* não-01, quando presente, foi o único enteropatógeno identificado. Suspeitou-se que a infecção teve caráter invasivo, porque leucócitos foram vistos na maioria dos espécimens fecais positivos para *V. cholerae* não-01. As culturas mostraram beta hemólise, mas nenhuma produziu a toxina colérica. Apesar do pequeno número de cepas isoladas, esta observação indica que o Recife tem condições ecológicas permissivas ao *V. cholerae.*

Palavras-chave: *Vibrio, Vibrio cholerae* não 01, diarréia a víbrio.

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INCREASE OF *CAMPYLOBACTER* ISOLATION RATES USING AN ENRICHMENT MEDIUM

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SUMMARY

A semisolid enrichment medium for the isolation of thermotolerant species of *Campylobacter* was evaluated. With this medium the isolation rate of *Campylobacter spp.* from human, dogs and hens fecal samples was increased in 2.7, 6.7 and 20% respectively. After the enrichment period, contaminating flora was significantly reduced in the subcultures made in Skirrow's medium.

Key words: *Campylobacter*, enrichment medium, isolation rates.

INTRODUCTION

Thermotolerant species of *Campylobacter* (mainly *C. jejuni* and *C. coli*) are important agents of diarrhea in human beings. They also have been isolated from a great variety of animals all over the world(1).

Several selective culture media have been described in the literature for direct isolation of these bacteria (6). Some enrichment media have also been proposed (8, 12) in order to increase the isolation of *Campylobacter*. However the results obtained are not consistent and no recomendation have been proposed for routine use of these media.

In the present work we describe the performance of an improved enrichment medium for *Campylobacter*.

MATERIAL AND METHODS

We studied 400 fecal samples corresponding to 150 rectal swabs obtained from normal children; 150 dog feces collected from the streets and 100 hens cloacal samples.

All samples were seeded directly onto Skirrow (SK) agar plates (13) and into an enrichment medium consisting of: (Formule/I) Brucella broth (Difco) 28 g; agar (Difco) 1.5 g; sodium metabissulfite (Merck) 0.5 g; sodium pyruvate (Merck) 0.5 g; ferrous sulphate (Merck) 0.5 g; trimethoprim (Sigma) 10 mg; rifampicin (Sigma) 15 mg; colistin (Sigma) 10.000 IU; amphothericine (Squibb) 10 mg and defibrinated horse blood 30 ml.

SK plates were incubated at 42°C for 48 h under microaerobic conditions in a GasPak system w/o catalyst. Enrichment cultures were incubated aerobically at the same temperature for 24 h and then, subculture on SK. Subcultures were incubated at the same microaerobic conditions described above.

Suspected colonies were identified morphologically (Gram stain) and biochemically using the differential tests proposed by Lior (9).

RESULTS

Using direct plating it was possible to obtain 85 isolates of *Campylobacter spp.* After the enrichment period 199 isolates were obtained increasing the isolation rate up to 8.5% (Table 1). Direct plating allowed us to detect 10 isolates from human feces; 40 from dog and 35 from hen feces. Enrichment procedures increased the isola-

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TABLE 1 - Isolation rates (%) of thermotolerant *Campylobacter* species using direct plating and enrichment methods.

Samples origin	Isolation Rates		Increase in isolation rate
	Direct plating	Post-enrichment	
Human n=150	10 (6.7)	14 (9.3)	4 (2.7)
Dogs n=150	40 (26.7)	50 (33.3)	10 (6.7)
Hens n=100	35 (35.0)	55 (55.0)	20 (20.0)
TOTAL N=140	85 (21.2)	119 (29.7)	34 (8.5)

tions by 2.7% , 6.7% and 20% respectively.

The use of enrichment media reduced the presence of background fecal flora in the plates. By direct plating, contamination was present in 60% of the cultures. After enrichment, contamination was reduced to 38.2%.

C. jejuni biotypes I and II were the most frequent isolates (29.4% and 34.4% respectively) and were present in the three group of samples studied. *C. jejuni* biotype III was isolated only from hens and was the less frequent isolate (1.7%). *C. coli* biotypes I and II were isolated from human and hens samples (14.3% and 20.2% respectively), but not from dog feces.

DISCUSSION

In the past years various authors have employed enrichment procedures to improve *Campylobacter spp.* isolation (2, 8, 10, 11, 14). All of them found higher isolation rates following selective enrichment. Only Gilrichst & col. (5) did not find it advantageous to use enrichment media.

Our overall results (8.5% increase) are similar to those obtained by Chan and McKenzie (6%) (2) and Skirrow and Benjamin (5.6%) (14), but lower to those obtained by Hutchinson and Bolton (14%) (8) and Ribeiro et al. (40.7%) (11). All of the previous authors worked with only human diarreic feces. In the human fecal samples our results are lower (2.7%) probably to the fact that we worked only with healthy children in which the number of *Campylobacter* cells per g of feces could be low, as it has been described to occur in convalescent patients (3, 12).

Despite the small increase of positive cases we believe that use of enrichment procedures may be better to evaluate the frequency of healthy carriers of *Campylobacter spp.* In a previous study, using direct plating on SK, we found 4% of intestinal carriers of *Campylobacter spp.* among children of 1 month to 6 years of age (3). In this work, the carrier rate increased from 6.7% by direct plating to 9.3% when using enrichment medium.

The isolation rate in dog stools after enrichment was increased by 6.7%. In this group of samples the number of viable cells could be low due to the exposure to unfavourable environmental conditions (e.g. sunshine, dessication). This is reflected by the fact that only 26.7% of the samples were positive by direct plating, while in a previous study (4) 51.4% of the samples were positive in fresh stools obtained from live dogs.

Surprising was the 20% increase in the isolation rate of *Campylobacter spp.* from hens samples. Martin et al. (10) showed that the use of an enrichment procedure for poultry fecal specimens was of little advantage. However, our results suggest that the use of an enrichment medium improves the isolation of *Campylobacter spp.* specially when the suspected number of viable *Campylobacter* cells is low. This instance may occur in some animals, in convalescent patients, in late or delayed specimens or in samples exposed to damaging environmental factors for the bacterial cells (7, 8, 12).

Using the enrichment medium we observed a great reduction in the presence of fecal contaminating flora. This seems to be an advantage because the competing microflora is usually high, specially in animals samples were fungi could also be present, and overgrowth may occur hiding the presence or inhibiting the growth of *Campylobacter* colonies on the plates. Similar observations were made by Martin et al. (10).

The semisolid enrichment medium used in this study is easy to prepare, does not require special incubation equipment and increases the isolation rate of *Campylobacter spp.* allowing a better understanding of the frequency of these zoonotic bacteria in humans and in animal reservoirs. Having in mind the characteristics of this enrichment medium, we are using it also as a transport medium for both human and animal fecal samples and for pure strains of *Campylobacter spp.* with good results.

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RESUMO

Aumento de freqüência de isolamento de *Campylobacter* usando um meio de enriquecimento.

Foi avaliada a capacidade de um meio de enriquecimento semi-sólido para o isolamento de *Campylobacter*. Com a utilização deste meio, freqüências de isolamento de *Campylobacter spp.* em fezes de origem humana, de cães e de galinhas aumentaríam em 2,7, 6,7 e 20% respectivamente. Após o período de enriquecimento, observou-se nas subculturas redução significativa da flora contaminante.

Palavras-chave: *Campylobacter*, meio de enriquecimento, freqüência de isolamento.

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HEMAGGLUTINATION PATTERN OF *CORYNEBACTERIUM DIPHTHERIAE*[1]

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SUMMARY

C. diphtheriae showed differences in reactivity with chicken, cat, monkey, goose, human, guinea-pig and sheep erythrocytes. Eight different hemagglutination (HA) patterns were observed and related to sucrose fermentation biotypes. Sucrose-fermenting strains did not agglutinate chicken, cat and monkey erythrocytes. The HA of *C. diphtheriae* was not inhibited by changing condition of growth, alcohol-ether, heat, trypsin, HCl, NaOH and carbohydrates treatments.

Key Words: *Corynebacterium diphtheriae*, diphtheria, hemagglutination.

INTRODUCTION

The meaning of the prevalence of sucrose fermenting *C. diphtheriae* biotype in our community has not yet been determined (5). The role of adhesive activity in *C. diphtheriae* infection has been thoroughly discussed (3). However, little is known about the adherence mechanisms of the organism (3, 15, 18, 19, 23).There are some apparent differences in the sheep erythrocytes HA and adhesive properties to glass surfaces of the biotypes related to sucrose fermentation activity (19). The use of HA patterns, particularly in combination with response to erythrocytes from different species, as well as biochemical characteristics is likely to be important epidemiologically (2). However, there have been no reports which relate HA patterns to biotype or to the site of infection of *C. diphtheriae*.

Hemagglutinins of some organisms show differences in reactivity that are dependent on the species of erythrocytes used (4, 10, 12, 14, 20). Previous studies have also demonstrated that the HA can be modified by treatment of the microorganisms with physical and chemical agents. Erythrocytes may also be blocked by the compounds which mimic the receptors concerned (14, 17, 22).

No further investigations on the agglutination activity of diphtheria bacilli with respect to other red blood cells, besides sheep erythrocytes, have been conducted as yet. In the present study we report HA patterns of strains of *C. diphtheriae* and relate results to biotype and source of the isolates. In addition, different compounds were tested for their capacity to inhibit sheep cells agglutination used in *Corynebacterium* HA assays.

MATERIAL AND METHODS

Strains of *C. diphtheriae* were isolated and identified in our Laboratory Rio de Janeiro, Brasil through tests described elsewhere (6,7) including

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the investigation of fluorescence under the ultraviolet light, the presence of pirazine-carboxilamidase enzyme (PYZ) (9, 26) and "in vitro" virulence test (single radial immunodiffusion method) (6, 7, 8). *C. diphtheriae* var. *mitis* CDC-E8392 and var. *intermedius* CDC-7920 were provided by the National Centers for Disease Control, Georgia, USA.

Bacteria were grown on nutrient agar medium supplemented with 5% calf serum for 24 or 48h at 37°C (27). The ability of bacteria to agglutinate erythrocytes was determined according to the microtechnique: conical bottom well microtiter plates were used to determine HA titers (Linbro/Titertek, Linbro Division Flow Laboratories, Inc. Hamden, Conn). Bacterial suspensions were prepared in PBS, pH 7.2, (turbidity equivalent to an optical density of 0.8 at 570nm). Five serial twofold dilutions of the suspension of microorganisms were made in PBS so that 25µl remained in each well. An aliquot of 25µl of the 0.25% erythrocytes suspension was then added to each well. The trays were shaken for 30 s and incubated at 37°C for 3-4 h. The final titer was taken to be intermediate between the last clear agglutination pattern and the following questionable pattern (18).

Agglutination of erythrocytes from different species was determined for 22 toxigenic strains of *C. diphtheriae* including the following biotypes: i) twelve non-sucrose-fermenting strains (*C. diphtheriae* var. *mitis* CDC-E8392, *C. diphtheriae* var *intermedius* CDC-7920; 3 isolated from throats and 7 from skin lesions); ii) ten sucrose-fermenting strains (8 from throats and 2 from skin lesions). Erythrocytes from the following species were used: sheep, chicken, cat, monkey, goose, guinea-pig and human (A, B, O Rh+ and O Rh-).

Suspension of a sucrose fermenting strain (number 241) and a non sucrose fermenting strain (number 222) were subject of the following treatments: i) 100°C/2h; ii) trypsin (Difco) 200µg/ml at 37°C for 30 min; iii) 1% (v/v) formaldehyde for 24h at 4°C; iv) 1 N HCl and 0.5N NaOH for 60 min at 37°C and v) ethyl-ether-ethanol (1:1, v/v). The treated cell suspensions were centrifuged, washed with PBS, and assayed for HA using sheep erythrocytes. Microorganisms cultivated on i) nutrient-agar containing 5% sucrose; ii) nutrient broth with 1% "Tween" 80; iii) on media containing glycerol (1.5% glycerol, 1% yeast extract and 0.4% NaCl) described by Ioneda & Silva (13) and iv) serially subcultivated in nutrient-broth and on agar plates were also submitted to HA test.

Various carbohydrates were also tested to detect specific inhibitors for *C. diphtheriae* HA: 25µl bacterial suspension was mixed with 0.25% sheep erythrocytes with 0.5% (w/v) D. galactose, L-arabinose, trehalose; 0.25% D-mannose and 0.065% L-rhamnose (2).

RESULTS AND DISCUSSION

Hemagglutination by bacterial cells serves as a useful model for bacterial attachment to epithelial cells, since similar mechanisms are probably involved (22) HA of *C. renale* has been studied using several erythrocytes. These organisms only adhere to trypsinised sheep cells (12).

The HA of *Corynebacterium* group D2 has also been studied. A recent report is the first study of the adhesion of CD2 to urinary tract epithelial cell and mucous and the agglutination of erythrocytes by this bacteria. These properties were correlated with origin (urine or skin) of the isolated CD2 strains. Difference of adhesion and HA tests between the two groups of strains of different CD2 origins was not statistically significant (21).

C. diphtheriae agglutinates non-trypsinized sheep cells and this HA it is not dependent on its toxin production (18, 19). In contrast to *C. renale* and diphtheroids isolated from lesions of erythrasma (20), diphtheria bacilli agglutinates several erythrocytes species other than sheep cells: chicken, cat, monkey, goose, guinea-pig and human red cells (Table 1).

Eight different HA patterns were observed for *C. diphtheriae*. Some strains (26.3%) agglutinated all erythrocytes used. Three or four erythrocytes species were agglutinated by 21% of the organisms. There were strains (21%) that agglutinated only guinea-pig cells. We also observed a strain (5.2%) that did not show HA. Guinea-pig and human erythrocytes showed higher titers and larger number of positive reactions for both sucrose-fermenting and non-fermenting biotypes.

In the case of CD2 only a few urinary strains and none of the health skin strains had hemagglutinins acting on human and guinea-pig erythrocytes. No further investigations with respect to other red blood cells have been conducted as yet (21), CD2 it is not the only diphtheroid capable of HA non trypsinized red cells. Other corynebacteria isolated from thoroats HA sheep erythrocytes, and show adhesive properties to solid surfaces (20).

Three skin isolates not included in Table 1 were hemolytic for all erythrocytes tested except for sheep red cells.

Two distinct groups of strains were observed:

TABLE 1 - Agglutination of erythrocytes from various animals by *C. diphtheriae*

HA pattems	Strains	Sources of erythrocytes and hemagglutination titer								
		Chicken	Cat	Monkey	Goose	Sheep	Human O-	Human O+ and A	Human B	Guinea-pig
	Sucrose-Fermenting-biotype									
I	239	0	0	0	0	0	0	0	0	0
II	9,13(*) and 61	0	0	0	0	0	0	0	0	16
III	236	0	0	0	0	0	2	1	2	0
IV	31 and 52	0	0	0	2	0 and 1	0	1	1	1
V	233 and 241	0	0	0	8	1 and 0	1	1	1	16
	Non-sucrose-fermenting-biotype									
VI	202(**)	L	2	1	4	4	2	1	2	L
VIII	6 and 222(**)	1	2	1	1	2	4	8	16	16
VIII	17	2	2	2	2	4	2	4	16	16
VII	3,10,65,(**) and 90	1	2	8	L	2	16	16	16	16
VIII	Biotypes:*mitis* and *intermedius*(***)	4	4	2	16	4 and 16	8	16	16	16
	Hemagglutination activity (%)	47,37	52,63	52,63	52,63	63,16	68,42	78,95	78,95	84,21

(*) skin origin; (**) throat origin; (***) (CDC-E8392) and (CDC-7920); (L) Hemolysis

one that did not agglutinate chicken, cat, and monkey erythrocytes in any opportunity and, a second group that agglutinated all kind of red blood cells. These differences were related to the bacterial biotypes and the site of infection by *C. diphtheriae*. HA patterns I to V were exhibited by strains of the sucrose-fermenting biotype and HA patterns VI to VIII by non fermenting biotype strains. HA patterns may also differ among identical biotype strains.

Previous investigation suggests an association between biotypes and the site of infection of *C. diphtheriae* strains, indicating a degree of tissue tropism among these organisms (19).

Differences in the HA between strains of the two sites of infections were observed (Table 1), when most of the skin isolates exhibited stronger HA for all erythrocytes tested while strains isolated from throat showed weaker HA.

Although our studies suggest a tropism of sucrose-fermenting biotype to buccal mucosa it should be noted, however, that sucrose-fermenting strains may adhere to skin epithelial cells, and non-fermenting strains adhere to oral epithelial cells. A skin, sucrose-fermenting (number 13) strain showed weak HA while some throat, non-fermenting (numbers 202,222 and 65) strains agglutinated all erythrocytes tested.

The finding is consistent with previous observations when strains isolated from skin were able to adhere to oral epithelial cells, but in a lower degree to that observed with strains isolated from throats (1,3).

The HA for sheep erythrocytes was stable after heat treatment with the non fermenting strain (Table 2). After incubation with trypsin HA increased for both biotypes. Treatment of *C. diph-*

TABLE 2 - Effects of culture conditions and various treatments of two throat strains of sheep erythrocytes on hemagglutination activity of *C. diphetheriae*

Treatments or culture conditions (**)	Hemagglutination titers Sucrose fermenting biotype (strain 241)	Non-sucrose Fermenting biotype (strain 222)
Microorganisms		
NONE	0	2
Heat	16	2
NaOH; HCl	2	2
Trypsin	1	16
Formaldehyde;	0	2
Tween 80 and Sucrose		
Glycerol	8	8
Alcohol-ether	2	1
Erythrocytes		
Carbohydrates (***)	1	2

(**) Experiments were performed in duplicate.
(***) D-galactose, L-arabinose, threhalose; D-mannose and L-rhamnose

theriae strains with formaldehyde did not affect the HA of the two biotypes. The sucrose-fermenting strains began to show HA after treatment with HCl and NaOH. Cultivation on media containing glycerol enhanced HA of strains with or without sucrose-fermentation activity. "Tween 80" did not affect the HA original features of the strains. Alcohol-ether treatment of the sucrose-fermenting strain enhanced its HA and reduced of HA of the non-sucrose fermenting strain.

The HA remained unchanged when the microorganisms were cultivated on media containing sucrose. Phase variation did not occur when *C. diphtheriae* was serially subcultivated on nutrient broth or an agar plates. Strains able or not of pellicle formation exhibited HA.

The cellular receptor for *E. coli* type 1 pili is thought to be D-mannose, and that for *V. cholerae* adhesin is thought to be L-fucose. A cellular receptor for corynebacterial adhesion is not yet defined. The adherence on the sheep erythrocytes by *C. renale* was not inhibited by six carbohydrates and 11 amino acids (10, 24, 25).

None of the following compounds showed inhibitory effects on the agglutination of sheep erythrocytes by *C. diphtheriae*: D-galactose, L-arabinose, trehalose, D-mannose and L-rhamnose (Table 2).

Differences occured between HA of sucrose-fermenting and non-fermenting strains. HA of diphtheria bacilli, as well as CD2 and *C. renale*, was not reversible in the presence of D-mannose (21, 24). The sucrose-fermenting bacteria showed enhanced HA for all carbohydrates tested as observed with *E.coli* (25).

Filamentous structures described as pili or fimbriae were observed by Yanagawa and Honda (27), however non-piliated *C. diphtheriae* strains also showed HA when trypsinized sheep erythrocytes were used (15).

A surface molecule, possibly a lipid compound, may be involved in HA of microorganisms. Common lipids were also observed in whole-cell preparations of diphtheria bacilli strains (11).

The presence of another non-hemagglutinating surface molecule may block the HA of the sucrose-fermenting strain, and may be also responsible for the stronger ability of adherence to glass surfaces (19). Different protein compounds may be involved in the adherence mechanisms of *C. diphtheriae* since trypsin enhanced the HA of both biotypes, while heat, NaOH, HCl treaments enhanced only the HA of the sucrose-fermenting strain (Table 2).

