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BIOTYPE AND LIOR'S SEROGROUP OF CAMPYLOBACTER JEJUNI AND CAMPYLOBACTER COLI ISOLATED IN SÃO PAULO, BRAZIL

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Kinue Irino

SUMMARY

Lior's schemes were used for biotyping and serogrouping enteric *Campylobacter* strains isolated during seven year period in São Paulo, Brazil. Frequency of *Campylobacter* was evaluated concerning its distribution according to the age group and proportion of other enteropathogens. Prevalence of 61,33% was observed in relation to *C. jejuni* biotype I, followed by 24,22% of *C. jejuni* biotype II and 9,76% of *C. coli* biotype I. Serotyping was performed in 84,94% of strains, using 46 antisera. The predominante serogroups were 36 (16,89%), 33 (9,13%) and 9 (6,85%). The remaining strains were classified into 23 different serogroups. *C. jejuni* and *C. coli* were isolated 67 times (23,51%) in coinfection and 62,81% of these strains were isolated from children aged under 2 years old.

Key words - *C. jejuni*, *C. coli*, serogroup, biotype

INTRODUCTION

With the implementation of new selective stool culture techniques developed by Dekeyser (9), Butzler (7) and Skirrow (27), *Campylobacter jejuni* and *Campylobacter coli* has been recognized as an important cause of bacterial diarrhoea in humans, equalling or exceeding *Salmonella* and *Shigella* in prevalence.

It is now known that there are several species of *Campylobacter* and that the major pathogen of humans is *C. jejuni*. Infections due to *C. coli* which are closely related to *C. jejuni*, appear to share many clinical and epidemiological characteristics.

The source of infection for humans is thought to be the massive reservoir of *C. jejuni* and to a lesser extent *C. coli* in the animal population (1,2,4).

Epidemiological evidence has linked human *C. jejuni-C. coli* infections to the consumption of contaminated food, especially poultry, unpasteurized milk, and water, as well as to contact with domestic animals (3,4,5,26).

To confirm these suspected routes of sources and transmissions, effective discrimination between strains is required. Since the bacteria have only a few biochemical reactions that may be routinely used in clinical laboratory it is expected that biotyping alone, will not be adequate for epidemiological purposes. Interest has, therefore, focused on the use of serotyping methods for epidemiological tracing.

Several different serotyping schemes have been described (16,23). Lior (18) developed a serotyping scheme for *C. jejuni*, *C. coli* and *C. laris* based on a rapid slide agglutination technique, us-

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ing live bacteria and absorbed antisera for detection of heat-labile antigenic factors.

The increasing frequency of *C. jejuni-C. coli* isolations from sporadic cases of human enteritis in Brazil (8,10,22,25), created the need of devising a possible mean of serological identification of strains for epidemiological purposes.

MATERIALS AND METHODS

From January 1983 through December 1989, 7652 faecal samples were cultured in *Campylobacter* selective medium consisting of 5% sheep-blood Trypticase soy agar (TSA), containing, per millilitre, 10 ug vancomycin, 5 ug trimethoprim, 2,5 IU of polymyxin B, 2,0 ug amphotericin B, 15 ug cephalothin, and supplemented with 0,5% of yeast extract and FBP. Plates were incubated at 42°C in microaerophilic atmosphere (19) and were examined after 48h for *C. jejuni* and *C. coli* growth.

Slide smears from suspected *Campylobacter* colonies were stained with crystal violet. Identification was based on catalase and oxidase production, growth in 1% glycine, growth at 42°C but not at 25°C, hydrogen sulfide production with lead acetate strips, sensitivity to nalidixic acid and resistance to cephalothin, and presence or absence of hippurate hydrolysis. Hippurate-positive strains were classified as *C. jejuni*, and negative ones as *C. coli*.

The biotypes of all the isolates were determined by biotyping scheme of Lior (17). For heat-labile antigens detection, Lior's scheme was used.

The reference strains for 46 serotypes had been kindly supplied by Dr. Lior (Laboratory Centre for Disease Control, Ottawa, Ontario, Canada).

The reference strains were first plated on TSA supplemented with 0,5% of yeast and containing 5% sheep-blood, incubated at 37°C in an oxygen-reduced atmosphere produced by Anaerocult C (Merck). Several smooth colonies from each plate were subcultured in Brucella broth and after incubation at 37°C for 24h, 3 ml of the culture were spread on plates (15x150mm) with supplemented TSA. After incubation at 37°C for 48h, the growth was harvested in 0,5% formalin-phosphate-buffer saline (PBS) pH 7,2. The suspension was washed three times, tested for sterility and adjusted to 10.10^{10} bacteria/ml.