Although pili play a role in HA and adherence of *C. renale*, non-piliated strains adhere effectively to bovine bladder epithelial cells (24).

The molecular nature of adherence in CD2 is unknown but there is no evidence of a fimbrial nature because of the absence of correlation between piliation and the adherence pattern (21). A recent study on adherence of several skin corynebacteria to human epithelial cells demonstrates that a number of adhesins and receptors are involved in this adhesion (23).

The fact that different patterns of HA were found with diphtheria bacilli suggests that more than one hemagglutinin might be implicated.

The experiments we performed suggest biochemical differences among *C. diphtheriae* hemagglutinins. It may represent a multifatorial adhesive property of the organism. These differences may be also related to the prevalence of one biotype over the other.

RESUMO

Padrões de hemaglutinação do *Corynebacterium diphtheriae*

O bacilo diftérico apresentou atividade hemaglutinante para hemácias de pinto, gato, macaco, ganso, humanos, cobaio e carneiro. Observamos oito padrões hemaglutinantes que foram relacionados com o comportamento bacteriano frente a sacarose. Os microrganismos de biotipo fermentador não hemaglutinaram hemácias de pinto, gato e macaco.

A aglutinação de hemácias de carneiro, pelo bacilo diftérico, não sofreu inibição após a variação das condições de cultivo, nem após o tratamento do microrganismo com diversos agentes como: calor, álcool, éter, tripsina, HCl, NaOH e açúcares.

Palavras-chave: *Corynebacterium diphtheriae*, difteria, hemaglutinação.

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OCCURRENCE OF *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI* IN RETAIL RAW CHICKEN MEAT AND GIBLETS IN SÃO PAULO, BRAZIL

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SUMMARY

The prevalence of *Campylobacter jejuni* and *Campylobacter coli* in two hundred refrigerated raw chichen meat samples, purchased at retail markets in São Paulo, Brazil, was studied. Samples included whole carcasses, meat parts and giblets (gizzards, livers, hearts and feet). *Campylobacter* was detected in 13,5% of the samples. The highest incidence was observed in meat parts (8%). Among the isolated strains, 86% were *C. coli* and 14% were *C. jejuni*.

Key Words: *Campylobacter* spp, raw chicken meat, raw chicken giblets

INTRODUCTION

Campylobacter jejuni and *Campylobacter coli* are common causative agents of diarrhea in children and adults in developing countries, including Brazil (9, 11, 19, 23, 32). *Campylobacter* species are widespread in the animal kingdom, but poultry have been implicated as the main source of *Campylobacter* infections in human because of the ubiquity of the microorganism in the intestines, carcasses and processed meat (4, 5, 12, 22). Several reports concerning contamination of poultry have been published in many countries (2, 7, 10, 12, 14, 20, 22, 25, 26, 28, 29). In Brazil, researches conducted by Levi & Ricciardi, 1982 (17), Leitão *et al.*, 1986 (16), and Almeida & Serrano, 1987 (1) detected *Campylobacter* in 78,7%, 62,2% and 47,5% of the surface samples obtained from fresh poultry carcasses, respectively.

Several researches have demonstrated that *Campylobacter* is relatively sensitive to chilling, storage at ambient temperature and freezing, resulting in the reduction of the initial contamination of carcasses (8, 24, 29, 30). For this reason, this study was conducted in order to determine the prevalence of *C. jejuni* and *C. coli in* retail raw chicken meat and giblets, in São Paulo, Brazil.

Sampling – A total of 200 refrigerated raw chicken meat samples were purchased at randomly selected retail markets of São Paulo city, Brazil. These samples included: 5 whole raw chicken carcasses, 82 raw chicken meat parts (including breasts, wings, thighs and necks) and 113 raw giblets (including gizzards, livers, hearts and feet). No information was available concerning processing and maintenance conditions of samples before sale. The samples were transported to the laboratory in ice-cooled containers and examined within two hours.

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Treatment of samples – Twenty-five grams of each sample (except whole carcasses) were asseptically homogenized with 100 ml of Selective Enrichment Broth (SEB), constituted by Tryptic Soy Broth (Difco) supplemented with yeast extract (2g/1), sodium citrate (1 g/1) and antibiotics (vancomycin, 0,01 g/1; trimethoprim, 0,005 g/1, polymyxin B, 2.500 IU, amphotericin B, 0,002 g/1, cephalotin, 0,015 g/1) (Campilofar, Cefar, SP), ferrous sulfate (0,25 g/1), sodium metabisulfite (anhydrous) (0,25 g/1) and sodium pyruvate (anhydrous) (0,25 g/1). After homogenization, the mixture was transferred to an erlenmeyer of 250 ml capacity.

Whole carcasses were sampled by swabbing 100 cm^2 of the breast with a swab premoistened in SEB. The swab was transferred to an erlenmeyer of 250 ml of capacity containing 100 ml of SEB (21; Park, personal communication).

Culture techniques – SEBs were incubated at 42°C for 48 h, in anaerobic jars, under microaerophilic conditions (Anaerocult C, Merck). Each broth was streaked on the surface of plates of *Campylobacter* Selective Agar (Merck) supplemented with 5% of defribrinated horse blood, vancomycin (0,01 g/1), trimethoprim (0,005 g/1), polymixin B (2500 IU), amphotericin B (0,002 g/1) and cephalotin (0,015 g/1). Plates were incubated at 42°C for 48h in microaerobic atmosphere (Anaerocult C, Merck). Wet mount preparations of isolated colonies showing typical growth (smooth, convex, translucent, colorless to cream-colored and pinpoint to 2-4 mm in diameter or often spread colonies) were examined with a phase-contrast microscope for corkscrew motion. Colonies stained with 10% cristal-violet were observed for characteristic morphology (curved, s-shaped or spiral).

Colonies with characteristic morphology and motility were cultured on Bacto Fluid Thyoglycollate Medium without Dextrose (Difco), supplemented with 0,16% Agar, at 42°C for 48 h and, then, transferred to Blood Agar plates (Difco) and to Tryptic Soy Broth (Difco) supplemented with yeast extract (2 g/1), sodium citrate (1 g/1), ferrous sulfate (0,25 g/1), sodium metabisulfite (0,25 g/1) and sodium pyruvate (0,25 g/1). Blood Agar Plates were used to test resistance/ sensitivity to cephalotin (30 ug) and nalidixic acid (30 ug) and oxidase production. Broths were used to test glucose fermentation, catalase production, nitrate reduction, H_2S production, growth in 1% glicine, 3,5% NaC1, growth at 25°C, 37°C and 42°C, according to Park *et al.*, 1984 (21).

Confirmed *Campylobacter* colonies were biotyped on the basis of three metabolic tests: sodium hippurate hydrolysis, H_2S production and DNA hydrolysis (18, 27).

RESULTS AND DISCUSSION

In this study, *Campylobacter jejuni* and *Campylobacter coli* were detected in 13.5% of the raw chicken meat and giblet samples (Table 1). Their prevalence in meat samples (whole carcasses and meat parts) was a little superior to that observed in giblets (8.0% and 5.5%, respectively).

Several studies carried out in Brazil, as well

TABLE 1 – Occurrence of *Campylobacter jejuni* and *Campylobacter coli* in raw chicken meat and giblets.

Group of samples	No. of samples	No. of positive samples	positivity (%) A(*)	B(**)	C(***)
whole carcasses	5	1	0.5	20.0	1.2
meat parts	82	15	7.5	18.3	17.2
Subtotal	87	16	8.0	-	18.4
gizzards	50	6	3.0	12.0	5.3
livers	32	0	0.0	0.0	0.0
hearts	27	4	2.0	14.8	3.5
feet	4	1	0.5	25.0	0.9
Subtotal	113	11	5.5	-	9.7
Total	200	27	13.5	-	-

(*) No. of positive samples / total No. of samples x 100
(**) No. of positive samples / No. of samples in the same group x 100
(***) No. of positive samples / No. of samples of meat or giblets tested x 100

TABLE 2 – Biotypes of *Campylobacter jejuni* and *Campylobacter coli* isolated from raw chicken meat and giblets.

	No. of strains	biotypes according to					
		Skirrow & Benjamin, 1980		Lior, 1984			
		1	1	I	II	III	IV
C. jejuni	14	14 (100%)	0	3 (21.4%)	11 (78.6%)	O	O
C. coli	86	NA (*)	NA	41 (47.7%)	45 (52.3%)	NA	NA
Total	100						

(*) not aplicable

as in other countries, have shown that the incidence of *C. jejuni* and *C. coli* in poultry carcasses and giblets examined immediately after evisceration is high, varying form 60% to 100% (7, 10, 12, 14, 16, 17, 22). However, the frequency is much lower (15% to 20%) in food samples that have been stored frozen or under refrigeration, for varying periods of time (10, 15, 29), These results confirm the ones obtained in this study, and both are in agreement with the fact that campylobacters are sensitive to freezing temperatures commonly used for storage of poultry before trading (7, 10). Stern *et al.*, 1985 (29) demostrated that freeze / thaw treatment of chicken carcasses reduced 100 times the number of detectable campylobacters. On the other hand, *Campylobacter* survives better in foods at refrigeration than at freezing temperatures (3, 31). Our results suggest that the samples of carcasses and/or giblets may have been submitted to a freezing step during their processing.

As far as laboratory methodology is concerned, it must be taken in account that recent studies have demonstrated that employment of high incubation temperatures (42°C) and the use of excessive amount of selective agents (antibiotics) may cause failure in the recovery of injured campylobacters from foods (Park, personal communication).

With respect to biochemical properties of the *Campylobacter* strains, it was observed that 86% of them were *Campylobacter coli* and 14% were *Campylobacter jejuni* (Table 2). Among *C. jejuni* strains, 100% of them belonged to biotype I of Skirrow & Benjamin's scheme (27) and 78,6% to biotypes II of Lior's scheme. There is no apparent reason for the higher incidence of *C. coli* than of *C. jejuni*. Biotyping results are in agreement with those of Rosef *et al.*, (25), but in contrast with those of Cabrita, 1989 (6), and Jorgensen & Olsen, 1989 (13). In both studies, a predominace of *C. jejuni* biotype I of Lior's scheme ocurred. Unfortunately, due to lack of information about biotypes and other characteristics of strains associated to diarrheal disease in Brazil, it's very difficult to establish the relationship between the strains isolated from poultry and those isolated from feces.

The significance of contamination of raw chicken meat and giblets with *Campylobacter jejuni* and *Campylobacter coli* will be better appraised after the establishment of the pathogenicity of the contaminating strains.

RESUMO

Ocorrência de *Campylobacter jejuni* e *Campylobacter coli* em carnes e miúdos de frango crus, comercializados em São Paulo, Brasil

Estudou-se a incidência de *Campylobacter jejuni* e *Campylobacter coli* em duzentas amostras de carne e de miúdos de frango crus, coletados em supermercados da cidade de São Paulo, Brasil. As amostras incluíram carcaças inteiras, pedaços de carne e miúdos (moelas, fígado, coração e pés). *Campylobacter* foi detectado em 13,5% das amostras estudadas. Maior incidência foi observada nos pedaços de carne. Das 100 cepas de *Campylobacter* isoladas, 14 foram caracterizadas como *C. jejuni* e 86 como *C. coli*.

Palavras-chave: *Campylobacter* spp, carne de frango, miúdos de frango crus.

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MEASUREMENT OF ANTIBODIES AGAINST FOOT-AND-MOUTH DISEASE VIRUS IN A CRUDE ANTIGEN SUSPENSION AND AGAINST THE VIA ANTIGEN BY THE QUANTITATIVE COMPLEMENT FIXATION TEST IN BUFFALOES

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SUMMARY

Antibodies against viral infection associated (VIA) antigen and against crude suspensions of strains "O_1" Campos, " A Venceslau", "A_{24}" Cruzeiro and "C_3" Indaial of foot-and-mouth disease virus (FMDV) acquired by natural passive immunization were measured by the complement fixation test in buffaloes. Three animals were periodical tested for the presence of antibodies from birth to 6 mouths of age. The results showed that it is perfectly possible to measure antibodies against the VIA antigen and against the different strains of FMDV in buffaloes with a large safety margin, a characteristic that is not observed in cattle when the direct method is utilized.

Index Terms: Foot-and-mouth disease, complement fixation, buffaloes.

INTRODUCTION

Indian buffalo (*Bubalus bubalis*) breeding has been growing at a fast rate in Brazil, with a notable increase in the population of this species. Cattle and buffaloes are frequently raised on the same property, with close contact between the two species. With respect to foot-and-mouth disease (FMD), it is know that buffaloes rarely show clinical manifestation of the disease (17), though there is evidence that they participate in the epidemiological chain of FMD in cattle. Few studies, however, have been carried out on FMD among buffaloes.

Because of the absence of clinical signs of FMD among buffaloes, allied to the evidence of the participation of these animais in the epidemiology of the disease among cattle, serologic tests for the detection of antibodies against foot-and-mouth disease virus (FMDV) and the "Probang" test for FMDV isolation become extremely important in the identification of carrier buffaloes. The method consist in recover the oesophageal-pharingeal fluid samples from animals by means of a cup probang passed through the mouth into the pharynx (23).

In cattle, antibodies against FMDV are usually measured by serum neutralization relatively complex and therefore impractical for aplication to large numbers of animals.

The complement fixation test (CFT) for the same purpose is also impratical because cattle do not fix normal guinea pig complement when FMDV is used as an antigen (2, 9, 10, 13, 14, 15, 21, 22). The CFT, however, can be used for the detection of FMDV antibodies in buffaloes, as demonstrated by Samara (16).

With the discription of the virus-infection-associated (VIA) antigen by Cowan & Graves (4), the determination of VIA antibodies by agar gel immunodiffusion has been widely utilized to detect animals with FMDV replication. After the technique (VIA test) was standardized by McVicar & Sutmoller (7), important recommendations for the interpretation of the results were made by

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Pinto & Garland (11) and by the Pan-American Foot-and-Mouth Disease Center (3).

By applying the VIA test to African buffaloes (*Syncerus caffer*), Pinto & Garland (11) demonstrated the greater sensitivity of this test in relation to the "Probang" test in the identification of infected animals. Several studies have demonstred that Indian buffaloes respond to FMDV by producing antibodies against the VIA antigen (5, 8, 17, 20).

On the basis of the data reported in the studies cited above, the objective of the present investigation was to compare the agar gel double diffusion test with a direct CFT method in the determination of antibodies against the VIA antigen of FMDV. An attempt was also made to determine a possible relationship between detectable levels of VIA antigen antibodies and crude antigen FMDV suspensions in buffaloes using the same direct CFT method.

MATERIAL AND METHODS

Blood sera

Different blood serum samples were obtained from 3 animals bled periodically from birth to weaning, i.e., immediately after birth, before suckling colostrum and at the following times after suckling: 12, 24, 48 and 72 hours: 1, 2 and 3 weeks; 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0 months. According to farm records, the mothers of these animals had been vaccinated against FMD more than seven years before only on two ocasions (4 and 8 months of age), and had been exposed to an outbreak of the disease 3 years before, when type "A" FMDV had been isolated and identified (17).

Antigens

Strains of subtypes "O_1" Campos, "A_{24}" Cruzeiro, "A Venceslau" and "C_3" Indaial were cultured on BHK-21 clone 13 cells and the viral suspensions were used as antigens for the CFT.

The antigen suspension of strain "O1" was also used for the preparation of the VIA antigen by the technique of Lobo *et al.* (6).

The FMDV used to prepare the VIA antigen can be any type because it is not type specific. The chomatography column was made by mixing 1.0 gm Sephadex A 50 (Pharmacia, Uppsala, Suecia) with 1.500 ml virus suspension. The column was washed with a solution containg 0,02M tris-buffer, 0.15 M NaCl, pH 7.6 to elude the 140 S and 12 S fractions. After which the VIA antigen was eluted with 0.02 M tris-buffer, 1.0, M NaCl, pH 7.6.

Complement Fixation Test

The 50% hemolysis technique of Bier & col. (1) was used to determine complement fixing antibody titers against the above viral strains and against the VIA antigen. Noninfected cell suspensions were used as controls to determine possible cross-reactivity of the crude antigen suspensions. From inicial 1/5 diluition the serum samples were diluted at double dilution until resch 1/5.120 in duplicate with the tubes being kept on ice. Each diluition were mixed individually with the FMDV antigens "O_1", Campos, "A_{24}" Cruzeiro, "A Venceslau", "C_3" Indaial and a non infected cell suspension. After incubation for 18 hours at 4°C, it was added the hemolytic system and the mixture was reincubated at 37°C for 30 minutes. Then the tubes were centrifuged and the degree of hemolysis determined by the method of grafic reading. The complement fixing antibody titer was the range of the both duplicated diluitions expressed as decimal logarithm.

Agar Gel Double Diffusion Test

Anti-VIA antigen precipiting antibodies were determined qualitatively by the procedure discribed by Pinto & Hedger (12). The immunodiffusion test was mode by the method of double-immunodiffusion. It was used purified agar 1.5% (W/V) in 0.02 M Tris-buffer, 0.15M NaCl, pH 7.6. The wells (1 central and 6 equidistants) 4 mm long and 5 mm apart were cut into the agar. The antigen was placed into of central well. Two positives hyperimmune sera were placed into of two opposite wells. In the others four wells the sample of serum were placed to be tested. The presence of the lures of precipitation was checked out daily in the plates incubated at environment temperature for 5 days.

RESULTS

Determination of Precipiting Antibodies

In Table1 are presented the results of three female Indian buffaloes at the end of gestation were first selected so that one of them was strongly reactive, and the other two were not, to the agar gel double diffusion test for the detection of

TABLE 1 - Determination of precipiting antibodies response to VIA antigen in serum sample take from buffalo calves and their dams.

Animal Number	Serum from Dams			Serum from Buffalo Calves		
Sample	1	2	3	1	2	3
Before parturion	+	-	-	-	-	-
Parturiom: 0 h.	+	-	-	-	-	-
Pos partum: 12 h.	+	-	-	+	±	-
24 h.	+	-	-	+	±	-
48 h.	+	-	-	+	±	-
72 h.	+	-	-	+	±	-
1st week	+	-	-	+	-	-
2st week	+	-	-	+	-	-
3st week	+	-	-	+	-	-
1.0 month	+	-	-	+	-	-
1.5 month	+	-	-	+	-	-
2.0 month	+	-	-	+	-	-
2.5 month	+	-	-	+	-	-
3.0 month	+	-	-	±	-	-
3.5 month	+	-	-	±	-	-
4.0 month	+	-	-	±	-	-
4.5 month	+	-	-	±	-	-
5.0 month	+	-	-	-	-	-
5.5 month	+	-	-	-	-	-
6.0 month	+	-	-	-	-	-

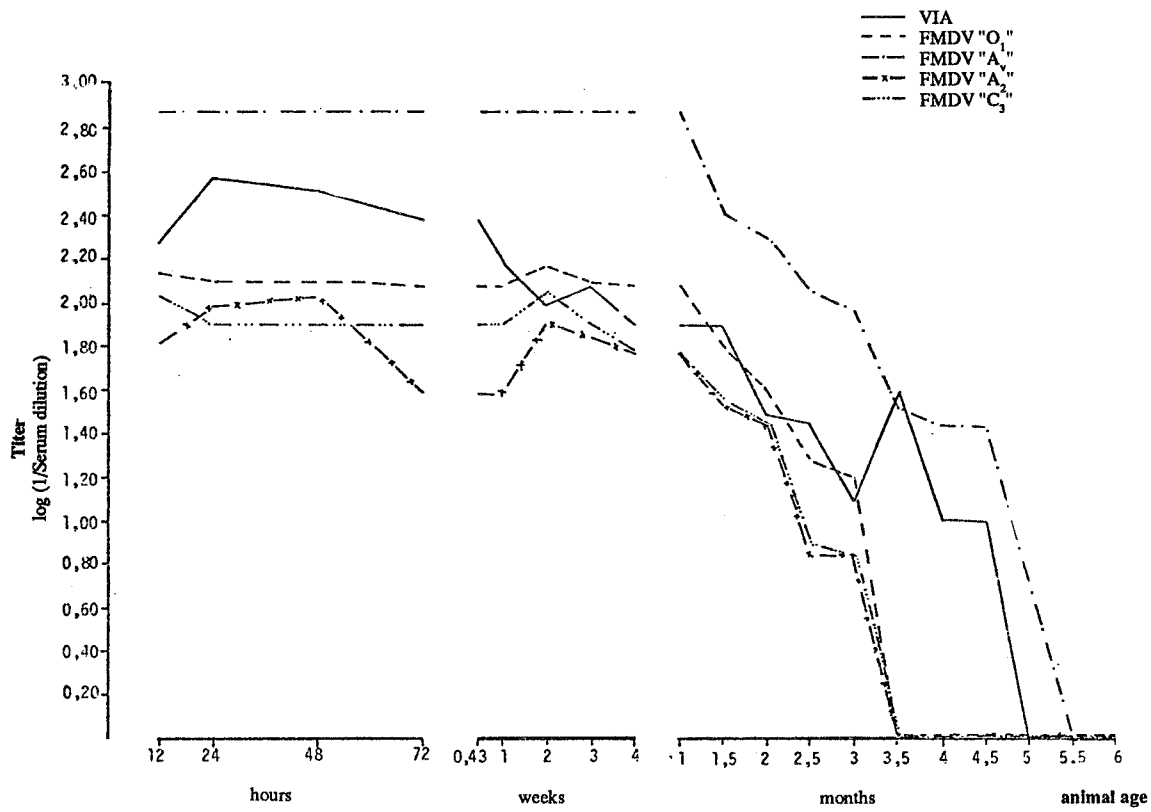

FIGURE 1 - Complement fixing antibody (Y) titers (log 1/serum dilution) against strains "O_1" Campos, "A Venceslau", "A_{24}" Cruzeiro and "C_3" Indaial and the VIA antigen of foot-and-mouth-disease virus determined on different days of life in calf nº. 1.

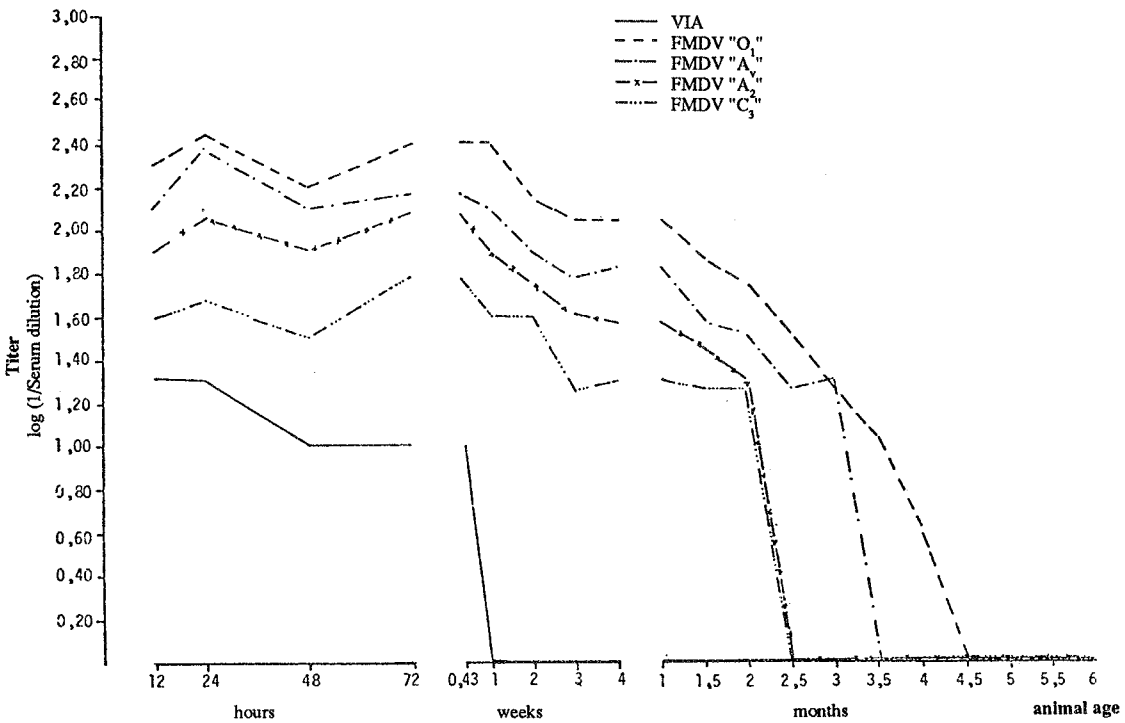

FIGURE 2 - Complement fixing antibody (Y) titers (log 1/serum dilution) against strains "O_1" Campos, "A Venceslau", "A_{24}" Cruzeiro and "C_3" Indaial and the VIA antigen of foot-and-mouth disease virus determined on different days of life in calf nº. 2.

anti-VIA antigen antibodies. When the test was performed on the respective calves, none of them showed the presence of these antibodies before suckling colostrum.

In the sample collected 12 hours after suckling, calf no. 1 (born to the dam that reacted to the VIA antigen), was also reactive, and this reactivity persisted in the subsequente samples for up 4.5 monts.

Calf no. 2 (born to one of the nonreactive dams), contrary to expectation, reacted up to 72 hours after birth, no longer reacting thereafter.

Calf n.3 (born to the other nonreactive dam), did not react to the test at any throughout the experiment. The above results, as well as data obtained for other animals, have been described in detail by Samara *et al.* (18).

Complement-Fixing Antibody Titers

Titration data referring to complement-fixing antibodies against the viral strains and the VIA antigen are presented as decimal logarithms in the figures.

Animal no. 1 (Figure 1) had the highest titer and duration (lowest detectable titer) of antibodies against the " A Venceslau" strain (2.87 at 12 hours and 1.43 at 4 and 4.5 months of age) than against the "O_1", "A_{24}" and "C_3" FMDV strains (2.14 at 12 hours, 2.03 at 12 hours and 1.20, 0.84 and 0.84 at 3.0 months of age). This same animal showed the highest titer (2.57) for anti-VIA antigen antibodies at 24 hours and the lowest detectable titer (0.1) at 4.5 months.

Animal no. 2 (Figure 2) showed the highest titer (2.45) at 24 hours and the lowest (0.60) at 4.0 months of age for antibodies against the "O1" strain. Titers for the other viral strains were 2.38 at 24 hours, 2.08 at 72 hours and 1.78 also at 72 hours of age for "A Venceslau", "A24" and "C3", respectively. The lowest detectable titer was 1.30 at 3 months, 1.28 and 1.26 at 2 months of age for a "A Venceslau", "A_{24}" and "C_3", respectively. The highest titer of anti-VIA antigen antibodies (1.32) was determined at 12 hours and the lowest (1.0) at 72 hours after birth.

Figure 3 shows the results for animal no. 3. Differences in antibody titers for the different viral strains were small, with values of 2.15, 2.20, 2.12 and 2.10 being obtained at 24 hours of age for "O_1", "A Venceslau", "A_{24}" and "C_3", respectively. No anti-VIA antigen antibodies were detected in any of the sample collected from this animal.

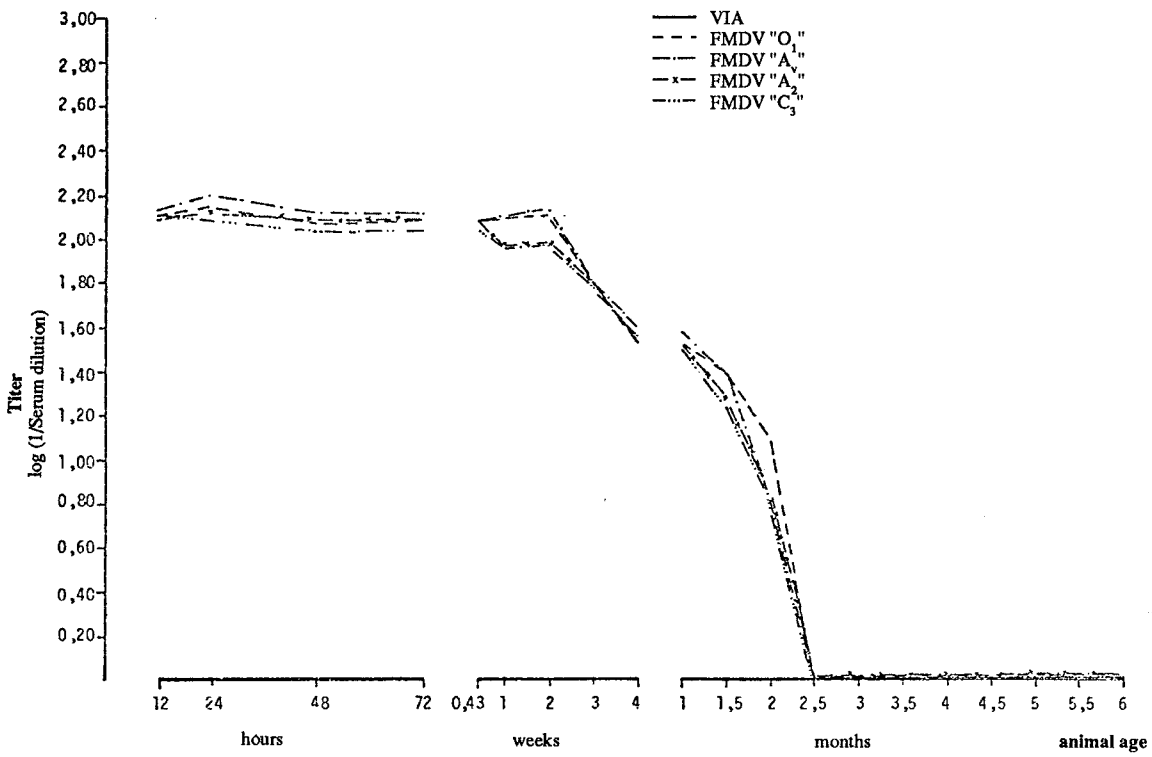

FIGURE 3 - Complement fixing antibody (Y) titers (log 1/serum dilution) against strains "O_1" Campos, "A Venceslau", "A_{24}" Cruzeiro and "C_3" Indaial and the VIA antigen of foot-and-mouth disease virus determined on different days of life in calf nº. 3.

DISCUSSION

The results of anti-VIA antigen antibody quantification obtained in the present study by the complement fixation test in buffalo sera indicate that technique can be used to greater advantage than the qualitative agar gel double diffusion technique.

So, analysis of the results of titration of buffalo sera at different times in the animals life shows life coherence between the complement fixing colostral antibodies for the virus and for the VIA antigen of FMDV. When these antibodies were titrated in calf no. 1, the curves for the strains studied were quite different, though the reactions decreased in all of them, with higher titers and duration for the FMDV strain "A Venceslau". Decreasing reactions for the VIA antigen also occurred concomitantly, their duration being very close to that of the reaction with the "A Venceslau" strain of FMDV. Since these are colostral antibodies, all indications are that the mother of this calf probably underwent a viral replication of the "A" type of FMDV, though the possibility that the calf maintains also antibodies of vaccine origin should not be ruled out.

Antibody titration in calf no. 2 also showed a distinct curve for each viral strain, the highest titer and duration being for the "O_1" strain of FMDV. In this particular case there was also a decreasing reaction to the VIA antigen, which, however, was of short duration, demonstrating that the mother, even though nonreactive by double diffusion or by the complement fixation test, was able to transfer antibodies to the so at levels detectable by the two test used. For this reason, the etiology of the probable viral replication to which the mother was subjected is not clear.

Antibody titration in calf no. 3, which was also the can of a dam not reacting to the VIA antigen, showed not well distinct reactions for the various strains studied and undetectable anti-VIA antigen antibody titers. We believe that this was an animal that did not undergo viral replication.

The evidence of previous infection in the mothers of the first two animals is reinforced not only by the CFT results for each viral strain and for the VIA antigen of FMDV, but also by the presence of some form of positive reactivity in the double diffusion reaction, as also reported by other investigators in

studies on infected buffaloes (5, 8, 17, 19, 20).

The CFT used in the present study (1) was not more sensitive than agar gel double diffusion (7) for the detection of anti-VIA antigen antibodies. The great advantage of the test, however, was the quantification of these antibodies, which had always been considered very complex in view of the antigenic composition of the VIA antigen.

Even though in the present investigation we worked with field animals, we did not note the occurrence of heterotypic cross-reactions. The objective was to show the possibility of quantifying anti-VIA antigen antibodies by the CFT, and the results were very promising, opening perspectives for future studies for the improvement and standardization of this technique for routine use.

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RESUMO

Medidas de anticorpos anti-virus da febre aftosa empregando suspensões de antígenos brutos e VIA, pelo método quantitativo de fixação do complemento, em búfalos.

Anticorpos contra o antígeno associado a infecção viral (VIA) e contra suspensões antigênica brutas das estirpes "O_1" Campos, "A". Venceslau, "A_{24}" Cruzeiro e "C_3" Indaial do vírus da febre aftosa (VFA), adquiridos através da imunização natural passiva em bubalinos, foram determinados pela reação de fixação de complemento. Três animais foram testados periodicamente para a presença dos anticorpos até os 6 meses de idade. Os resultados mostraram que é perfeitamente possível determinar pela técnica direta de fixação de complemento, anticorpos contra o antígeno VIA e contra diferentes estirpes do VFA em búfalos, de forma satisfatória, característica esta não observada em bovinos.

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ROTAVIRUS AND ADENOVIRUS IN DIARRHEIC AND NON-DIARRHEIC FECES OF CHILDREN, IN ARARAQUARA, SP, BRAZIL

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SUMMARY

The presence of rotavirus and adenovirus has been investigated in normal and diarrheic stools of 280 children younger than five years, in Araraquara – SP, Brazil. From the 280 fecal samples, 140 were collected from children with acute diarrhea and 140 from children without gastroenteritis symptoms – control group. It was used an immunoenzymatic assay (EIARA) for detection of both viruses. In addition, it was used polyacrylamide gel electrophoresis (PAGE) for rotavirus. 8.6% of diarrheic feces were positive for rotavirus using EIARA and 7.9% using PAGE. This virus was not detected in feces of the control group. Adenovirus was detected in 2.9% of diarrheic feces and in 1.4% of controls. The results show that rotavirus is an important agent of the infant diarrhea in Araraquara, SP.

Key Words: Rotavirus, Adenovirus, diarrhea.

INTRODUCTION

Several viruses have been associated with human gastroenteritis, e.g. rotavirus, Norwalk virus, adenovirus, calicivirus, astrovirus e coronavirus. However only the first three show medical significance so far (4).

The first association of rotavirus with infantile gastroenteritis was reported in Australia, when viral particles were observed in duodenal biopsies from children with gastroenteritis by thin-section electron microscopy (3).

Rotavirus infections are more frequent in children between 6 months and 2 years old and are less frequent in older than 5 years children and in adults (4, 8, 9). The infection can be mild or severe, being frequently characterized by watery diarrhea, vomit and fever (9).

The cultivation of rotavirus in cell culture is not a routine and electronic microscopes are not available in most of the laboratories so, a variety of methods have been developed for detection of rotavirus in feces, e.g. complement fixation (6), indirect immunofluorescence (5), counter immunoelectroosmophoresis (7), latex agglutination (12), Staphylococcal co-agglutination (21), detection of viral RNA by polyacrylamide gel electrophoresis (16, 18), enzyme immunoassay (12, 16, 17) and recently hybridization with DNA probes (1, 16).

Adenovirus 40 and 41 have been recently associated with infantile gastroenteritis (11, 13).

The cultivation of this virus, like rotavirus, is not easy so other methods are being used in its detection in feces, e.g. enzyme immunoassay (13, 17) and hybridization with DNA probes (11, 15).

The purpose of this study was to verify what is the participation of rotavirus and adenovirus in the etiology of the infantile diarrhea in Araraquara – SP, Brazil.

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MATERIAL AND METHODS

Fecal samples – 280 fecal samples of children from 0 to 5 years, receiving medical care at SESA–USP in Araraquara, were collected from March 1986 to December 1987. 140 of these samples were from children with gastroenteritis symptoms (diarrheic group) and 140 were from children without these symptoms within the last 30 days (control group).

Fecal samples were suspended at 10-20% in phosphate buffered saline (pH 7.4) and clarified by centrifugation at 3,000 rpm for 15 minutes. Supernatants were stored at -20°C for no longer than one month.

Detection of viruses – Rotavirus and adenovirus were detected by using a combined enzyme immunoassay (EIARA) as described by Pereira *et al.* (17). In addition, genomic RNA of rotavirus was detected by polyacrylamide gel electrophoresis (PAGE) (18).

RESULTS

Rotavirus was detected in 8.6% of diarrheic feces using EIARA and in 7.9% using PAGE. This virus was not detected in feces of the control group. Adenovirus was detected in 2.9% of feces of the diarrheic group and in 1.4% of control ones. Results are shown in Table 1.

Table 2 shows the distribution according to the age of positive cases for rotavirus and adenovirus within the diarrheic group.

TABLE 1 - Frequency of rotavirus and adenovirus in feces of children in Araquara – SP.

Group of children	Samples collected	Positive Samples		
		Rotavirus		Adenovirus
		EIARA Nº (%)	PAGE Nº (%)	EIARA Nº (%)
Diarrheic	140	12 (8.6)	11 (7.9)	4 (2.9)
Control	140	—	—	2 (1.4)

TABLE 2 - Distribution according to the age of positive cases for rotavirus and adenovirus within the diarrheic group.

Age (months)	Tested	Samples	
		Positive	
		rotavirus (%)	adenovirus (%)
0 ⊣ 3	7	—	—
3 ⊣ 6	27	5 (18.5)	2 (7.4)
6 ⊣ 2	38	—	1 (2.6)
12 ⊣ 18	27	5 (18.5)	1 (3.7)
18 ⊣ 24	15	1 (6.7)	—
> 24	26	1 (3.8)	—

DISCUSSION

Rotavirus was detected in 8.6% of diarrheic feces and it was not detected in feces of the control group. This result is lower than those related by many workers in diferent cities in Brazil (2, 10, 14, 19, 20). In spite of being low, this result shows that rotavirus is an important agent in the infantile diarrhea in Araraquara.

Adenovirus was detected in 2.9% of feces in the diarrheic group and in 1.4% of the control group. The value observed in the diarrheic group is lower than those of other workers once again. Adenovirus was detected twice more in the diarrheic group than the control group but the absolut values are low for any interpretation of these results. In a similar work, Linhares *et al.* (14) have detected adenovirus in 4.0% of feces of the diarrheic group and in 4.8% of feces of the control group.

Two methods were utilized in the detection of rotavirus and they have shown a similar eficiency (Table 1). In one sample rotavirus was only detected by EIARA. Pereira *et al.* (18) have related the same situation and the sample was confirmed as positive by immunoelectron microscopy. The utilization of more than one method for detection can result in a more secure diagnoses of rotavirus infection.

According to the age, the distribution of positive cases within diarrheic group (Table 2) was in agreement with other workers being more frequent among children between 3 months and 2 years. In spite of the low number of positive cases, the frequence of adenovirus was higher among children between 3 and 18 months. This result shows a little difference to that related for Perez *et al.* (19).