White New Zeland rabbits were immunized by 5 injections with increasing doses of 0,5-2,0 ml at 3-4 days intervals. After 7 days the last injection, the sera were tested for agglutinins and if the titer was satisfactory, the animal were bled. The sera were maintained at 4°C with merthiolate (1:10.000).

Each serum was absorbed with the homologous strain heated for 2h at 100°C and was tested with all strains for cross-reactions. If a cross-reaction occurred, it was eliminated by absorption with heterologous unheated strain.

Nine pools were formed with 46 antisera and the working dilution were determined as that which was observed slide agglutination of the homologous strain within one minute.

Before serotyping the test strains were subcultured 5-10 times or more (rough strains up to 15-20 times). The serotyping technique consisted of a slide agglutionation test with 0,1% DNase (Boehringer-Mannheim) as the diluent. The pool sera, unabsorbed sera and absorbed sera were used successively.

All faecal specimens were also cultured for *Salmonella*, *Shigella*, and enterophathogenic *E. coli*.

RESULTS

During 7 years period 1983-1989, 285 (3,72%) isolates of thermotolerant *Campylobacter* species were recovered. Identification of 285 *Campylobacter* isolates showed that 239 (83,86%) were *C. jejuni* and 34 (11,93%) *C. coli*. *Campylobacter* sp. were identified in 16,48% among positive cases and mixed infection with *Campylobacter* sp. and other bacterial pathogens were found in 67 of 285 cases (23,51%).

Most (62,81%) of the *Campylobacter* sp isolations were from children under two years of age (Table 1). *C. jejuni* biotype I was most common among isolates (61,33%) followed by *C. jejuni* II (24,22%), *C. coli* I (9,76%, *C. coli* II (3,13%), and *C. jejuni* III (1,17%). One strain of *C. jejuni* IV was found in this study.

TABLE 1 - Age distribution of *Campylobacter* isolated during period 1983-89

Age	Number	Percentage
0 -11 mo	111	38,95
12 - 23 mo	68	23,86
2 - 4 y	29	10,17
5 - 20 y	16	5,62
> 20 y	41	14,38
unknown	20	7,02

Enteropathogenic *Escherichia coli* was the most commonly isolated bacterial pathogen (33,58% among positive cases). Other common bacterial pathogens (Table 2) included *Shigella* (20,43%), *Salmonella* (18,18%), enterotoxigenic *E. coli* (5,99%), and enteroinvasive *E. coli* (5,34%).

TABLE 2 - Percentage of enteropathogenic bacteria isolated during period 1983-1989

Bacteria	Percentage
Enteropathogenic *E. coli*	33,58
Shigella	20,43
Salmonella	18,18
Campylobacter	16,48
Enterotoxigenic *E.coli*	5,99
Enteroinvasive *E.coli*	5,34

TABLE 3 - Distribution of *Campylobacter jejuni* and *Campylobacter coli* serogroups according Lior's scheme

Serogroup	Total nº of strains	% of total
Lio 1	3	1,37
Lio 2	8	3,65
Lio 4	13	5,93
Lio 5	1	0,45
Lio 6	1	0,45
Lio 7	6	2,74
Lio 8	6	2,74
Lio 9	15	6,85
Lio 10	8	3,65
Lio 11	3	1,37
Lio 12	1	0,45
Lio 13	8	3,65
Lio 16	1	0,45
Lio 17	9	4,21
Lio 19	6	2,74
Lio 21	12	5,48
Lio 22	1	0,45
Lio 27	8	3,65
Lio 28	1	0,45
Lio 29	2	0,91
Lio 31	1	0,45
Lio 32	1	0,45
Lio 33	20	9,13
Lio 35	4	1,82
Lio 36	37	16,89
Lio 44	10	4,56
Untypeable	22	10,04
Rough	11	5,02

Of 219 strains tested, 186 (84,94%) were typable by 46 antisera of Lior typing scheme; they were associated with 26 serotypes listed in Table 3. The remaining 33 strains either did not react with any of the sera (10,04%) or were rough and were simultaneously agglutinated by many antisera even after 20 transfers on TSA (5,02%).

DISCUSSION

The reported number of 285 (3,72%) isolates of *Campylobacter* represents only a small fraction of infections that occurred. The magnitude of the problem of campylobacteriosis is difficult to assess because faecal cultures are obtained from only a fraction of persons who have diarrhoeal illnesses. Nevertheless, by comparing *Campylobacter* isolations rates with those of other pathogens, a relative estimate of incidence can be made.

We found the highest isolation rates of *Campylobacter* among infants, which is consistent with the age distribution found in low-income populations where diarrhoeal disease are endemic (11,13). The results of the present study suggest that children are exposed to *C. jejuni* and *C. coli* at an early age in developing countries.