Rotavirus infections occur more frequently during the winter in countries with temperate climate; in tropical countries the frequency is the same throughout the year, not showing a characteristic seasonal pattern (9). Although the number of available samples in our study varied from month to month and, thus, the seasonal pattern of rotavirus infection could not be properly assessed. The number of positive cases from rotavirus was higher during the winter.

This is the first study that relates the ocurrence of rotavirus and adenovirus in feces of children in Araraquara and the association of these viruses with the infantile diarrhea in the region.

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RESUMO

Ocorrência de rotavírus e adenovírus em fezes diarréicas e não diarréicas de crianças, em Araraquara – SP, Brasil.

Procurando verificar a participação de rotavírus e adenovírus na etiologia da gastrenterite infantil, 280 amostras de fezes de crianças da faixa etária de 0 a 5 anos foram coletadas em Araraquara – SP, sendo 140 de crianças com diarréia e 140 de crianças do grupo de controle. A pesquisa desses vírus foi realizada através de um ensaio imunoenzimático (EIARA) e adicionalmente, rotavírus foi pesquisado através da eletroforese em gel de poliacrilamida (PAGE). No grupo diarréico, rotavirus foi detectado em 8,6% das amostras usando EIARA e em 7,9% usando (PAGE). Nenhuma das amostras do grupo controle se mostrou positiva para esse vírus. Adenovírus foi detectado em 2,9% das amostras do grupo diarréico e em 1,4% das fezes do grupo controle. Os resultados mostram que os rotavírus participam ativamente da etiologia da diarréia infantil em nossa cidade.

Palavras-chave: Rotavírus, Adenovírus, diarréia.

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DETECÇÃO DE ROTAVÍRUS EM DIARRÉIAS CRÔNICAS E GASTRENTERITES AGUDAS INFANTIS EM BELO HORIZONTE, MG

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RESUMO

Foi realizado um estudo em crianças atendidas no Hospital das Clínicas da UFMG, tendo sido o rotavírus detectado em amostras fecais de 36 (17,14%) crianças não hospitalizadas, em um grupo de 210 crianças com diarréia; e em 14 (56,0%) crianças, de um grupo de 25 crianças hospitalizadas com gastrenterite aguda. As amostras fecais foram testadas em ELISA e os perfis eletroforéticos do RNA foram determinados em gel de poliacrilamida. Entre 42 amostras de vírus analisadas, três diferentes eletroforetipos foram observados, com marcada predominância de um tipo.

Palavras-chave: Rotavírus, diarréia, gastrenterites.

INTRODUÇÃO

A gastrenterite aguda é uma doença muito comum nas crianças, que ocorre nas formas epidêmica e endêmica. É sabido que essa síndrome ocupa o segundo lugar nas doenças infantis mais freqüentes, sendo suplantada apenas pelas infecções do trato respiratório. A etiologia viral das gastrenterites agudas infecciosas não bacterianas foi esclarecida apenas nos últimos 17 anos, através do exame de espécimes clínicos humanos em microscopia eletrônica (1,2). Até o presente momento, os rotavírus, os vírus Norwalk e os adenovírus entéricos são reconhecidos como os agentes etiológicos mais importantes em gastrenterites humanas(4). A identificação e caracterização dos vírus que causam as gastrenterites, bem como uma clara compreensão de uma epidemiologia e imunologia, são necessárias para que se possa evitar sua transmissão e se obter vacinas eficientes para o seu controle.

MATERIAL E MÉTODOS

Amostras de fezes - Foram colhidas amostras de fezes de crianças com menos de dois anos de idade, em três diferentes situações clínicas:

1. 210 crianças com diarréia aguda, mas que não foram hospitalizadas;
2. 25 crianças com gastrenterite aguda, hospitalizadas com os seguintes sintomas: desidratação, vômito e febre, em estado de subnutrição. Esse grupo de crianças teve suas fezes colhidas no primeiro dia, antes de serem submetidas à reidratação; e depois, no 5º e no 30º dias;
3. 124 crianças com diarréia crônica, cujas fezes foram enviadas ao Laboratório Central do HC da UFMG com suspeita de parasitose intestinal. Nessas crianças foi constatada a presença de um a quatro dos seguintes parasitas intestinais: *Strongyloides, Enta-*

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moeba coli, Ascaris lumbricoides, Necator americanus, Schistosoma mansoni, Trichocephalus trichiurus e Giardia lamblia. Todas as amostras de fezes foram suspensas em solução salina tamponada a 10-20% e centrifugadas a 4.000 G durante 5 minutos, sendo o sobrenadante conservado a -20°C.

Ensaio imunoenzimático (EIARA) - Foram usados kits cedidos pelo Dr. Hélio G. Pereira, da *FIOCRUZ.* A técnica utilizada foi a descrita por Pereira & col.(11).

Eletroforese em gel de poliacrilamida (PAGE) - Também foi adotada a técnica empregada por Pereira & col.(10).

RESULTADOS

Dos três grupos de crianças examinadas, obteve-se os seguintes resultados:

1. de 210 crianças com diarréia comprovou-se a presença de rotavírus nas fezes de 36 crianças (17,14%), através dos testes de EIARA e/ou PAGE;
2. no grupo de 124 crianças com diarréia crônica não se detectou rotavírus nas fezes de nenhuma criança, tendo sido utilizado apenas o teste de ELISA;
3. no grupo de 25 crianças, que foram acompanhadas durante 30 dias, obteve-se os resultados da Tabela 1.

TABELA 1 - Amostras de rotaváirus nas fezes de crianças, colhida s no 1, no 5 e no 30 dias após a manifestação clín doença.

Amostras de fezes	Número	Positivas*	%
No dia 1	25	14	56,0
No dia 5	25	3	12,0
No dia 30	25	3	12,0
Total	75	20	26,6

* Em testes de EIARA e PAGE

Para a determinação do tipo eletroforético, adotou-se a classificação sugerida por Lourenço & col.(8). A grande maioria das amostras obtidas pertencia ao tipo cbfa; duas amostras eram do tipo cbda; e uma amostra era do tipo bada.

Entre as amostras de vírus analisadas(42), 7 apresentaram mais de 11 bandas.

DISCUSSÃO

A análise dos resultados obtidos com o exame das fezes de crianças diarréicas em três graus distintos de severidade demonstra a participação do rotavírus de maneira também distinta. Naqueles casos em que o diagnóstico clínico foi de parasitose intestinal não foi constatada a presença de vírus em um só caso: portanto, os sintomas podem ser atribuídos apenas àqueles parasitas encontrados nas fezes. No segundo grupo, em que havia diarréia aguda, já se verificou a presença do vírus em 17,14% dos casos, o que corresponde com os dados da literatura. Peres & col..(12) encontraram 21,05% de casos positivos para rotavírus em 152 crianças com diarréia aguda; Linhares & col.(6) encontraram 33,1% em 369 crianças com diarréia aguda. No terceiro grupo, cuja sintomatologia era mais grave, exigindo tratamento ambulatorial com reidratação oral nas primeiras 24 horas, houve um aumento bastante significativo da presença do vírus, subindo para 56,0%. É interessante notar que os primeiros relatos sobre os rotavírus revelam também uma alta incidência do vírus em crianças com um quadro clínico agudo de gastrenterite. Bishop & col. (1,2) encontraram 78,57% de rotavírus em extratos fecais, e 66,6% em biópsia duodenal de crianças através de microscopia eletrônica, em um grupo de 14 e outro de 9 crianças, respectivamente.

Vários estudos têm sido feitos com o objetivo de se classificar sorotipicamente os rotavírus, sabendo-se hoje que há no mínimo 4 sorotipos. A divisão original em dois subgrupos, I e II, baseada em provas de fixação do complemento e ensaios imunoenzimáticos, foi mantida. Paralelamente ao desenvolvimento da classificação dos rotavírus em sorotipos e subgrupos, vários pesquisadores têm envidado esforços para classificá-los mediante a mobilidade dos segmentos RNA de seus vírions em eletroforese. A despeito da extensa diversidade entre os tipos eletroforéticos já encontrados e, portanto, de diferentes propostas de classificação baseadas nessa diversidade, há dois modelos distintos de migração de RNA: o "curto" e o "longo", associados com os subgrupos I e II, respectivamente(9). Chanock & col.(3) acham que é improvável que a eletroforetipagem possa ser utilizada como uma forma de classificação geral, mas que ela pode ser útil como informação adicional em epidemiologia, já que diferentes tipos eletroforéticos podem predominar em determinadas regiões. A questão crítica é se essa diversificação reflete diversidade antigênica. Se assim for, então,

isto terá um profundo efeito na estratégia a ser empregada na prevenção da doença, com a introdução de vacinas.

O estudo eletroforético das amostras analisadas revelou a grande predominância de um tipo, o cbfa, tipo "longo".

O encontro de sete amostras com mais de 11 segmentos sugere a presença de mais de um vírus. Segundo Estes & col.(5), uma infecção dual pode resultar do aparecimento de uma nova amostra de vírus devido a uma redistribuição dos genes.

No grupo de 25 crianças que necessitaram de tratamento ambulatorial, três crianças ainda excretavam vírus no 5º e no 30º dias. Este fato sugere que essas crianças passaram a ser portadoras do vírus, pois não apresentavam quaisquer sintomas da doença. Linhares & col.(7) encontraram cinco crianças sem sintomas com o vírus no primeiro ano de vida.

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SUMMARY

Detection of rotavirus in stools of diarrhoeic children in Belo Horizonte, MG, Brazil

Rotavirus was detected in faecal samples from 36 (17.14%) of 210 diarrhoeic out-patient children, and in 14 (56.0%) of 25 diarrhoeic in-patient children. This latter group had their samples collected on days first, 5th and 30th. Faecal samples were used as a 10-20% suspension in PBS, stored at -20° C until the moment of testing. These samples were tested by ELISA technique, used as a double-antibody sandwich assay.

Capture sera were obtained from adult goats, immunized with SA 11 virus. Detector sera were produced in adult guinea pigs, immunized with human rotaviruses Wa or DB strains. Conjugate was obtained by coupling of peroxidase with purified IgG from serum of a rabbit immunized with guinea pig IgG.

The electrophoretypes of the dsRNA were determined in polyacrylamide gel electrophoresis. Viral RNA was extracted from crude faecal suspensions by treatment with SDS, followed by deproteinization with phenol-chloroform. After centrifugation, the pellet was treated with 5M urea, 3% 2-mercaptoethanol and 0.001% bromophenol blue.

Electrophoresis was carried out according to the technique used in FIOCRUZ. Gels were stained by silver impregnation. Any pattern in which at least the four first bands were clearly visible were considered as positive.

Out of the 25 in-patient children three continued to excrete virus through the 5th and 30th days. Among 42 virus samples analysed by electrophoresis, three different classes of electrophoretypes were observed, with a marked predominance of one class.

It was also observed that stools from seven children revealed genome profils with more than 11 segments, which points towards a dual infection. It is suggested that new virus strains could appear by reassortment of the genes present in the faecal samples. Rotavirus antigen was not detected by ELISA in stools from 124 chindren with chronic diarrhoea. They had intestinal parasitosis.

Key Words: Rotavirus, diarrhoea, gastroenthoeritis

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EMPREGO DE ALGUMAS FONTES NITROGENADAS NA OBTENÇÃO DE BIOMASSA DE *OSCILLATORIA LIMNETICA*

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Sunao Sato
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RESUMO

Foi realizado um estudo do rendimento de biomassa e de proteínas totais da cianobactéria *Oscillatoria limnetica* em meios sintéticos com diferentes fontes de nitrogênio. Utilizaram-se nitrato de potássio, uréia e cloreto de amônio, em cinco diversas concentrações cada um. A melhor produção de biomassa da *O. limnetica* foi conseguida em meios contendo nitrato de potássio a 2,57 g/L, porém com uma oferta de 1,0g/L, obtiveram-se resultados bastante favoráveis, assim como com a uréia e o cloreto de amônio em baixas concentrações. As concentrações proteicas determinadas ficaram na faixa de 30,3 - 82,7%. Conclui-se que os valores satisfatórios de biomassa e proteína podem ser obtidos com concentrações econômicas de nitrato no meio de cultura, ou empregando-se baixas concentrações de uréia e amônio, a um custo ainda mais reduzido.

Palavras-chave: *Oscillatoria limnetica*; biomassa, cianobactéria.

INTRODUÇÃO

As cianobactérias representam uma das fontes alternativas de proteínas e sua utilização como complemento alimentar já foi ensaiada em condições experimentais e industriais.

A maior experiência disponível relaciona-se à *Spirulina maxima*, mas muitas investigações foram realizadas também com espécies do gênero *Oscillatoria* (5, 9, 10)

O gênero *Oscillatoria* ainda não é aproveitado em termos de suprimento alimentar, mesmo sabendo-se que contém alto teor proteico, em parte por apresentar teores de DNA/RNA extremamente elevados. Este gênero não possui um sabor atraente e algumas espécies, como a *Oscillatoria brevis* (4) excretam compostos de odor desagradável como a geosmina.

As "Oscillatorias" são cianobactérias formadas apenas de células vegetativas que possui tricomas livres, solitários, pluricelulares, retilíneos ou com inflexões.

As necessidades nutritivas e a tolerância ao oxigênio permitem às cianobactérias desenvolverem-se nas camadas ricas de O_2 dos ambientes aquáticos, próximos à interface ar-água. Porém algumas cianobactérias crescem junto com as bactérias vermelhas e verdes em ambientes aquáticos ricos em H_2S. É o caso da *O. limnetica* que exibe tanto fotossíntese oxigênica quanto anoxigênica.

Uma das principais dificuldades para a ampla utilização dessas fontes de biomassa diz respeito aos custos de produção elevados, devidos em parte à necessidade de consumo de fontes nitrogenadas dispendiosas.

Neste trabalho, definiram-se fontes experimen-

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tais de nitrogênio visando a diminuição de custos de produção, e o estabelecimento de novas opções para a produção de biomassa a partir da *O. limnetica*.

MATERIAL E MÉTODOS

Estudou-se uma cepa de *O. limnetica* proveniente do "Centro di Studio dei Microorganismi Autotrofi" da Universidade de Florença, Itália.

A cepa foi ativada por repicagem em tubos contendo o meio de cultivo (6) em ágar, mantidos por uma semana em estufas a 30°C e 3 Klux de intensidade luminosa e, em seguida, conservados a temperatura ambiente e luz do dia.

TABELA 1 – Composição do meio de cultura utilizado

	g/L	Solução de microelementos	mg/L
NaCl	0,92	$SnCl_2 . 2H_2O$	304
Na_2SO_4	1,88	$MnCl_2 . 4H_2O$	173
K_2HPO_4	0,50	$CoCl_2 . 6H_2O$	194
Na_2CO_3	8,89	$SeCl_2 . 2H_2O$	146
Na_2HCO_3	15,15	$BaCl_2 . 2H_2O$	2846
$CaCl_2 . 2H_2O$	0,05	LiCl	293
KNO_3	2,57	$CuSo_4 . 5H_2O$	186
$MgSO_4 .7 H_2O$	0,25	$NiSO_4 . 6H_2O$	717
		$Na_2MoO_4 . 2H_2O$	121

Sol. de Fe-EDTA - 1mL
Sol. de microelementos - 1mL

A composição do meio (6) básico utilizado é mostrada na Tabela 1, com exceção da fonte nitrogenada que variou com os experimentos e são mostrados na Tabela 2.

TABELA 2 – Fontes de nitrogênio e suas concentrações

Fontes de nitrogênio	Concentrações (g/L)
KNO_3	0,5 – 1,0 – 2,57 – 5,0 – 10,0
$(NH_2)_2CO$	0,1 – 0,3 – 0,5 – 0,75 – 1,0
NH_4Cl	0,1 – 0,2 – 0,3 – 0,5 – 0,8

O pH inicial do meio era 9,5± 0,2. Para crescimento em nitrato utilizava-se este pH e para meios contendo uréia ou amônio o pH era ajustado para 8,4 ± 0,2 (11) com solução de ácido clorídrico diluído 1:3. Para a obtenção da concentração celular de inóculo desejada foram usados erlenmwyers de 500mL com 40% do meio, agitados a 100rpm, 30°C e 3,5 Klux.

Para a obtenção da biomassa usaram-se erlenmeyers de 500ml com 160 ml de meio a 40 ml de inóculo. Este inóculo, preparado como descrito no parágrafo anterior, correspondia a uma massa de 50mg/L.

Os erlenmeyers eram colocados em "shakers" por 10 dias com temperatura, agitação e intensidade luminosa iguais aos usados para o crescimento dos inóculos.

Valores de pH e biomassa foram determinados diariamente.

A concentração da biomassa era medida em espectrofotômetro Procyon SC 90 em comprimento de onda 625 nm. O teor proteico total determinado no final do ensaio, foi medido pelo método de Kjeldahl (2, 3) verificando-se o teor de nitrogênio total e convertendo-o em proteína por simples multiplicação fatorial.

RESULTADOS

KNO_3 como fonte nitrogenada – Na biomassa produzida no meio KNO_3, o rendimento mais vantajoso consubstanciou-se naquele contendo 2,57 g/L, porém é conveniente assinalar que também com ofertas consideravelmente mais baixas (0,5 e 1,0 g/L), os resultados foram apenas ligeiramente afetados, com menos de 20% de diferença na biomassa.

Não houve fase lag de crescimento e em todas as concentrações a fase estacionária foi atingida no oitavo dia (Figura 1).

O pH do meio de cultura durante o ensaio praticamente não se alterou. Variou de 9,8 no início

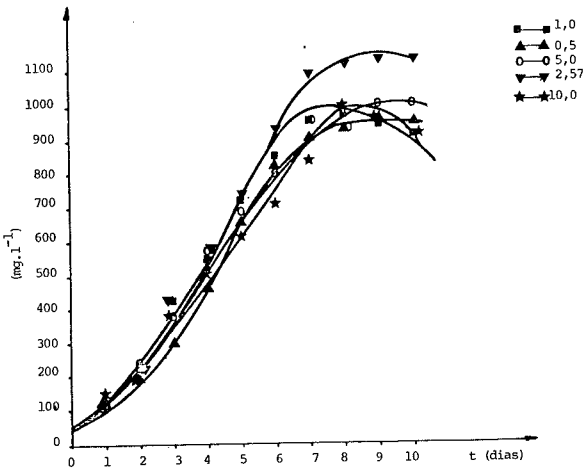

FIGURA 1 — Concentração de biomassa da *Oscillatoria limnetica* em função do tempo, para diferentes concentrações de KNO_3 (g/L).

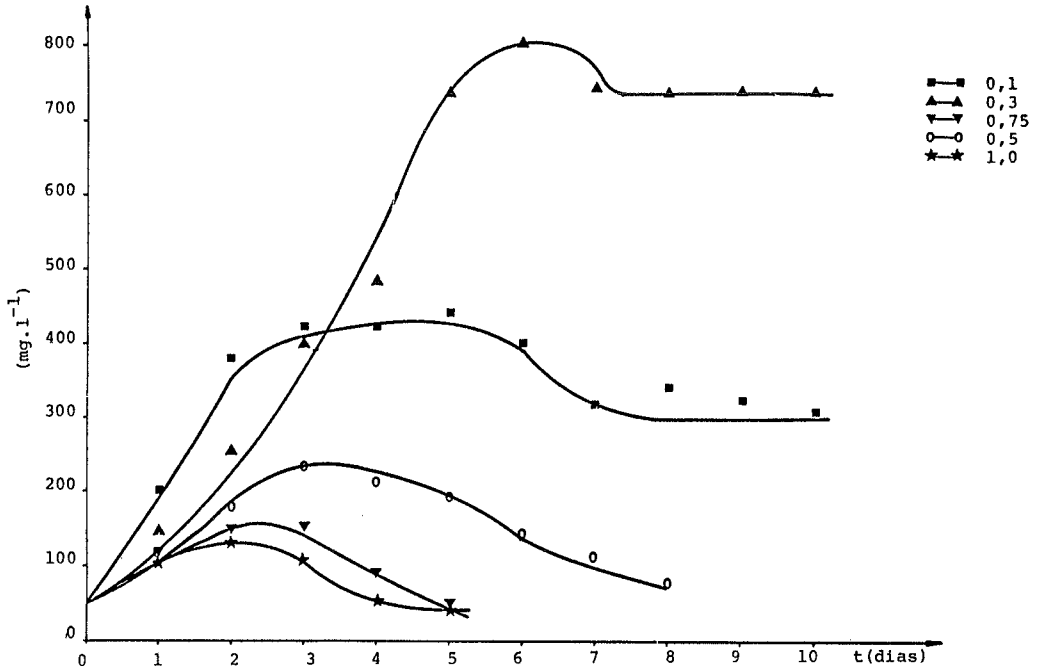

FIGURA 2 — Concentração de biomassa da *Oscillatoria limnetica* em função do tempo, para diferentes concentrações de Uréia (g/L).

do experimento até 10,6, para todas as concentrações de KNO_3 utilizadas.