It is difficult, however, to compare isolation rate in different age groups since the sampling frequency may vary. The apparent high isolation rate in infants may be due to a distortion caused by oversampling relative to adults.

It has now established on basis of DNA hybridization studies that the hippurate-positive strains (*C. jejuni*), and hippurate-negative strains (*C. coli*) represent two different species. *C. coli* accounts for only about 3 to 5% of the *Campylobacter* isolates from cases of human gastroenteritis (28). The relative contribution of *C. coli* in our study (11,93%) was not significantly different from that reported in Sweden (14) and Norway (15) but lower than that reported in China (13), Central African Republic (11), and in Yugoslavia (24).

In our finding concerning other enteric bacteria, EPEC were more frequently isolated. *Shigella*, *Campylobacter*, and *Salmonella* were isolated with similar frequencies. These data indicate that *C. jejuni* and *C. coli* is a significant cause of gastroenteritis in São Paulo, and thus, laboratories should seriously consider the possible presence of this organism when culturing stool specimens.

It has been reported that *C. jejuni* and *C. coli* is frequently encountered in mixed infections with other known bacterial enteropathogens (2,8,13). In this study 67 of 285 case (23,51%) of *Campylobacter* enteritis was coinfected with other enteropathogenic bacteria.

Bukholm & Kapperud (6) reported that *Salmonella*, *Shigella*, and *E. coli* strains produce synergistic interactions that facilitate the invasion by *C. jejuni* in cell cultures. However the effect exerted by the coinfectants at the host cell remain unclear.

A reproducible, sensitive, and well-standardized typing system is critical to the successful investigation of outbreaks caused by *C. jejuni* and *C. coli*. A successful scheme should be capable of typing the majority of strains associated with sporadic infection.

The percentage of serotypable strains detected in the present study (84,94%) was not significantly different with the results reported for strains from Canada, Bangui, USA, and Germany (12,18,21,29). Our finding of 26 serotypes among 186 typable isolates may be explained by the fact that all strains were from sporadic cases of *Campylobacter* enteritis.

Serotype 36, the most frequently identified serotype in São Paulo, is also one of the most predominant in France (20), USA (21), Central African Republic (12), and Germany (29). The serotype 33, the second most frequent in our study, was reported in Germany, only.

The serogrouping scheme of Lior is easy to apply, consisting of a slide agglutination, similar to that used to type other organisms such *Salmonella*. However, the scheme is difficult to implement because absorption of the sera is necessary and the other problem is the need for multiple subcultures before successfully typing strains which may have been stressed by storage.

On the other hand, once the antisera have been prepared, direct agglutination is simpler, which explains its preference by an increasing number of investigators.

C. jejuni biotype I and II were the majority of types found, 61,33% and 24,22%, similar to the reported in other countries (17,20,29).

The integration of the biotyping and serotyping scheme resulted in a further differentiation of *C. jejuni* and *C. coli* isolates, specially of the common serotypes. The subdivision of these serotypes provides additional epidemiological information.

In our study, 15 of 26 serotypes identified were associated with two or more biotypes. Thus, the bio-serotypes most frequent were *C. jejuni* biotype I/serotype 36, *C. jejuni* I/33 and *C. jejuni* I/9.

Serotyping in sporadic cases of *Campylobacter* enteritis does not have important medical implications but should be performed for epidemiological purposes.

RESUMO

Biotipos e sorogrupos de Lior de *C. jejuni* e *C. coli* isolados em S. Paulo-Brasil

Foi avaliada a freqüência de *Campylobacter* sp no período de 1983 a 1989, quanto à sua distribuição por grupo etário e ao percentual em relação a outros enteropatógenos. As cepas isoladas foram classificadas em biotipos e sorogrupos, segundo as metodologias descritas por Lior. Foram utilizados 46 anti-soros preparados com cepas de referência recebidas do "National Reference Service for *Campylobacter*" do Canadá. Neste período foram isoladas 285 cepas de *Campylobacter* sp., correspondendo a 3,72% do total de amostras de fezes analisadas, sendo 83,86 % de *C. jejuni* e 11,93% de *C. coli*, das quais 62,81% foram isoladas de crianças com menos de dois anos de idade. Em 67 vezes (23,51%), *C. jejuni* e *C. coli* foram isolados em infecções mistas. Houve prevalência do *C. jejuni* biotipo I com 61,33%, seguidos de *C. jejuni* biotipo II com 24,22% e *C. coli* biotipo I com 9,76%. Foram sorotipadas 84,94% das cepas isoladas, havendo predominância dos sorogrupos 36 (16,89%), 33 (9,13%) e 9 (6,85%). As demais foram classificadas em 23 diferentes sorogrupos; 22 cepas (10,04%) não puderam ser tipadas com os 46 anti-soros utilizados e outras 11 (5,02%) se apresentaram autoaglutinantes.