Uréia como fonte nitrogenada – Percebe-se uma resposta muito satisfatória com baixas concentrações de uréia.

A produção de biomassa em meio com 0,3 g/L foi o dobro da obtida em meios com 0,1 g/L. Não houve fase lag de crescimento, sendo que a fase estacionária foi atingida no sexto dia do experimento, com uma excelente concentração de biomassa (Figura 2).

No meio em que a concentração de uréia foi 0,1 g/L a fase estacionária se iniciou no segundo dia, prolongando-se por mais três dias, seguidos de declínio.

A variação de pH do meio de cultura contendo diferentes concentrações de uréia no crescimento da *O. limnetica* foi de 8,8 a 10,6, denotando uma elevação progressiva desta medida.

NH_4Cl como fonte de nitrogênio – Com a utilização de NH_4Cl no meio de crescimento observou-se que, mesmo em altas concentrações deste sal, não houve despigmentação da cultura podendo-se efetuar leituras no espectrofotômetro até o final do experimento.

A *O. limnetica* teve seu melhor desempenho na concentração de 0,5 g/L, na qual chegou ao final do ensaio sem atingir a fase estacionária. Nota-se também uma fase lag de crescimento. A segunda melhor produção de biomassa ficou com o nível de 0,3 g/L (Figura 3). O comportamento do pH já mencionado reproduziu-se nestes meios acidificados sem modificações importantes, e sem influência apreciável das concentrações de nutrientes.

Teor proteico da biomassa – O conteúdo proteico de *O. limnetica* foi maior nos meios com KNO_3 a 0,5 g/L, uréia a 0,3 g/L e NH_4Cl a 0,5 g/L (Tabela3).

TABELA 3 – Porcentagem proteica obtida nos vários ensaios

Fonte de N	Conc. (g/l)	% de Proteínas Totais
	0,5	78,5
	1,0	70,2
KNO_3	2,57	63,8
	5,0	46,4
	10,0	30,3
Uréia	0,1	64,8
	0,3	79,8
NH_4Cl	0,3	52,6
	0,5	82,7

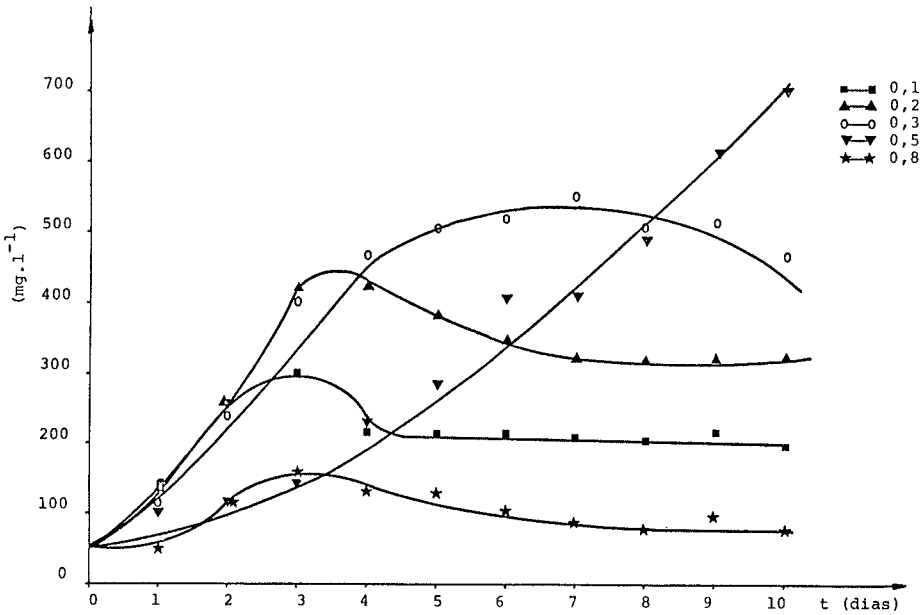

FIGURA 3 — Concentração de biomassa da *Oscillatoria limnetica* em função do tempo, para diferentes concentrações de NH_4CL (g/L).

DISCUSSÃO

O meio de cultura utilizado no crescimento da *O. limnetica* por outros autores (5, 8) difere do nosso, embora valendo-se de nitrato de amônio como fonte principal de nitrogênio, e de constituintes minerais comparáveis aos do meio Paoletti *et al.* (6). Temos adotado para o crescimento da *O. limnetica* o mesmo meio recomendado para *Spirulina*, uma vez que estas cianobactérias crescem juntas em lagos naturais(9).

Após o carbono, e também deixando-se de lado o hidrogênio e o oxigênio, que podem ser obtidos da água, o nitrogênio é quantitativamente o elemento mais importante da matéria seca das células das algas.

Quando o nitrogênio é fornecido na forma oxidada de nitrato ele precisa primeiramente ser reduzido para ser incorporado em moléculas orgânicas.

Em meios contendo diferentes concentrações de KNO_3 a *O. limnetica* desenvolveu-se bem, registrando-se a sua maior produção de biomassa no meio contendo 2,57 g/L.

A segunda fonte de nitrogênio investigada, a uréia, tem um custo bastante inferior ao do nitrato, além do que cada molécula de uréia fornece dois átomos de nitrogênio enquanto cada molécula de KNO_3 apenas um.

A uréia normalmente precisa ser hidrolisada antes que seu nitrogênio possa ser incorporado às células das algas (1).

A *O. limnetica* teve seu melhor crescimento em meio contendo a uréia a 0,3 g/L. Com valores superiores a esta concentração de uréia no meio não obtivemos boa produção de biomassa, sugerindo um fenômeno de inibição.

A terceira fonte nitrogenada utilizada foi o cloreto de amônio, que pode ser eficientemente utilizado pela maioria das algas (7).

Quando em proporções adequadas, o nitrogênio é rapidamente assimilado pela alga carente do mesmo, até quatro a cinco vezes mais rapidamente do que por células normais. Entretanto, a concentração de cloreto de amônio a 0,8 g/L foi inibitória ao crescimento do microrganismo, sendo que a melhor produção ficou com a concentração de 0,5 g/L.

Em alguns dos nossos ensaios não observamos a fase lag (indução) de crescimento, por iniciarmos o experimento com uma pequena quantidade de inóculo, sendo que os microrganismos já vinham crescendo em meio completo.

Note-se que os inóculos, que estavam sendo cultivados em meio de KNO_3, não passaram por exaustão do nitrogênio acumulado antes de serem testados com nutrientes ou concentrações diferentes.

Todavia, não é provável que este fato tenha interferido significativamente na análise e interpretação das curvas de crescimento estudadas nesta investigação, exceto no que se refere à redução

da fase lag em algumas culturas. Conforme já demonstrado todos os dados foram aqui examinados de forma comparativa, o que tende a anular variáveis comuns a todos eles. Outrossim, diante do pequeno inóculo utilizado e da rápida velocidade de crescimento obtida na maioria das curvas, o nitrogênio armazenado na fase pré-inoculação não poderia repercutir prolongadamente sobre o comportamento do microrganismo.

Uma análise dos valores de pH revela que graças aos tampões presentes no meio de cultura, as oscilações desta variável foram sempre modestas, ao longo dos ensaios, nunca alcançado níveis superiores a 11,0 que poderiam ser tóxicos para a cultura.

A *O. limnetica* apresentou o seu maior conteúdo proteico, de valor 82,73%, em meio com cloreto de amônio a 0,5 g/L. Este valor excede um pouco as médias disponíveis na literatura, mas vem ao encontro das proporções alcançadas com outros meios, a saber o de uréia a 0,3 g/l (79,75%), e analogamente o de KNO_3 na concentração de 0,5 g/L (78,5%).

A análise dos resultados expostos permite as seguintes conclusões:

O desenvolvimento deste organismo em meios utilizando uréia, teve sucesso na presença de até 0,3 g/L e com cloreto de amônio de 0,5 g/L, observando-se intolerância a estas fontes e inibição do crescimento com proporções mais elevadas.

Em todas as etapas analisadas a produção de biomassa mostrou-se mais expressiva no meio de KNO_3 a 2,57 g/L, mas com índices geralmente favoráveis, sobretudo nos primeiros dias do ensaio, para os meios à base de nitrato de potássio com 1,0 g/L, uréia com 0,1-0,3 g/L e amônio 0,3-0,5 g/L.

Observaram-se concentrações proteicas de *O. limnetica* na faixa de 30,3 - 82,7%. Os valores mais elevados de proteína associaram-se a culturas mais concentradas neste substrato, quando da introdução de uréia e amônio.

Como decorrência das considerações anteriores, ficou demonstrada a possibilidade de cultivo da *O. limnetica* em meios contendo fontes e concentrações nitrogenadas diversificadas e bastantes econômicas, sem prejuízo do rendimento de biomassa e proteína desta cianobactéria.

SUMMARY

Use of different nitrogen sources in the production of biomass of *Oscillatoria limnetica*.

Analysis of the production of biomass and of the total protein concentration obtained with the cyanobacteria *Oscillatoria limnetica* was undertaken, employing synthetic media and three different nitrogen sources. The selected substances were potassium nitrate, urea and ammonium chloride, in five concentration for each nutrient. The best yield of biomass corresponded to the mixture containing 2.57 g/L of KNO_3. Nevertheless, with a reduction of salt, to 1.0 g/L, almost as good results of biomass could be observed, as well as with urea and ammonium in low proportions. Protein concentrations were distributed in the range of 30.3 - 82.7%. It is concluded that satisfactory rates of biomass and protein can be reached with economical amounts of potassium nitrate, or with low concentrations of urea and ammonium salt, at more favorable cost.

Key Words: *Oscillatoria limnetica*; biomass; cyanobacteria.

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EFEITO DA CONCENTRAÇÃO DE FOSFATO NA SOLUBILIZAÇÃO DE FLUORAPATITA POR *ASPERGILLUS NIGER*

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RESUMO

A síntese da fostatase ácida em *Aspergillus niger* foi controlada pela concentração de fosfato inorgânico do meio de cultura. A adição de fluorapatita reprimiu a produção da enzima devido à solubilização do fosfato insolúvel pelo fungo. A produção da fosfatase e a solubilização da fluorapatita diminuiram com o aumento da concentração de fosfato solúvel, chegando a atingir níveis nulos para uma concentração saturante de fosfato. Quando *A. niger*, cultivado em meio pobre em fosfato, foi transferido para meio rico, verificou-se a repressão da solubilização. Por outro lado, a transferência de micélio de meio rico em fosfato para meio pobre contendo fluorapatita estimulou a solubilização. Não foi possível relacionar a produção de ácidos totais ou a queda do pH, na presença ou ausência de fluorapatita e o processo de solubilização. Aparentemente, o processo de solubilização é controlado pelos níveis de fosfato exógeno de modo similar às fosfatases.

Palavras-chave: *Aspergillus niger*, controle de solubilização, fluorapatita fosfato solúvel, fosfato de rocha.

INTRODUÇÃO

Do total de fósforo existente no solo, 95 a 99% está na forma indisponível às plantas (7). A transformação desse fósforo orgânico ou inorgânico pelos microrganismos em uma forma assimilável pelas raízes das plantas é da maior importância agrícola e econômica. A mineralização do fósforo orgânico ocorre graças às fosfatases, enzimas que propiciam o rompimento hidrolítico de ligações éster de fosfato (3). O estudo dessas enzimas foi relatado em diferentes microrganismos onde foi constatado um mecanismo de controle pela concentração de fosfato solúvel, sendo reprimidas em concentração alta de fostato e desreprimidas em concentração baixa (9, 12, 14, 15). Da mesma forma, as fosfatases estão sujeitas a uma regulação pela fonte de carbono e pelo pH do ambiente (14).

A solubilização de fosfatos inorgânicos decorre da produção de ácidos orgânicos ou inorgânicos, formados principalmente pelos microrganismos ou ainda exsudados pelas raízes das plantas (1, 7). Os ácidos orgânicos são mais efetivos no processo de solubilização, graças ao seu efeito direto ou de quelação dos cátions dos fosfatos insolúveis (17, 19). Em adição, maior quantidade de fósforo solubilizado foi obtida quando se utilizou uma fonte de nitrogênio na forma de amônio que na forma de nitrato ou orgânica (4, 5). Foi demonstrado também, em *Aspergillus niger*, que em concentração baixa de fosfato, maior quan-

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tidade de ácido foi produzida por unidade de peso micelial que em concentração alta de fosfato (11, 22). Em *Erwinia herbicola*, comprovou-se que fosfato bicálcico ou hidroxiapatita foram solubilizados quando a concentração de fosfato do meio de cultura foi baixa. Os autores sugerem a existência de um mecanismo de indução ou repressão da solubilização regulado genéticamente em função da concentração de fosfato solúvel externo, de modo similar ao sistema de controle das fosfatases em *Escherichia coli* (6).

Assim, este trabalho propôs estudar a influência da concentração de fosfato solúvel, adicionado ao meio de cultura, sobre a solubilização de fluorapatita por *Aspergillus niger*, relacionando esses resultados à produção de fosfatase ácida.

MATERIAL E MÉTODOS

O fungo *Aspergillus niger* van Thiegham utilizado correspondeu à linhagem 26, anteriormente caracterizada (13). As condições de cultivo, obtenção e conservação das culturas estoque e a obtenção dos inóculos foram descritos em trabalho anterior (5).

O fungo foi cultivado em placas de Petri de 15 cm de diâmetro contendo 50 ml de meio de cultura (18), à temperatura de 30°C pelo período de 5 dias, a não ser em ensaios específicos. Concentrações limitantes de fosfato (KH_2PO_4) foram 200 µM e, concentrações saturantes de, 2000 µM. A fonte insolúvel utilizada foi a fluorapatita contendo 32,8% (p/p) PO_4^{3-}, na concentração de 3 g/l(5).

Terminado o período de incubação, as culturas foram filtradas em papel de filtro Whatman nº 1, para separar o micélio do meio de cultura. O micélio foi seco em estufa à temperatura de 98°C por 24 horas para determinação do peso seco. No filtrado, determinou-se o teor de fosfato solúvel (2), a atividade da fosfatase ácida (14) e a acidez titulável. Esta última foi determinada pela titulação de 10 ml do filtrado com solução de NaOH 0,05N até pH 7,0. Uma unidade de atividade da enzima foi definida como a liberação de 1 µmol de p-nitrofenol por hora e, a atividade específica, foi expressa em unidades por mg de micélio peso seco.

Para verificar o efeito de concentrações crescentes de fosfato solúvel na produção de fosfatase ácida e solubilização do fosfato insolúvel, empregou-se um procedimento indireto, que consistiu em cultivar o fungo em meio de cultura, a princípio, isento de fluorapatita. Após o período de cultivo, uma alíquota de 10 ml do filtrado foi esterilizada e incubada com 1,6 g/l de fluorapatita por 5 dias.

Nos ensaios de transferência de micélio, o fungo foi cultivado em meio de cultura contendo fosfato saturante e 3 g/l de fluorapatita. Após 1 dia de crescimento, o micélio foi asseticamente isolado por filtração, lavado e redistribuído em placas com 3 g/l fluorapatita e fosfato saturante. Em outro conjunto de ensaios, o fungo foi cultivado em meio de cultura contendo fosfato limitante e 3 g/l de fluorapatita. Após 1 dia de crescimento, adicionou-se fosfato para se obter uma concentração saturante em parte das placas, prosseguindo-se a incubação do fungo pelo tempo estabelecido.

RESULTADOS

A Tabela 1 resume os resultados de crescimento de *A. niger* em meio de cultura contendo fosfato limitante. A maior parte do fosfato, ou seja 96,1% foi consumida logo após 1 dia de cultivo, acarretando por isso uma redução no crescimento do fungo. O teor de ácidos totais e a quantidade de fosfatase ácida produzidos aumentaram com o tempo de cultivo.

Quando se adicionou fluorapatita (3 g/l) ao

TABELA 1 – Produção de fosfatase ácida por *A. niger* cultivado em meio contendo fosfato limitante.

Tempo de cultivo (dias)	Micélio (mg/ml)	pH final	Acidez titulável (µE/ml)	Fosfato solúvel (µg/ml)	Fosfatase ácida (U/h.mg)
0	ND	3,6	0,9	16,03	ND
1	0,67	2,4	3,8	0,62	2,90
2	5,37	2,2	8,7	0,57	4,70
3	3,77	2,2	11,9	0,34	5,62
4	3,17	2,1	12,4	ND	5,71

ND= não detectado

TABELA 2 – Acúmulo de fosfato solúvel por *A. niger* cultivado em meio contendo fosfato limitante e fluorapatita (3 g/l).

Tempo de cultivo (dias)	Micélio (mg/ml)	pH final	Acidez titulável (µE/ml)	Fosfato solúvel (µg/ml)	Fosfatase ácida (U/h.mg)
0	ND	4,7	0,6	20,69	ND
1	0,98	2,8	2,3	60,99	0,22
2	4,23	2,3	7,9	116,21	0,16
3	5,01	2,3	9,8	130,49	0,12
4	5,85	2,2	10,4	138,74	0,09

ND= não detectado

meio de cultura contendo fosfato limitante (Tabela 2), obteve-se uma resposta metabólica diferenciada da obtida na Tabela 1. Constatou-se maior disponibilidade de fosfato solúvel, proveniente da solubilização da fluorapatita, favorecendo, em consequência, maior crescimento do fungo. A atividade enzimática foi severamente reprimida.

Variando-se a concentração de fosfato solúvel no meio de cultura em presença de fluorapatita, foram obtidos os resultados apresentados na Fig. 1. Com o aumento do teor de fosfato, diminuíram progressivamente as quantidades de fosfato solubilizado até atingir um valor nulo para uma concentração saturante de fosfato solúvel. De modo contrário, o crescimento do fungo foi aumentando pelo menos até a concentração de fosfato de 1500 µm, quando foi observada uma diminuição do peso seco de micélio. A produção de ácidos totais diminuiu no início mas foi aumentando a partir da concentração 500 µM de fosfato solúvel.

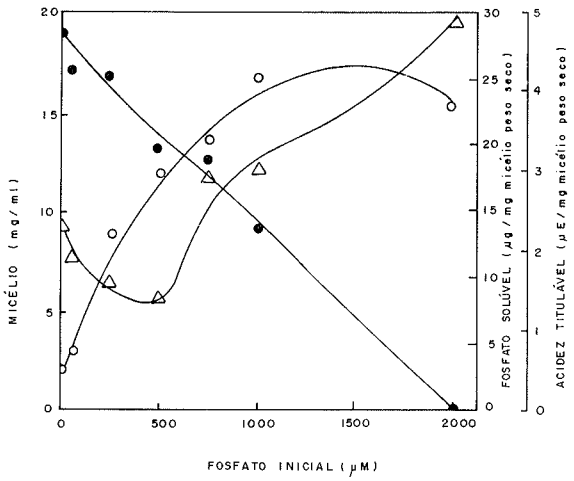

FIGURA 1 – Efeito de concentrações crescentes de fosfato solúvel sobre a solubilização de fluorapatita. Peso seco do micélio (o); fosfato solúvel (•); acidez titulável (▲).

Como não se detectou atividade enzimática nessas condições, foram realizados ensaios em que se suprimiu de início a fluorapatita para se verificar o efeito da concentração de fosfato sobre a produção de fosfatase ácida (Fig. 2). Após o crescimento do fungo, adicionou-se fluorapatita ao meio de cultura isento de micélio a fim de comprovar o efeito da concentração de fosfato na solubilização do fosfato insolúvel (inserção Fig. 2). Como se esperava, tanto a produção da enzima como a solubilização da fluorapatita diminuíram com o aumento da concentração de fosfato solúvel do meio de cultura.

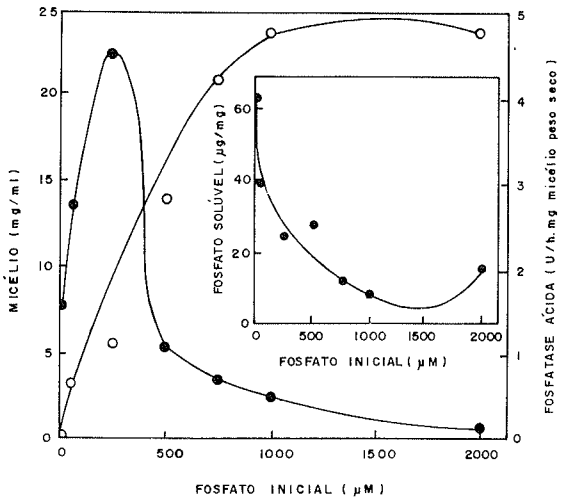

FIGURA 2 – Efeito de concentrações crescentes de fosfato solúvel sobre a produção de fosfatase ácida e solubilização indireta de fluorapatita (inserção). Peso seco de micélio (o); atividade da fosfatase ácida (•).

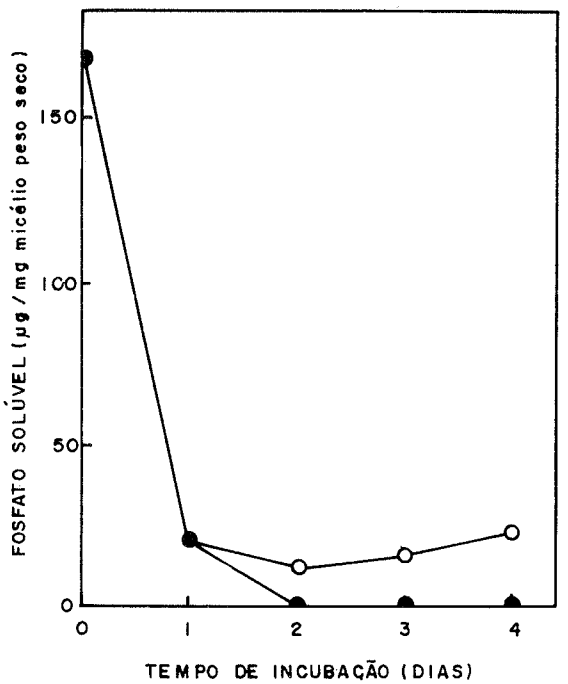

FIGURA 3 – Estímulo da solubilização de fluorapatita em uma cultura de *A. niger* crescido em fosfato saturante e transferido para meio isento de fosfato. Sem fosfato (o); com fosfato (•).

A solubilização de fluorapatita na ausência de fosfato solúvel é mostrada na Fig. 3. O fungo foi cultivado em meio de cultura contendo fosfato saturante por 1 dia e, a seguir, transferido para placas contendo meio de cultura e fosfato de rocha, porém sem fosfato solúvel. Observou-se que a trans-

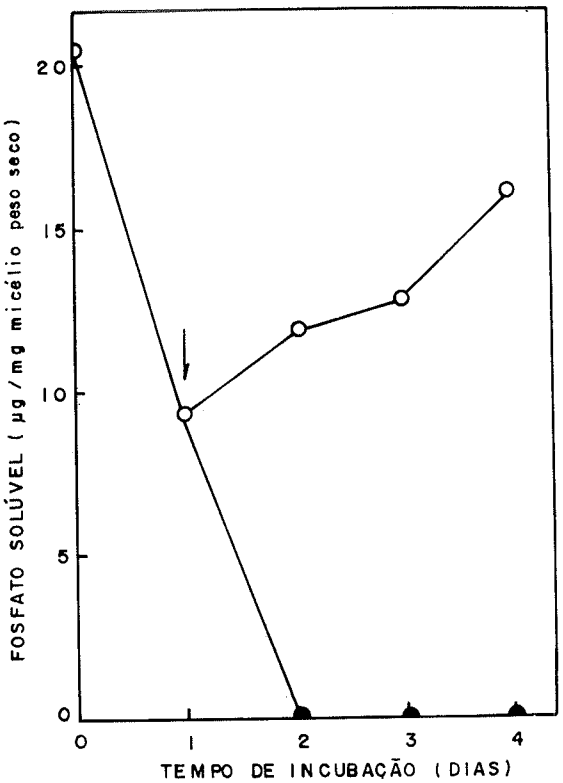

FIGURA 4 – Repressão da solubilização de fluorapatita em uma cultura de *A. niger* crescido em fosfato limitante e transferido para fosfato saturante. Fosfato limitante (o); fosfato saturante (•).

ferência do micélio para uma concentração de fosfato nula favoreceu a solubilização. De modo contrário, o fungo foi cultivado em concentração limitante de fosfato + fluorapatita por 1 dia, adicionando-se, a seguir, fosfato de modo a se obter uma concentração saturante em parte das placas (Fig. 4). Constatou-se uma drástica redução da solubilização pelo aumento da concentração de fosfato solúvel no meio de cultura.

DISCUSSÃO

Os ensaios relatados aqui constituem parte de um intensivo programa de pesquisa sobre a solubilização de fosfatos insolúveis. Neste trabalho, ficou evidenciado que a solubilização de fluorapatita por *Aspergillus niger* pode estar sujeita a um mecanismo dependente da concentração de fosfato solúvel inorgânico adicionado ao meio de cultura.

Como referência foi determinada a atividade de fosfatase ácida, enzima reconhecida por ser estritamente ou parcialmente repressível por concentrações altas de fosfato exógeno em *A. niger* (8).

Corroborando relatos de Komano (9), foi comprovada a secreção de fosfatase ácida em meio de cultura contendo concentrações limitantes de fosfato. Em adição, a produção dessa enzima foi aumentada quando o fosfato foi consumido pelo fungo em crescimento. Contudo, ao se adicionar fluorapatita ao meio de cultura, a produção da enzima foi severamente restringida em consequência do aumento da concentração de fosfato solúvel. Esse aumento foi proporcionado pela ação de ácidos orgânicos, secretados pelo fungo, sobre a fluorapatita, liberando fosfato solúvel, através de um mecanismo plenamente conhecido na literatura (5, 20).

Quando se comparou os valores de fosfato solúvel adicionado ao meio de cultura com o total (adicionado + solubilizado), constatou-se (dados não incluídos) que a partir da concentração inicial de 250 µM de fosfato solúvel, a proporção de fosfato solubilizado foi diminuindo até chegar a zero para uma concentração inicial saturante. A redução do crescimento observada, nesse ponto (Fig. 1), mostrou que não houve consumo excessivo de fosfato pelo fungo e que tratou-se, provavelmente, de uma repressão do fenômeno de solubilização. Também, em consequência do aumento da concentração de fosfato do meio de cultura, constatou-se a repressão da atividade da fosfatase ácida. Esse efeito tem sido mencionado em inúmeros fungos (9, 14, 15). Um dos mais estudados foi *Neurospora crassa* onde se acredita que a síntese da enzima seja decorrente de um processo em cascata entre genes localizados em diferentes loci e ativados por substâncias reguladoras (10). A presença de fosfato interromperia, então, esse processo. Por esse mecanismo, em condições desrepressivas, uma família de enzimas relacionadas ao metabolismo do fósforo seria sintetizada (12). Goldstein & Liu (6), que estudaram o processo em *Escherichia coli* e *E. herbicola*, admitem a possibilidade da produção de um agente de solubilização que seria produzido em quantidades variáveis dependendo da espécie microbiana. Considerando o aspecto de economia celular, não se pode descartar a possibilidade de um mecanismo de solubilização em *A. niger*, controlado da mesma forma que o relatado para as fosfatases de *N. crassa* (10).

Os resultados apresentados nas Figs. 3 e 4 comprovam as especulações anteriores de que a presença de uma fonte de fósforo solúvel inibiria o mecanismo de solubilização e, sua ausência, o favoreceria. Em adição, verificou-se ainda nesses ensaios que, uma vez esgotado o fósforo adicionado ao meio de cultura (dados não mostrados), o

teor de fosfato solúvel começou a aumentar como resposta ao processo de solubilização do fosfato de rocha então desencadeado.

Pelos resultados obtidos, não foi possível relacionar a produção de ácidos totais e nem a queda do pH com a solubilização na presença de fluorapatita (Tabela 2 e Figura 1) quando comparados com os ensaios em que a mesma foi omitida (Tabela 1 e Figura 2). Dados de literatura não são conclusivos quanto à influência desses fatores na solubilização (5, 13, 16, 17, 21).

SUMMARY

Effect of phosphate on the solubilization of fluorapatite by *Aspergillus niger*

A study was conducted on the effect of soluble phosphate concentration on the solubilization of fluorapatite by the mold *Aspergillus niger*. Fluorapatite is a rock phosphate contaning 32,8% PO_4^3. The mold was cultivated in Petri dishes containing 50 ml culture medium with low (200 µm) or high (2000 µm) soluble phosphate and 3 g/1 fluorapatite. The production of acid phosphatase by *A. niger* was stimulated in low phosphate medium in the absence of fluorapatite. However, when fluorapatite was added to the medium, the enzyme was severely repressed probably due to the enhancing soluble phosphate concentration. This was the result of the dissolution of the insoluble phosphate by organic acids secreted by the mold leading to an increased content of soluble phosphate. Acid phosphatase production and fluorapatite solubilization were decreased when the concentration of soluble phosphate was enhanced, reaching pratically null levels at Pi 2000 µm. Similar results were obtained in a separate assay when a culture medium filtrate was incubated with fluorapatite (1.6 g/1) for 5 days, confirming the effect of soluble phosphate concentration on solubilization but not the result of phosphorus consumption by the mold. In addition, when soluble phosphate was exhausted, the solubilization process was triggered, with a consequent increase in soluble phosphate in the medium. When the mycelium was transferred from a low phosphate medium to a high phosphate medium, solubilization was repressed. Conversely, when the mycelium was transferred from a high phosphate medium to a low phosphate medium, solubilization was stimulated. It was not possible to correlate the production of total acids or the fall in pH with the presence or absence of fluorapatite and the solubilization process. Apparently, the process of solubilization by the mold is governed by external phosphate levels as also observed for phosphatases.

Key Words: *Aspergillus niger*, fluorapatite solubilization, soluble phosphate.

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AMINO ACID COMPOSITION OF SINGLE AND MIXED FUNGAL CULTURES GROWN IN SUGAR CANE VINASSE

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Sâmia Maria Tauk[1]

SUMMARY

The amino acid composition of pure and mixed cultures of *Cryptococcus laurentii* and *Aspergillus niger* grown in sugar cane vinasse medium was evaluated. The amino acid determination was carried out by ion exchange chromatography. The profiles showed that the *A. niger* biomass presented higher values that those ones in *Cr. laurentii* biomass for each amino acid. However, the mixed culture of those microorganisms presented even higher contents of the same amino acids. The mixed culture biomass was deficient in cystine in comparison to the FAO reference protein; glutamic acid and proline in comparison to the casein; and methionine in comparison to the meat, wheat, milk and egg proteins. However, the results obtained indicated that the mixed culture of *A. niger* and *Cr. laurentii* may be a good source of protein.

Key Words: Fungi, vinasse, amino acid.

INTRODUCTION

The sugar and alcohol production has originated several by products such as vinasse, bagasse, filter cake and the alcohol fermentative yeast. The vinasse (or stillage) has received great attention for its potential uses (4, 12). Microorganisms have been cultivated in vinasse for microbial protein production mainly fungi. The potential use of mixed cultures of yeast and filamentous fungi has been studied (3), presenting good results for biomass/protein production associated with biochemical oxygen demand (BOD) decrease.

In this paper, we report the amino acid profiles of pure and mixed cultures of *Aspergillus niger* and *Cryptococcus laurentii* grown in sugar cane vinasse supplemented with nitrogen, phosphorus and carbohydrate under cultural conditions previously established (Ceccato-Antonini & Tauk, in press), trying to evaluate the mixed culture of those microorganisms as source of protein (SCP).

MATERIAL AND METHODS

Microorganisms – The strains of *A. niger* and *Cr. laurentii* were isolated from vinasse and soil, respectively, both maintained in stock slants of Sabouraud-dextrose agar (9).

Medium composition, inocula and cultural conditions – The composition of vinasse medium was sugar cane vinasse, molasses by 8.0 gl^{-1} of total carbohydrate; ammonium sulfate by 1.0 gl^{-1} of nytrogen and phosphoric acid by 0.04 gl^{-1} of phosphorus (C:N:P proportion was 20:3:0.1). The initial culture pH was adjusted to 4.6 with 1N NaOH solution. 500-ml erlenmeyers flasks containing 100 ml of vi-

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nasse medium were then pausterized at 85C for 20 minutes and after being cooled to room temperature.

The inocula were prepared as following: 0,5 x 0,5 cm squares of fungal mycelium and loops of yeast cells from three to four-day stock slants were transferred separately to erlenmeyer flasks containing the vinasse medium and kept at 30°C for 48 and 24 hours for *A. niger* and *Cr. laurentii* respectively, under rotational agitation at 250 rpm. Each inoculum was represented by 7,5 % vol/vol in relation to the volume of the medium in the mixed culture and by 15% vol/vol in the pure cultures.

After inoculation, the four replicates were maintained at 30°C for 24, 72 and 72 hours for *A. niger*, *Cr. laurentii* and the mixed culture, at 250 rpm.

Analytical procedures – The samples of *Cr. laurentii* cultures were centrifuged at 25,000 g for 20 minutes at 5°C, washed with distilled water and centrifuged again. Fungal cultures were vacuum-filtered. Mixed culture samples were both filtered and centrifuged. The biomass was estimated as dry weight at 105°C. Protein content was quantitated by the Kjeldhal method for total nitrogen multiplied by 6.25 (1). The amino acid determinations were carried cut by ion exchange chromatography (11). A high efficiency cation exchange resin (Durrum DC 6A) was used and the samples (two replicates) were detected simultaneously at 570 and 440 nm. The column temperatures were 55 and 75°C. The dried biomass and casein (Mococa trademark) were hidrolysed with 4N LiOH solution for 24 hours at 110°C for the tryptophan (TRY) analysis and with 6N HCl solution for 22 hours at 110°C for the others amino acids as following: alanine (ALA), arginine (ARG), aspartic acid (ASP), cystine (CYS), glutamic acid (GLU), glycine (GLY), histidine (HIS), isoleucine (ILE), leucine (LEU), lysine (LYS), methionine (MET), phenylalanine (PHE), proline (PRO), serine (SER), threonine (THR), tyrosine (TYR) and valine (VAL).

RESULTS AND DISCUSSION

The strain of *A. niger* grew best within 24 hours of cultivation in vinasse medium but presented the lowest protein content (in %) among the cultures. The yeast has showed a higher content of protein in the biomass. The mixed culture, however, presented intermediate value for protein but in a satisfactory level, which is 40% (Table 1).

The values found for the amino acids in *A. niger* biomass grown in vinasse medium were always higher than those ones in *Cr. laurentii* biomass in the same culture medium. However, the mixed culture of those microorganisms presented even higher contents in comparison to *A. niger* amino acid profile, which has showed that the utilization of the mixed culture is much more ad-

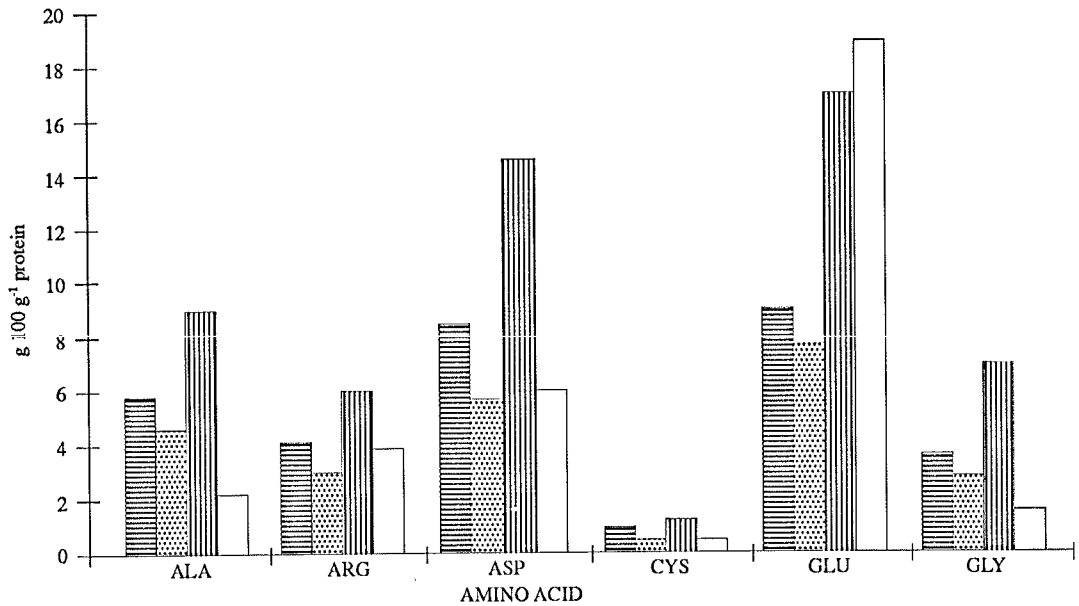

FIGURE 1 – Amino acid composition (g 100 g^{-1} protein) of casein (□); mixed (▥) and pure cultures of *A. niger* (▤) and *Cr. laurentii* (▦) grown in sugar cane vinasse medium.

TABLE 1 – Biomass (gl^{-1}) and protein (% wt wt^{-1}) means (n=4) and standard deviations of *A. niger*, *Cr. laurentii* and *A. niger* + *Cr. laurentii* cultures grown in sugar cane vinasse medium for 24, 72 and 72 hours, respectively.

Parameter	*A. niger*	*Cr. laurentii*	*A. niger* + *Cr. laurentii*
Biomass	8,19 ±2,91	5,82 ±1,35	3,15 ±0,65
Protein	34,18 ±2,21	57,62 ±1,02	40,91 ±4,04

TABLE 2 – Amino acid composition (g 100 g^{-1} protein) of conventional protein sources in comparison to the *A. niger* plus *Cr. laurentii* protein

Amino acid	Egg[2]	FAO[1]	Meat[1]	Milk[1]	Wheat[1]	*A. niger* + *Cr. laurentii*
Cystine	–	2.0	–	–	–	1.1
Isoleucine	6.7	4.2	3.3	4.3	3.5	7.0
Leucine	8.9	4.8	12.5	16.3	12.0	10.2
Lysine	6.5	4.2	8.3	7.4	2.0	8.3
Methionine	3.2	2.2	4.2	4.0	4.0	3.1
Phenylalanine	5.8	2.8	4.6	5.7	4.6	7.4
Threonine	5.1	2.8	4.6	4.6	2.5	7.5
Tryptophan	1.6	1.4	1.3	1.7	1.0	2.6
Valine	7.3	4.2	3.3	5.4	3.0	7.6

[1]Reference: (5)
[2]Reference: (10)

vantageous than the pure cultures of *A. niger* and *Cr. laurentii* (Figures 1, 2 and 3).