Palavras chaves: *C. jejuni*, *C.coli*, sorogrupo, biotipo.

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RELEASE OF EXTRACELLULAR POLYSACCHARIDE BY SPONDYLOSIUM PANDURIFORME (DESMIDIACEAE)

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ABSTRACTS

The filamentous desmid *Spondylosium panduriforme* (Heimerl) Teiling var. *panduriforme* f. *limneticum* has a large rigid mucilaginous sheath and releases great amounts of extracellular dissolved polysaccharide. Experiments on the kinetics release of labelled extracellular dissolved polysaccharide and on the labelling of the sheath polysaccharides were carried out during 28 hours the dissolved polysaccharide could be detected one hour after addition of C^{14} and its amount increased continuously with time even during dark period. Labelled sheath material could be detected half an hour after addition. The results show that the viscosity of the culture medium was not due to the dissolution of ageing sheaths. There are evidences that the origin of the dissolved polysaccharide could be from an active physiological process of production of this polymer.

Key words: *Spondylosium panduriforme*, extracellular dissolved polysaccharide, release kinetics.

INTRODUCTION

Relatively great amounts of dissolved polysaccharides are found in culture media of microalgae and frequently these media become viscous with ageing cultures. This fact is conspicuous in species with not well outlined or non consistent mucilaginous sheaths like Tetrasporales, several Cyanophyceae and Zygnemataceae. We can observe this fact also in *Cryptomonas* sp cultures (13). This also happens with species of Desmidiaceae which shed the primary wall like *Pleurotaenium trabecula*, in Volvocales and Chlorococcales which have sporangium wall autolysis, thus releasing polysaccharides (1, 2, 3, 14, 16, 17). It also occurs on species which have well outlined, rigid and non easily soluble sheaths like *Spondylosium panduriforme*.

Many works have shown that the release of polysaccharide in several taxa increases during the senescent culture phase or under stress conditions like nutrient limitation (5, 6, 8, 12, 17). This leads one to conclude that the origin of the dissolved polysaccharides would be a simple dissolution of ageing sheaths or by increasing its production only in ageing cultures.

This work is concerned to production of both extracellular dissolve polysaccharide and sheath polysaccharide.

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MATERIALS AND METHODS

Organism and Culture Conditions. Spondylosium panduriforme (Heimerl) Teiling var. panduriforme f. limneticum (West & West) was isolated from a marginal lagoon of the Mogi-Guaçu River, SP, Brazil and has been maintained in the freshwater microalgae culture collection at Federal University of São Carlos as 072CH-UFSCAR strain. The cells were grown in WC medium (7) under axenic condition at 20-22°C. The cultures were continuously aerated by gentle bubbling with filtered and moist air. Illumination was provided by 40W fluorescent tubes (100μE. m^{-2}. s^{-1}) and with a light/dark cycle of 12:12 hours. The experimental cells were in fast growth (logaritmic growth phase).

Labelling with C^{14}. To label the cells and dissolved material $NaC^{14}O_3$ (0.18μCi.ml^{-1}), New England Nuclear Corp., was injected at the beginning of the light period (07:00h) into a new preadapted culture in exponential growth (3.10^4 cells.ml^{-1}) in 3.0 liters medium.

Total Extracellular C^{14}*-labeled Matter.* To determine C^{14} into total dissolved excreted material, 35 ml of the cultures were sampled at regular intervals and centrifuged gently (800 - 100 rpm by 5 minutes in an universal centrifuge because the cells are larger than 100μm, including the sheaths). The supernatant was filtered through a 0.45μm pore membrane and was acidified with 1N HCl till pH 3.0 and vigorously aerated during 40 minutes to eliminate inorganic C^{14}. After this, the pH was adjusted to 7.0 and aliquots of 10 ml of the filtrate were transfered into scintillation vials and 10 ml of toluene based fluor were added for radioactivity measurements of total excretion. The remainder (25 ml) was concentrated five times at reduced pressure by a rotary evaporator at 40°C and subsamples of 3 ml from each concentrated sample were collected for gel filtration at Sephadex G-50 in a 1.6 x 62 cm column.

Toluene Based Fluor (Scintillation Cocktail): Toluene + Renex™ (7:6, used for aqueous samples) + 100 $mg.^{L-1}$ dimethyl POPOP (*1, 4-bis-2-*(4-*methil-5-phenyloxazolyl*))+ 3$g.L^{-1}$ PPO (*2,5 diphenyloxazol*). For filters with cells, 2:1 toluene/Renex™ was used.

Total Particulate Matter Labeled with C^{14}. To determine total particulate organic labeled matter (the whole cells) samples of 10 ml of the culture were filtered through 1.0 μm pore membrane to get easier and gently filtration since the cells are very large. The filters were washed with distilled water, dried and placed in scintillation vials with toluene based fluor as above but 2:1 of toluene/Renex.

Extraction of Sheath Products Labeled with C^{14}. 80 ml of the cultures were sampled at regular intervals and centrifuged. The cells were washed twice with fresh culture medium and fixed with 5% formaline for two hours. After this the cells were washed again and ressuspended in 80 ml of distilled water and kept at 55°C for 16h in order to extract the sheaths. The extraction was monitored by microscopical observations. The extracts were filtered through a 0.45 μm pore membrane and concentrated as above and dialyzed against distilled water in a Spectrapor dialysis tube with a cutoff of 12,000-14,000 daltons. Toluene was added to prevent microbial growth. After dialysis the sheath material was treated with ethanol and saline (15) to precipitate the polysaccharides. The sheath precipitates were dissolved in 30 ml of distilled water by heating overnight at 55°C. 10 ml of dissolved samples were then placed in scintillation vials with 10 ml toluene based fluor at 2:1 of toluene/Renex™.

Extracellular Polysaccharide Labeled with C^{14}. The supernatants (80 ml) of the centrifuged cells samples above were filtered through 0.45 μm pore membrane, concentrated five times, and after dialyzed they were precipitated as above to colect the extracellular dissolved polysaccharides. The precipitated polysaccharides were dissolved in 15 ml of distilled water then 10 ml of dissolved samples were placed in scintillation vials with 10 ml of 7:6 toluene/Renex™ toluene based fuor.

Radioactivity Measurements. The radioactivity was determined by a Packard TriCarb mod. 1550, with quench correction by automatic external standardization. DPM values for sheath polysaccharide and extracellular dissolved polysaccharide were corrected for 10 ml culture.

Controls. Samples taken both at time zero and at different intervals throughout the experiments were all fixed with 5% formaline and used as controls.

Carboydrate content was tested by the phenol-sulfuric acid method (4) and protein by the Lowry method (10).

RESULTS AND DISCUSSION

Labelled extracellular dissolved polysaccharide could be detected in culture medium one hour after the addition of $NaHC^{14}O_3$ meanwhile labelled capsular material could be detected after half an hour (Fig. 1). Sephadex G-50 gel filtration of total excreted material shows that the high molecular weight compounds excreted increase with time from first hour on (Fig. 2). Phenol-sulfuric acid as-

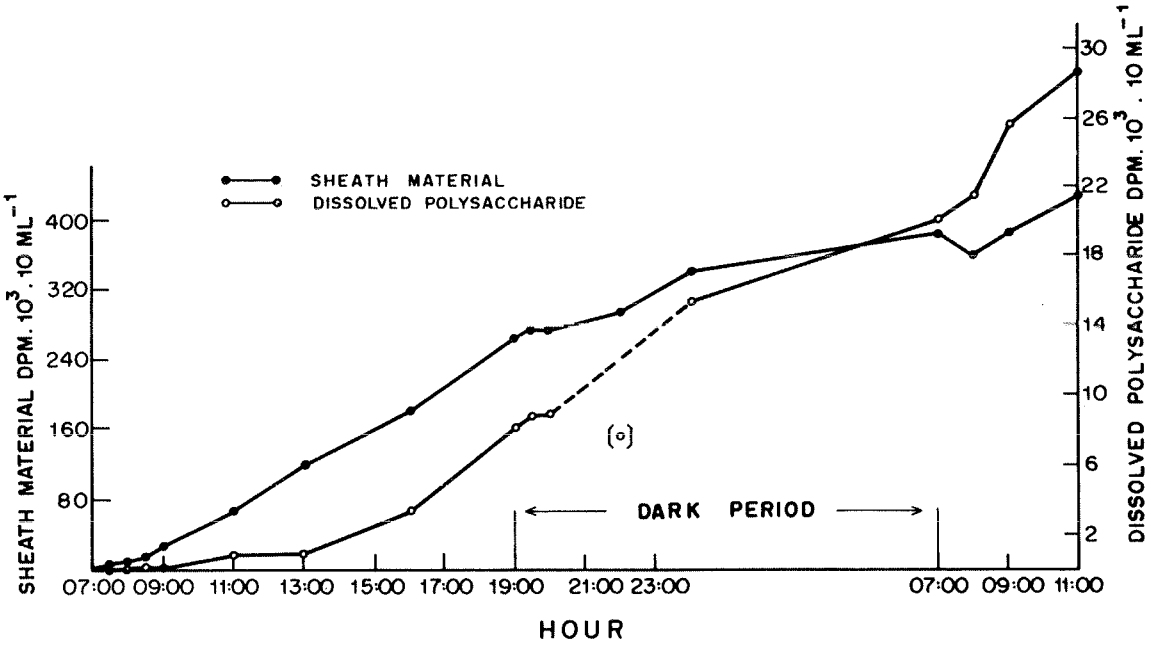

FIGURE 1 - C^{14}-incorporation into sheath material and extracellular dissolved polysaccharide. Desintegrations per minute (DPM) by 10 ml culture.