Phenylalanine, alanine and glycine contents were higher in the yeast biomass than in casein. The biomass of *A. niger* presented about 50% of the amino acids analysed (10 within 18) in higher amounts when compared to the casein, including the following essential amino acids: cystine, phenyalanine, isoleucine and threonine. Concerning to the mixed cultures biomass, lower contents were only found for glutamic acid and proline in comparison to casein (Figures 1, 2 and 3).

Except for cystine, the amino acid contents of the mixed culture were always higher than those for the FAO reference protein. When compared to the meat, milk and wheat proteins, leucine and methionine are also deficient as well as methionine in the egg protein (Table 2).

The amino acid profile of *A. niger* grown in vinasse was well compared to *Phanerochaete chrysosporium* (2) and *Fusarium oxysporum* (6) also grown in vinasse. It also presented higher values for phenylalanine, leucine, lysine, threonine, tryptophan and valine than the FAO reference protein (5).

The protein obtained from *Cr. laurentii* cultivation in vinasse did not present high contents of amino acids, as already verified for other yeasts (8). It was deficient in essential amino acids when compared to the FAO reference protein, and the amino acid profile was not better than those found for *Rhodotorula glutinis* in glucose (7) and *Sac-*

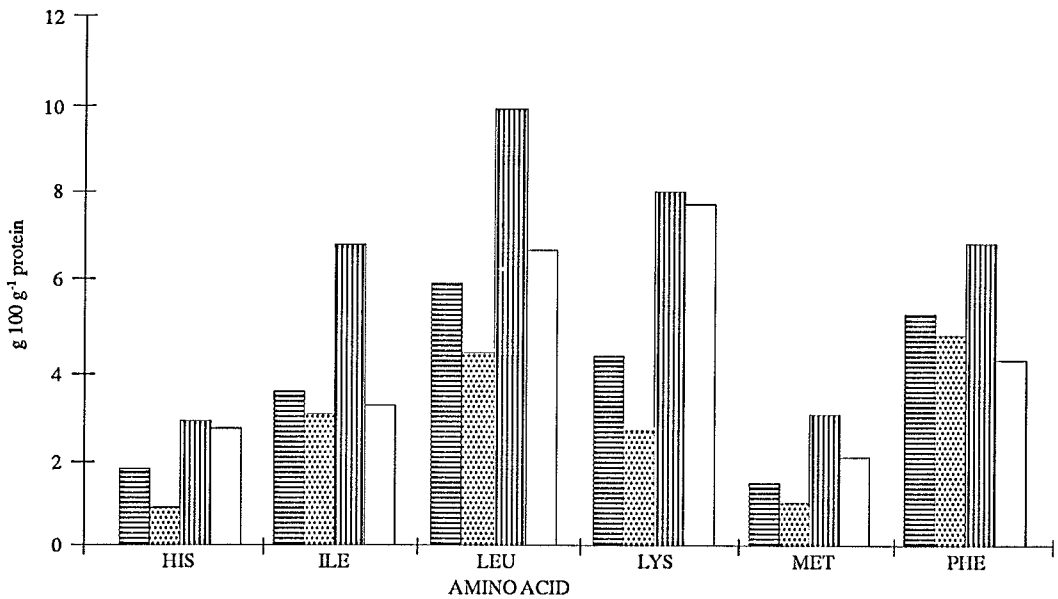

FIGURE 2 – Amino acid composition (g 100 g^{-1} protein) of casein (□); mixed (▥) and pure cultures of *A. niger* (▤) and *Cr. laurentii* (▨) grown in sugar cane vinasse medium.

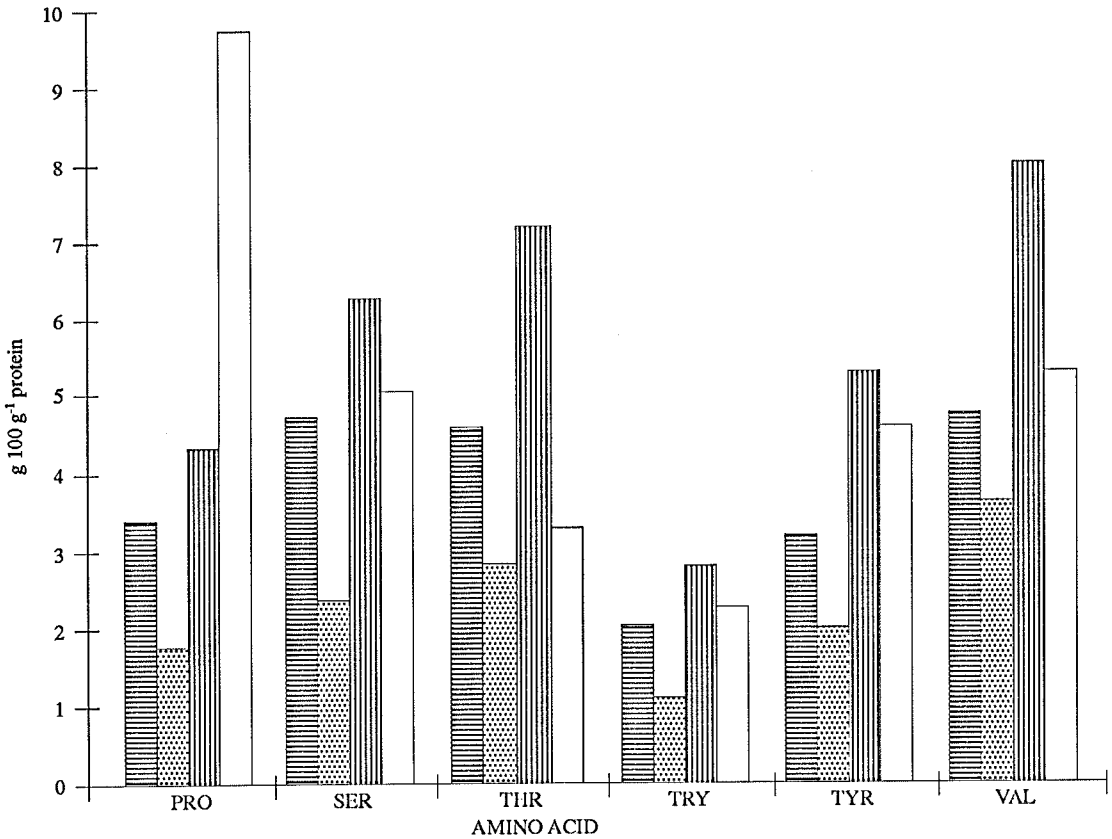

FIGURE 3 – Amino acid composition (g 100 g^{-1} protein) of casein (□); mixed (▥) and pure cultures of *A. niger* (▤) and *Cr. laurentii* (▨) grown in sugar cane vinasse medium.

charomyces fragilis in acidified cheese whey (8).

This paper has revealed a qualitative aspect on the utilization of mixed culture biomass of a yeast and a filamentous fungus grown in sugar cane vinasse medium. Many others aspects concerning the nutritional value of the biomass should be studied in order to evaluate the purpose of using that biomass as food or feed, but the results obtained have pointed out favorably to the mixed culture of *A. niger* and *Cr. laurentii* grown in vinasse medium for microbial protein.

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The authors wish to thank Dr. Claudete Rodrigues Paula, for the yeast identification; the "Centro Interdepartamental de Química de Proteínas", Faculdade de Medicina/USP - Ribeirão Preto for the amino acid analysis; and FAPESP (process nº 84/2123-8) and CAPES for the M. Sc. fellowships to S. R. Ceccato-Antonini and CNPq (process nº 300829/80) for the research fellowship to S. M. Tauk.

RESUMO

Composição de aminoácidos de culturas puras e mista de fungos desenvolvidas em meio de vinhaça.

Foi avaliada a composição de aminoácidos de culturas puras e mista de *Cryptococcus laurentii* e *Aspergillus niger* desenvolvidas em meio de vinhaça. Os perfis de aminoácidos, determinados por cromatografia de troca iônica, mostraram que a biomassa de *A. niger* apresentou valores superi-

ores àqueles apresentados por *Cr. laurentii* para cada aminoácido. No entanto, a cultura mista destes microrganismos apresentou teores ainda mais elevados de aminoácidos. A biomassa da cultura mista foi deficiente em cistina em comparação com o padrão de proteína recomendado pela FAO; ácido glutâmico e prolina em comparação com a caseína; e metionina em comparação com as proteínas de carne, trigo, leite e ovo. Os resultados mostraram ser a cultura mista de *A. niger* and *Cr. laurentii* uma boa fonte de proteína.

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COMPATIBILITY BETWEEN PESTICIDES AND THE FUNGUS *NOMURAEA RILEYI* (FARLOW) SAMSON

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Neiva Monteiro de Barros*

SUMMARY

The objective of the present study was to evaluate the action of some pesticides on the entomopathogenic fungus *Nomuraea rileyi*. Seven insecticides, one fungicide and one herbicide were used for this purpose. The study was carried out on plates containing the pesticides compared with control plates. Statistical analysis of the data showed significant differences among pesticides and doses tested, but not among strains.

Key Words: *Nomuraea rileyi*, pesticides.

INTRODUCTION

Much emphasis is being currently placed on programs of integrated pest control based on the use of chemical products compatible with biological products in order to lessen their impact on the agricultural ecosystem.

An important factor that may alter the success of entomopathogenic fungi is their compatibility with pesticides (7), which may affect the development of primary foci and consequently the outbreak of epizootic disease.

Most of the herbicides, fungicides and insecticides employed in soybean culture inhibit the growth of *Nomuraea rileyi* "in vitro", even at 1/10 the percentages recommended (4), and soybean treatment with combinations of fungicides and insecticides has shown a potential to delay and reduce the epizooties of the fungus (3).

Pesticides may affect the pathogen directly when applied together, or indirectly when the pathogen is applied in the presence of pesticide accumulation (2).

A higher rate of infection of *Anticarsia gemmatalis* (HÜBNER) with *Nomuraea rileyi* has been observed in fields where treatment with benomyl, benomyl plus methyl parathion and benomyl plus carbaryl had been interrupted (5).

Benomyl in combination with carbaryl apparently delayed the progress of the epizooties of the fungus within one week, whereas carbaryl alone had no effect on them (3).

Using commercially available pesticides, Terribile (9) reported that, at concentrations of 10 µg/ml, dimilin, carbaryl and karate did not inhibit the development of *Nomuraea rileyi* "in vitro", whereas the insecticides azodrin, ambush, thiodan and lannate had inhibitory effects on the development of the fungus starting from the concentration of 5 µg/ml, and the herbicide basagran and the fungicide benomyl were effective startig from the concentration of 10 µg/ml.

MATERIALS AND METHODS

Strains – The following strains of *Nomuraea rileyi*, maintained at the Institute of Biotechnolo-

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gy, University of Caxias do Sul, were used: NP 87521, PF 86202, Gu 87401 and SR 86151. The strains were originally isolated from *Anticarsia gemmatalis* and collected at Nova Prata, Passo Fundo, Guaíba and Santa Rosa, respectively, in the State of Rio Grande do Sul.

Pesticides – Insecticides: karate (cialotrin), Azodrin (monocrotophs), Sevin (carbaryl), Dimilin (diflubenzuron), Thiodan (endosulfan), Ambush (permetrin) and Lannate (metomyl). Fungicide: Benlate (benomyl). Herbicide: Basagran (bentazon).

Culture Media – The media used were SMAY and SMAY plus pesticides added so as to obtain concentrations of 1, 3, 5 and 10 µg/ml. Media were plated into Petri dishes maintained at room temperature and left to stand for 24 hours to permit diffusion of the product into the medium.

Inoculation and Incubation – Strains were inoculated into the culture medium by removing two disks 1.0 cm in diameter of SMAY containing *N. rileyi* mycelium which were transferred to plates containing SMAY and SMAY plus pesticides. The plates were incubated at 26°C for eight days and the diameters of the disks were measured. Each tratment was carried out in duplicate.

Statistical Analyses – Data were analyzed statistically by analysis of variance and by the Tukey test using a TIGER XT microcomputer (IBM-PC/XT type) with 640 Kb RAM and a 20 MB hard disk.

RESULTS AND DISCUSSION

The compatibility of the pesticides with the fungus *Nomuraea rileyi* was evaluated by comparing the diameter of mycelial disks obtained in SMAY with the diameter of disks obtained in SMAY plus pesticides. The results are presented in Figure 1.

To determine possible differences among the 4 strains tested, among the pesticides and among the doses, the data were submitted to analysis of variance. No significant differences in sensitivity to pesticides was detected among the strains. In contrast, significant differences were observed both for doses and for pesticides. In general, starting at the dose of 5 µg/ml, the development of *N. rileyi* was reduced, with the greatest differences being obtained between 10 and 1 µg/ml, 10 and 5, and between 5 and 1 µg. The pesticides most compatible with *N. rileyi* were Dimilin, Sevin and karate. This can be seen in Figure 1 which illustrates the variability among strains during exposure to the dose of 10 µg/ml for all pesticides tested.

As to insecticide compatibility with *N. rileyi*, Alves (1) also demonstrated that Dimilin at the dose of 10 µg/ml was compatible with the fungus. Thus, the confirmation of the low inhibitory effect of Dimilin on *N. rileyi* development demonstrates that the two can be used together (6, 11).

Field studies showed that pesticides reduce the mortality of *Anticarsia gemmatalis* induced by

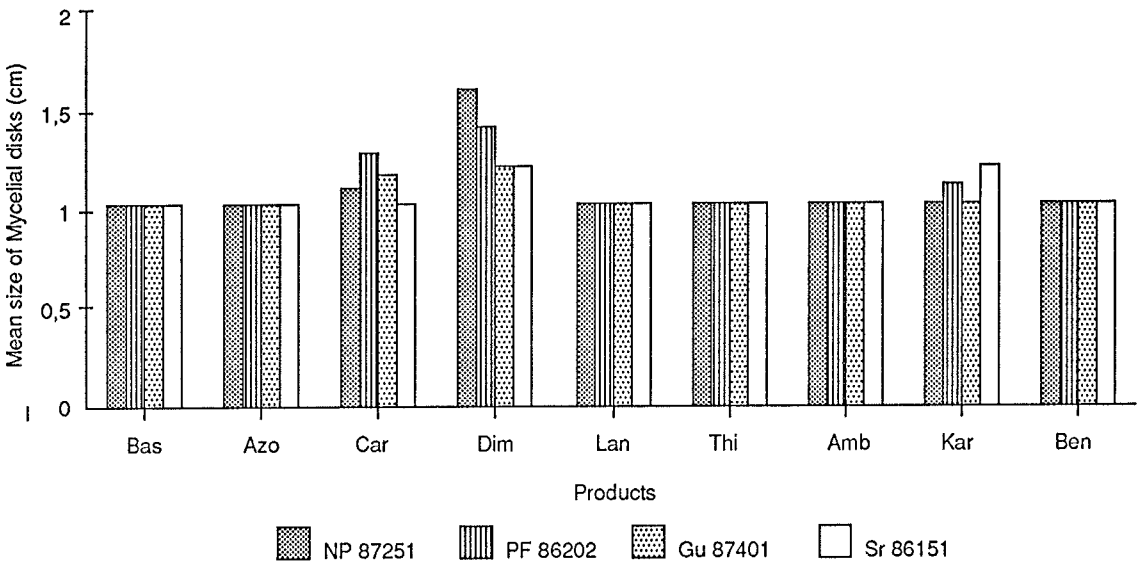

FIGURE 1 – Influence of pesticides (concentration 10 ppm) over *N. rileyi* fungi

N. rileyi; however, when carbaryl was applied alone it appeared not to harmful to the epizooties of the fungus (3).

It was confirmed that benlate has the ability of inhibiting the development of entomopathogenic fungi "in vitro" (4, 8, 10) even at the dose of 10 µg/ml (3).

In the present study we observed that azodrin and thiodan were not compatible with *N. rileyi*, although Alves (1) reported that both were compatible with the fungus at the dose of 10 µg/ml and Ignoffo *et al.* (4) reported that azodrin was one of the insecticides that most intensely inhibited the development of the fungus.

RESUMO

Compatibilidade entre agrotóxicos e o fungo *Nomuraea rileyi* (Farlow) Samson

Este trabalho teve o objetivo de avaliar a ação de alguns agrotóxicos sobre o fungo entomopatogênico *Nomuraea rileyi*, sendo utilizados, para isso, sete inseticidas, um fungicida e um herbicida. O estudo foi realizado em placas contendo agrotóxicos em comparação com placas controle. Através da análise estatística dos resultados foi possível verificar diferenças significativas entre os agrotóxicos e doses testadas, enquanto que entre as linhagens não foram verificadas diferenças significativas.

Palavras-chave: *Nomuraea rileyi*, pesticidas, agrotóxicos.

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A NEW MEDIUM FOR PRESUMPTIVE IDENTIFICATION OF *CRYPTOCOCCUS NEOFORMANS*

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U.M. Lanfer[2]

SHORT COMMUNICATION

SUMMARY

A culture medium obtained from banana peel (*Musa paradisiaca*) for rapid characterization of *Cryptococcus neoformans*, was described using its capability of production of melaninic compounds. Poliphenoloxidases existing in the banana peel was inhibited through a treatment with ascorbic acid and when necessary atmosphere of N_2, blocking the possible reactions which interfered negatively with the performance of the new medium. The culture medium is sensitive and inexpensive and has long storage life.

Key Words: Banana medium. *Cryptococcus neoformans.*

Based in the knowledge that *Cryptococcus neoformans* produces melaninic pigments when in the presence of substracts which contain ortho and paradiphenol (Chaskes, S.J.; Tyndall, R.L. J. Clin. Microbiol., 1, 509-514, 1978; Edberg, S.C.; Chaskes, S.J.; Alture-Werber, E.; Singer, J.M. J. Clin. Microbiol., 12, 332-335, 1980.; Shaw, C.W.; Kapica, L. Appl. Microbiol. 24, 824-830, 1972; Shields, A.B.; Ajello, L. Science, 151-208-209, 1966; Staib, F. – Z. Hyg., 148, 466-475, 1962) and that the banana peels contains these compounds under the form of dopa, dopamine, serotonine and nor- epinephrine (Marriott, J. – Crit. Rev. Food Sci. Nutrit. 13, 41-88, 1980; Purchio, A.; Souza, E.M.B.; Paula, C.R.; Gambale, W., Corrêa, B. – Abstracts, Xth Congress of the International Societh for Human and Animal Mycology, Barcelona, Spain, June 27-July 1, 1988), a new culture medium was developed with banana peel (*Musa paradisiaca*). The pulp and the peel of the fruit contains water, several types of sugar, starch, fibers, proteins, vitamines and lipids (Marriot, J. – Crit. Rev. in Food Sci. Nutrit. 13, 41-88, 1980; Palmer, J.K. In: The Biochemistry of fruits and their products. vol. 2, Academic Press, London, 1971; A.A.; Southgate, P.A.T. – The composition of foods, 4 edn., Her Majesty's Stationery Office, London, 1978; Polansky, M.M.; Murphy, E.W. – 3. A.M. Diet. Assoc., 48-109, 1966) which are ideal compounds for the elaboration of a nutritive culture medium. Besides this, the banana peel, in a climaterium, has average of 720µg of dopamine and 80µg of nor-epinephrine, and other catecolamines, per gram of humid tissue (Mariott, J. – Crit. Rev. Food Sci. and Nutrit. 13, 41-88, 1980).