says showed these compounds are polysaccharides and its protein content is 6.0%. Sheath material is around 96% polysaccharide and 3.8% protein.

The release of the extracellular dissolved polysaccharides was continuous throughout 28 hours including the dark period (19:00-07:00). The same happened with the labelling of sheath material. These results show that the kinetics release of extracellular dissolved polysaccharide is similar to that of total excreted, and both are closely related to total particulate carbon fixation but the increase of the dissolved material during dark period shows that the process was not a passive loss of the photoassimilated carbon (Fig. 3). Rapidly incorporation of C^{14} into sheath material without lag phase and its continuity even in dark period agrees with microscopical observations. Rarely we can see *S. panduriforme* dividing cells without mucilaginous sheaths.

The results for sheath labelling agree with those obtained for natural phytoplankton communities by authors which found that polimeric fractions are labelled without lag phase or with a very short one (9, 11, 18). Also, in the literature we can find references to a very fast labelling of excreted polymers like polysaccharides in rapidly growing cells of *Chaetoceros affinis* (Bacillariophyceae) (12).

We can conclude that the culture media viscosity, at least for *S. panduriforme*, is due to the accumulation of polysaccharides which are continuously released by an active process. The short lag phase to detect labelled extracellular polysaccharide reinforce the idea that the accumulation of this material into the medium does is not due to a passive dissolution of the ageing sheaths neither technical artifacts or stress conditions. Thus, the dissolved polysaccharide could be released by chemical transformation of the sheath material what agrees with the fact that there is a lag phase between sheath and dissolved polysaccharide labelling and that the ratio extracellular dissolved polysaccharide/sheath polysaccharide increases in a linear shape (Fig. 4).

RESUMO

Liberação de polyssacarídeo extracelular por *Spondylosium panduriforme* (desmidiaceae)

A desmidiacea filamentosa Spondylosium panduriforme tem uma bainha mucilaginosa relativamente grande e consistente. Usando C^{14} como marcador foi estudada a cinética de liberação de polissacarídeo extracellular solúvel e a marcação dos polissacarídeos da bainha durante 28 horas seguidas. O polissacarídeo solúvel pode ser detecta-

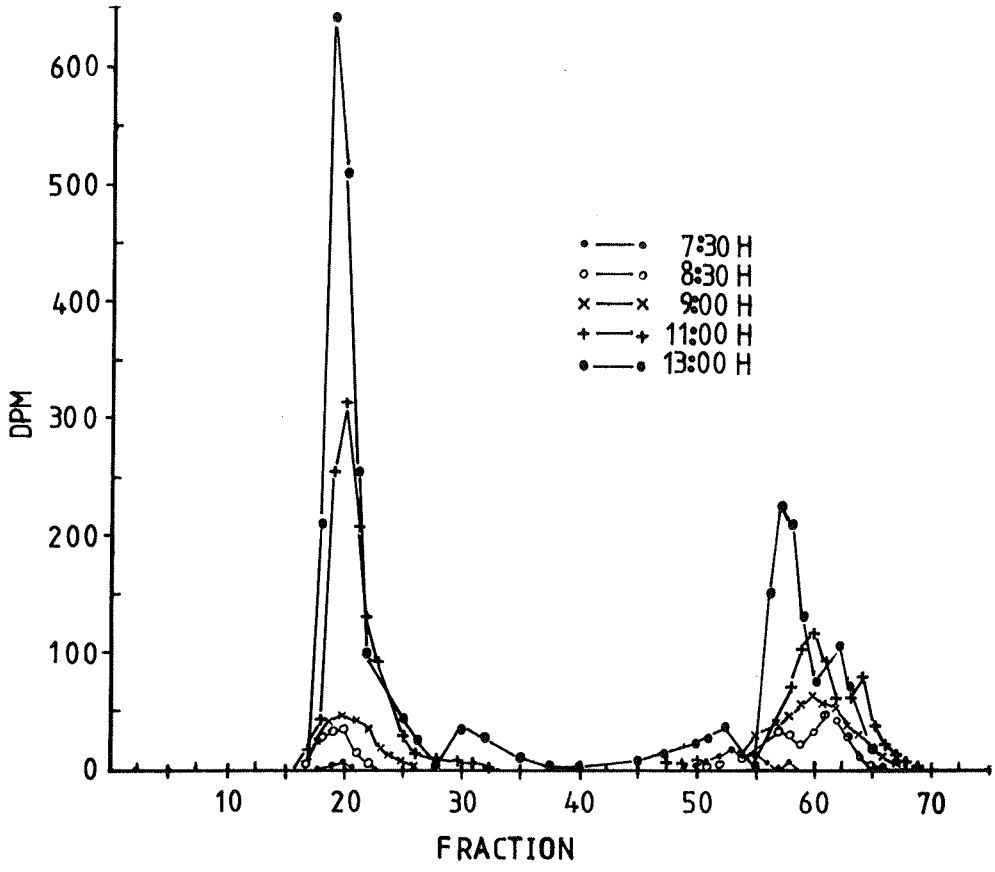

FIGURE 2- Gel-filtration (Sephadex G-50) of concentrated culture medium with C^{14}-labelled dissolved organic matter released by *S. panduriforme*. DPM by 2 ml fraction.

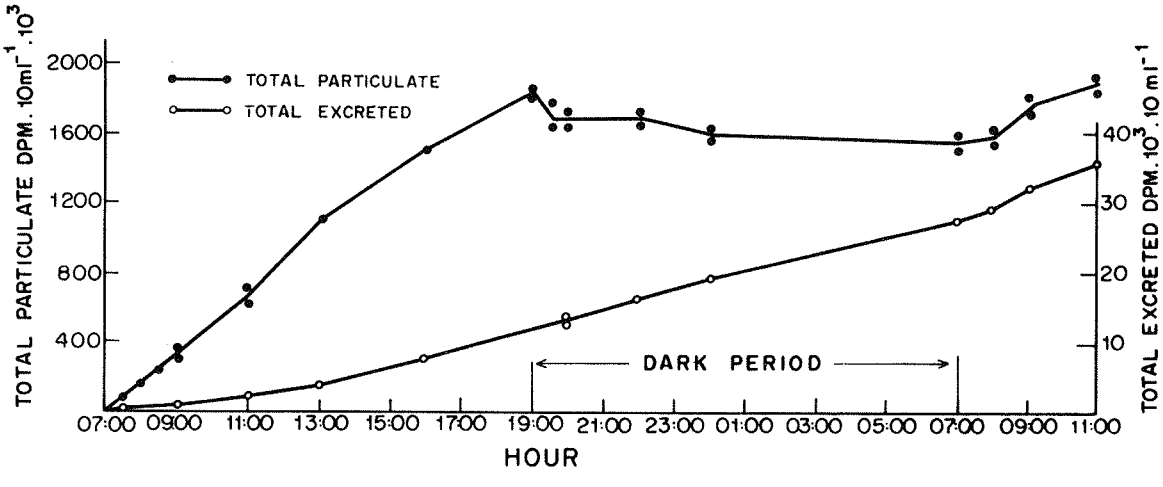

FIGURE 3- C^{14}-incorporation into total particulate (whole cell) and into total released dissolved organic matter. DPM by 10 ml culture.

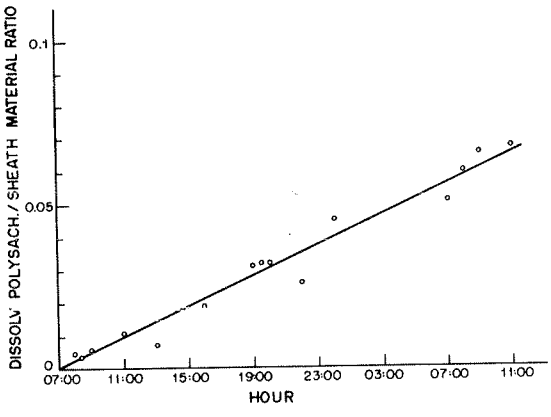

FIGURE 4- DPM ratio of extracellular dissolved polysaccharide and sheath polysaccharide.

do no meio de cultura uma hora após a adição do marcador enquanto o polissacarídeo da bainha pode ser detectado meia hora após. Os resultados mostram que a viscosidade do meio de cultura desta espécie não é devida à dissolução das bainhas de células velhas, como se pensava. Há evidências que o polissacarídeo solúvel pode ser produzido por um ativo processo fisiológico.

Palavras-chave: *Spondylosium panduriforme*, polyssacarídeo extracelular dissolvido, cinética de liberação.