For the initial preparation of the proposed cul-

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ture medium, it was used 250 g of banana peel in the beginning of the maturation process (colour yellowish-green) and without dark pigmentation. For the extraction of the phenolic compounds and others, these peels were added to a recipient containing 980ml of distilled water and 20ml of HCl (Merck) at 0.1%. The solution was kept in repose for 30 minutes, with eventual agitation. Then, it was heated to 45°C for more 30 minutes. The extract obtained was filtered by Millipore filtration. The filtrate was added to an agar solution at 4% (Difco) that had been sterilized by autoclaving at 121°C for 20 minutes and allowed to cool to 55°C. The volumes of extract of banana peel and agar were completed to 1.000ml (600ml of extract and 400ml of agar solution) and the final pH was adjusted to 5.5. (HCl - Merck - 0,1%).

The presence of enzymatic activity of phenolic compounds present in the substract could result in an oxidation of the extract produzing quinones, highly reactive and forming dark pigments by polimerization. These dark pigments have, at the same time, the property of binding to protein resulting in the floculation, turvation and darkening of the medium (Weaver, C. & Charley, H. – Journal of Food Science, 39, 1200, 1974).

In order to investigate the possible poliphenoloxidase activity of the banana extract obtained, the spectrophotometric analysis was used (Varian – DMS 80). The mixture of the incubation contained: 0.5ml of catechol (0.1M – Sigma), 3.0ml of phosphate buffer (0.1ml – pH 6.6), 0.5ml and 1.0ml of banana peel extract and 1ml of distilled water. The lectures were performed in 410nm. As it was expected, the presence of the enzyme in the extract interfered negatively in the performance of the culture medium. During the first five minutes, the oxidation reaction showed a certain linearity, being the velocity similar to the two volumes of the extract. To inactivate the enzymes of the substract, 20ml of the reduzing agent (ascorbic acid – Merck – 0,1%) was added during the acid extraction. For a long storage life the medium would be maintained in atmosphere of inert gas – N_2, on the repose phase, at 45°C. By the results obtained it was observed an effect of blocking to the reactions of darkening of the peel extract, not being detected a phenolasic activity of the same, after a new spectrophotometric analysis. The final preparation of banana medium is summarized on the Table 1.

TABLE 1 – Banana peel based medium (B. P. Medium)

Ingredient	Quantity
Solution 1* – Banana Extract	600ml
– Banana peel	250g
– HCl (Merck – 0,1%)	20ml
– Ascorbic Acid (Merck – 0,1%)	20ml
– Distilled water	960ml
Solution 2** – Agar solution	400ml
– Agar (Difco)	16g
– Distilled water	400ml

* = Repose for 30 min. at 25°C and plus 30 min. at 45°C. This extract is sterilized by filtration.

** = The agar solution is sterilized by autoclaving at 121°C for 20 min., cools to 55°C and then 600ml the sol. 1 was added.

The medium was tested with 39 strains of the *C. neoformans (serotypes* A, B, C and D), 5 strains of *C. laurentii, C. terreus, C. uniguttulatus, C. difluens* and *C. albidus;* 20 strains of *C. albicans,* 5 strains of *C. krusei, C. tropicalis, C. parapsilosis* and *C. guilliermondii* and 5 strains of *Rhodotorula rubra.* These cultures are maintained in the Mycology Section the Department of Microbiology ICB/USP, while the standard strains of *C. neoforman* (CBS132) and *C. albicans* (CBS562) were also used. The tests were done in triplicate, at temperature of 25°C and 37°C, considering as control the medium described by Chaskes & tyndall (Chaskes, S.J.; Tyndalll, R.L. – Microbiol, 1, 509-514, 1978.

All the cultures of yeasts grew well in the mentioned medium and only the cultures of *C. neoformans* produced a dark-brown pigment. The other species of *Cryptococcus* presented colonies with light or dark yellow color. The strains of *C. albicans* and other species of the genus *Candida* did not developed any pigment, and the strains of *R. rubra* presented only their characteristic carothenoid pigment, at 25°C and 37°C.

Twenty six samples (66.6%) of *C. neoformans* developed a characteristic pigment (dark-brown) in 24 hours or less, and 6 samples (15.3%) in 48hs, while 4 samples (10.2%) in 72hs. Three strains, one isolated from human liquor of unkown serotype, and two serotype A, one isolated from dog liquor and one from human sputum, only produced a characteristic pigment after 5 days, at 25°C and 37°C.

The stability tests of the culture medium were also done, from 5 to 5 days, maintaining the medium at room temperature, for until 30 days and in the refrigerator for until 60 days. The medium were maintained in the darkness. After the time of storage, the cultures of *C. neoformans* presented a good growth

and the characteristics pigment (dark-brown).

On the basis of these results, we believe that the new proposed medium constitutes a valid option to the mycologists for the isolation and rapid characterization of *C. neoformans* strains, which could be used in the laboratorial routine and as presumptive test. It is pointed out the quantity and disponibility of the basic material during the whole agricultural year.

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RESUMO

Novo meio de cultura para caracterização presuntiva de *Cryptococcus neoformans*.

Um novo meio de cultivo foi elaborado a partir de cascas de banana (*Musa paradisiaca*) para uma rápida e presuntiva caracterização de *Cryptococcus neoformans*, utilizando-se a sua capacidade de produzir compostos melanínicos. Este meio é sensível, de baixo custo e pode ser estocado por longo tempo.

Palavras-chave: meio de banana, *Cryptococcus neoformans*.

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A SIMPLE METHOD FOR STORAGE OF METHANOGENIC BACTERIA IN LIQUID NITROGEN

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SHORT COMMUNICATION

SUMMARY

Procedures for strict anaerobic methanogens culture preservation are described. Two different techniques were adopted: one for low redox potential maintenance into the culture flasks and other for cells conservation in liquid nitrogen.

Key Words: Methanogenic bacteria, cultures preservation

Isolation of methanogenic cultures requires special techniques for strict anaerobic manipulation. The roll-tube method developed by Hungate (1969) (6) is one of the most recognized techniques for manipulation of anaerobic bacteria, including media, stock solutions and inoculation procedures. The goal is to avoid the oxygen contamination and maintenance of redox potential below -300mV.

Preservation of pure methanogenic cultures by usual methods can require the utilization of anaerobic glove box (Edwards & McBride, 1975) (4), a piece of equipment that keeps high volumes of oxygen-free atmosphere inside it. Nevertheless, many laboratories do not have this equipment available for routine studies, and subcultivation using Hungate tecniques e.g. is the most common technique adopted. But systematical subcultivation can lead to losses of culture or species characteristics

Hippe (1984) (5) working at DMS (Deutshe Samlung van Mikroganismen), one of the most recognized institutions operating with methanogens collections, presented results about those species viability, after preservation from a few weeks to several years by subcultivation in enriched media and storage at 4°C. However, the best results were obtained after freezing and storage under liquid nitrogen.

This paper presents pratical procedures of storing pure methanogens cultures, joining the original techniques of microorganisms preservation under liquid nitrogen, described by Guerna (1981) in Cañhos (1984) (2), and the strict anaerobic bacteria cultivation methods used by CETESB (1984) (3) and adapted from Hungate (1969) (6) and Bryant (1972) (1).

The culture tested was *Methanobacterium* genus, isolated from granular sludge (Novaes *et al.*, 1988) (7) originating in a up-flow anaerobic reactor operating with domestic sewage (Vieira, 1984) (8). The culture grew and produced methane in a culture medium with sodium formate or hydrogen and carbon dioxide gases (80/20%).

The methanogenic stock culture was initially kept by subcultivation in a solid medium with sodium formate plus oxygen-free hydrogen and carbon dioxide (80/20%) gases, at a room temperature. For freezing procedures the stock culture was previously subcultivated in tubes with 5.0 ml of liquid medium (Novaes, *et al.*, 1988) (7) closed

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with rubber stoppers and incubated at 37°C. The cell growth was measured by the variation of liquid absorbance (Bausch & Lomb 710 Spectrophotometer) at a wave length as 600 nm. During the exponential phase of growth, the tubes were centrifuged for 15 minutes at 1,000 rpm (Fanem centrifuge). Supernatant volume of 3.0 ml was discharged of each tube and the pellet was homogenized with the residual liquid. Aliquots of 0.05 ml were transfered to glass ampoules (15.0 ml) containing 2.0 ml of liquid medium as indicated above plus 5% (v/v) of glycerol, under 100% oxygen-free carbon dioxide flow. Figure 1 shows the scheme of open ampoule with the bacterial culture plus glycerol under carbon dioxide flow.

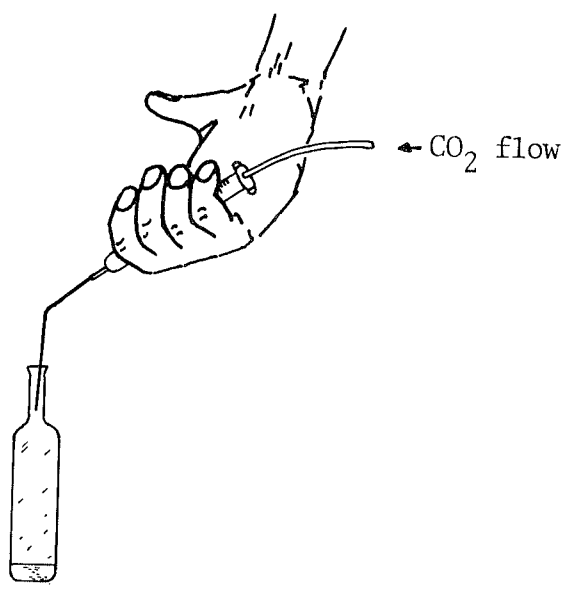

FIGURE 1 – Scheme of ampoule with the bacterial culture plus glycerol, under 100 % carbon dioxide flow.

The ampoules were closed with rubber stoppers, removing carefully the gas flow. Each ampoule was fastened on to a rotating base (70 rpm/min), near a flame torch directed towards just below the rubber stopper (Figure 2). When this region became red-rot, it was pressed using a tweezers, sealing the ampoules. These procedures must be done very fast in order to avoid the oxygen contamination.

The sealed ampoules were wrapped in paper sheets and first submitted to slow freezing: -20°C/60 min, -30°C/60 min and -70°C/18h. Then, the frozen material was storage under liquid nitrogen at -196°C.

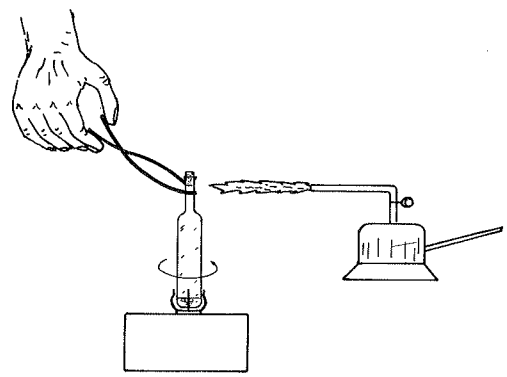

FIGURE 2 – Scheme of ampoule fastened on a rotating base, near a flame torch directed towards just below the rubber sttoper.

The culture viability was tested after one week, one month, 6 months and 18 months of incu bation time in liquid nitrogen. The ampoules were taken out from liquid nitrogen bottle and placed in water bath at 37°C/1min. Aliquots were transfered to liquid medium as indicated, under hydrogen plus carbon dioxide (80/20%). The cell growth and methane produced were verified after 5 days. The gas analysis were done by gas chromatography (CG-Cromatograph FI - Porapac Q Column). Morphological analysis of methanogens cells were done by light microscopy under phase-contrast and after Gram staining (Zeiss Universal Microscopy).

The characteristics of methanogenic cells after preservation in liquid nitrogen at different periods of incubation time were similar of those previously described by Novaes *et al.* (1988) (7): rod shaped cells, gram-negative, about 5.0 µm in length and around 30% of mean methane produced in the culture tube atmosphere.

The utilization of glycerol (5% v/v) was adequated and the freezing steps as well. Then, besides Guerna's method in Cañhos (1984) (2) recommends the decreasing temperature speed of 1°C per minute during the freezing steps, the results showed that this procedure can be avoided. But, it should be essential the cells quantitative evaluation after different periods of nitrogen liquid preservation.

According to Hippe (1984) (5) the method of freezing and storage methanogenic cells under liquid nitrogen is very appropriate for preservation of these microorganisms. In this way, the results presented in this note can contribute to simplify techniques of preservation cells used in research laboratories of anaerobic digestion technologies where microbiological studies are carried out on methanogenic cultures. Pure culture of this bacte-

ria are difficult to obtain and their preservation is essential. The procedures described can help preservation of other kinds of strict anaerobic bacteria.

RESUMO

Um método simples para manutenção de bactérias metanogênicas em nitrogênio líquido.

São descritos métodos e procedimentos de preservação de bactérias anaeróbias estritas produtoras de metano. Duas técnicas diferentes foram adotadas: uma para manutenção de baixo potencial de oxi-redução em frascos de cultivo e outra para conservação das células bacterianas em nitrogênio líquido.

Palavras-chave: bactérias metanogênicas; preservação de culturas.

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INFORMAÇÕES TÉCNICO-CIENTÍFICAS

REVISÃO DE LIVROS

BRUNT, A., CRABTREE, K. & GIBBS, A. (ed.) **Viruses of Tropical Plants.** CAB International + Australian Centre for International Agricultural Research/ A.C.I.A.R., xiv + 707p., Redwood Press Ltd., UK, 1990.

O livro relaciona os vírus isolados de plantas tropicais, sobre os quais já foram publicados trabalhos. Originou-se do projeto VIDE ("virus identification data exchange"), que utiliza o sistema DELTA de base de dados ("description language for taxonomy") para coletar informações sobre os vírus. A vantagem do sistema DELTA é que ele permite manusear todos os tipos de informações taxonômicas de uma forma útil e flexível. Os dados armazenados por este sistema são efetivamente transferidos para programas taxonômicos.

No capítulo introdutório (cap. 1, 6p.), são fornecidos esclarecimentos sobre o projeto VIDE, o sistema DELTA, a forma de utilizar e entender o livro e conceitos gerais sobre a sistemática de vírus de plantas. O capítulo 2 abrange as plantas hospedeiras naturais de vírus, nos trópicos; contém uma relação das espécies, por ordem alfabética do nome botânico e os vírus já isolados destas espécies (p. 7-50). A seguir, é apresentada uma relação das famílias e os vírus já descritos nas mesmas. (p. 51-62). O capítulo 3, que é o mais longo, e o grande enfoque do livro, discorre sobre a descrição dos vírus (p. 63-618). Está apresentado em ordem alfabética do nome vulgar do vírus. Tais nomes foram modificados, de acordo com sugestão apresentada no trabalho de Fenner (Fenner, F. (1976). Classification and Nomenclature of Viruses. 2nd Report of the International Committee on Taxonomy Viruses. *Intervirology*, 7:1-116), acrescentando-se, ao nome vulgar, o nome do grupo de vírus. Se o vírus está provisoriamente enquadrado em um grupo, coloca-se o nome do grupo entre aspas. Assim: tobacco streak ilarvirus (vírus "streak" do fumo, grupo ilarvirus); centrosema mosaic "potexvirus" (vírus do mosaico da centrosema, provavelmente um potexvírus). Para cada vírus descrito, há os seguintes itens: introdução, círculo de hospedeiras naturais e sintomas, transmissão e ecologia, distribuição geográfica, círculo de hospedeiras experimentais, estabilidade e concentração da partícula em extratos, purificação, morfologia, propriedades físicas, composição química, replicação, citopatologia, sorologia, relações de parentesco, diagnose, referências, colaborador(es) que forneceu(eram) os dados. O capítulo 4 (p. 619-687) apresenta a descrição dos grupos de fitovírus e uma tabela para a identificação dos grupos. Tais grupos são apresentados em ordem alfabética e, para cada um, também há as mesmas informações encontradas para os vírus individuais, como no capítulo 3 (introdução, círculo de hospedeiras naturais e sintomas, transmissão etc.). A tabela para a identificação do grupo de vírus (p. 687-689) é bastante interessante e leva em conta: a morfologia e o tamanho da partícula, a concentração na seiva, o coeficiente de sedimentação, o peso molecular do ácido nucléico e da proteína do capsídeo. De acordo com esta tabela, há 42 grupos de fitovírus, sendo que três têm nomes ainda não oficialmente aprovados pelo ICTV ("International Committee on Taxonomy of Viruses") e 6 ainda possuem o nome do vírus-tipo. No capítulo 5 - Apêndice (p. 691-707), há o questionário de coleta de dados sobre os fitovírus, para armazená-los em computador. As respostas ao questionário devem ser encaminhadas ao projeto VIDE (Research School of Biological Sciences, Australian National University, G. P. O. Box 475, Canberra City, A. C. T., 2601, Austrália). Na última página, os autores citam as fontes dos produtos para diagnóstico mencionados no texto (tais fontes são fontes comerciais, por ex., a "American Type Culture Collection", ou pessoas).

Trata-se de um livro extremamente importante para os virologistas de plantas. Os editores são virologistas conceituados. O primeiro (Alan Brunt) pertence ao "AFRC Institute of Horticultural Research", de Littlehampton, Reino Unido, e os outros dois (Karen Crabtree e Adrian Gibbs) trabalham no "Molecular Evolution and Systemics Group, Research School of Biological Sciences, The Australian National University", de Canberra, Austrália.

O projeto VIDE existe desde 1970 e começou com um banco de dados experimental sobre os vírus das leguminosas com financiamento do "Rural Credit Development Fund of the Reserve Bank of Australia". A partir de 1983, o projeto tem sido financiado pelo "Australian Centre for International Agricultural Research" e informações adicionais de vírus de leguminosas e cereais tropicais têm

sido incluídas no banco de dados. Um grande número de virologistas no mundo todo tem colaborado com o fornecimento de informações para o banco de dados do projeto VIDE e isso tornou possível a realização deste livro. A relação destes virologistas ("List of contributors") é apresentada nas páginas vii - xiv e consta de 171 nomes, incluindo os respectivos endereços. Há 4 fitovirologistas brasileiros entre estes colaboradores.

O projeto continua em desenvolvimento, mesmo após a publicação do livro, e os responsáveis solicitam a colaboração de todos os virologistas, de qualquer país do mundo, no sentido do envio de informações sobre vírus novos (ver cap. 5).

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Oral Candidosis. Ed. Lashman P. Samaranayake & T. Wallace Mac Farlane Wright, London, 1192 Butterworth & Co. Ltd.

Este livro editado recentemente apresenta grande importância no conhecimento atual da candidose, conforme o apresentador do mesmo, Prof. J. J. Pindborg, comenta. Os editores convidaram para escrever alguns dos capítulos, renomados nomes na área, os quais com sua experiência, contribuem para esclarecimento de alguns problemas relacionados a infecção por Candida.

Desde os aspectos básicos até os aplicados seus 14 capítulos apresentam-se bem elaborados e com numerosas referências além de ilustrações de ótimo nível.

Aspectos fundamentais como a biologia de espécies de Candida, ecologia e epidemiologia bem como fatores de virulência são comentados de forma compreensiva e objetiva.

Um capítulo de candidose e Aids, tão importante no contexto atual, é também apresentado e problemas relacionados as próteses dentárias e infecção por Candida, mereceram uma atenção especial dado ao grande desafio desta patologia na área da estomatologia.

Os autores nas suas notas finais concluem que graças aos conhecimentos atuais e a recente tecnologia, deverá ocorrer um grande avanço em relação a candidose bucal, resolvendo-se nos próximos anos alguns dos principais problemas ainda em questão.

Recomendamos este livro que vem preencher uma lacuna não só na área de Estomatologia e Microbiologia Bucal, mas de modo geral nas áreas básicas e aplicadas apresentando uma revisão compreensiva e atual desta micose tão prevalente em nosso meio.

Esther G. Birman
Prof. Assoc. do Departamento de
Estomatologia – FOUSP.

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