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COLONIZATION FACTORS AMONG ENTEROTOXIGENIC ESCHERICHIA COLI (ETEC) ISOLATED FROM DIARRHEIC CHILDREN IN OURO PRETO (MINAS GERAIS STATE) AND PAULÍNIA (SÃO PAULO STATE), BRAZIL

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SUMMARY

Eighty-seven enterotoxigenic *Escherichia coli* (ETEC) and 36 non-ETEC strains were examined for the presence of the colonization factors CFAI, CFAII(CS1-CS3) and CFAIV(CS4-CS6). All strains were isolated from cases of diarrhea among children under 12 years of age living in the cities of Ouro Preto (MG) and Paulínia (SP), Brazil. Two ETEC (2,3%) strains from Ouro Preto both producing STa enterotoxin, showed serological positive reactions with a-CFAI antiserum. No positive reactions were found with a-CFAII and a-CFAIV antisera, though several strains have shown D-manose resistant hemagglutination with at least one of the following erythrocytes: human, bovine and chicken.

Key Words: Colonization factors; ETEC; infant diarrhea.

INTRODUCTION

Presently enterotoxigenic *Escherichia coli* (ETEC), as well as enteropathogenic *E. coli* (EPEC) and rotavirus are among the most common agents involved in infant diarrhea in less-developed areas (17,21).

For human ETEC, as for most infectious microorganisms, the pathogenicity of this group of bacteria is associated with the expression of specific adhesin proteins that enable bacterial attachment to the host mucosal surface before tissue colonization (1). After colonizing the proximal small intestine, the critical site of host-parasite interactions, they elaborate heat-labile enterotoxin (LT) (6,7,13,15) and/or heat-stable enterotoxin (ST) (5,20,24).

The main human ETEC adhesins are designated as colonization factor antigens (CFAs) I and II (11,12). CFAII consists of a family of three immunologically distinct *E. coli* surface antigens (CS1, CS2 and CS3) (1). Thomas et alii (26) have reported the presence of a new colonization factor named CFAIV in the prototype strain E8775. CFAIV like CFAII is composed of three different coli surface antigens (CS4, CS5 and CS6). Other new putative colonization factors have also been described (1).

Through cooperative work with researchers from the Federal University of Ouro Preto (MG) and from the School of Medicine of Campinas University (SP) which has a small Hospital in Paulínia (SP), we selected 87 ETEC and 36 non-

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ETEC strains, which were isolated from cases of diarrhea among children under 12 years old. In these strains, we looked for the presence of CFA-I, CFA-II and CFA-IV antigens using the D-mannose resistant haemagglutination (23) and specific serological tests.

MATERIALS AND METHODS

Strains

From cases of diarrheal illness among children from the cities of Ouro Preto (MG) and Paulínia (SP), 123 strains of *E. coli* (table I) were selected to look for the CFA-I, CFA-II and CFA-IV colonization factors.

TABLE I - Enterotoxigenic phenotypes of the *E. coli* strains isolated from cases of diarrhea among children in the cities of Ouro Preto (MG, 1989) and Paulinia (SP, 1990).

Enterotoxigenic	Strain origin		
Phenotypes	Paulínia	Ouro Preto	Total
LT^+	30	17	47
STa^+	28	07	35
LT^+/STa^+	04	01	05
LT/STa^-	21	15	36
Total	83	40	123

Enterotoxin detection

The production of STa by these strains was verified through the infant mouse test (IMT) (10), by injecting intragastrically 0,1 ml of the supernatants taken from cultures grown at 37°C in CAYE medium. For the detection of LT the same supernatants were examined by the modified radial immune haemolysis test (28) and by the indirect hemagglutination test (22).

Antiserum preparation

Specific antisera were prepared against CFA-I and CFA-II (CS1, CS3). For this purpose, strains TR50/3 and 242 were used respectively, according the recommendations of Yano et al. (29). Cultures were grown in CFA liquid medium for 18h at 37°C, in a microfermentor. Then, cultures were centrifuged at 5.000 rpm for 30 min, and the sediment resuspended in phosphate buffer containing 1M NaCl. This suspension was incubated at 60°C for 20 min with intermittent shaking, in order to release fimbrial structures from bacteria.

This suspension was thereafter centrifuged and the pili, in the supernatant, were concentrated by precipitation with 60% ammonium sulphate, under constant stirring. After centrifugation, the supernatant was discarded, the sediment resuspended with PBS, pH 7,0, 0,05 M and dialyzed for 48h against the same buffer to which 0,5% of sodium deoxycholate (DOC) was added. After three days in this buffer, the dialysate was centrifuged, the DOC soluble fraction concentrated by ultrafiltration through a PM 10 membrane (Amicon) and applied to a column (2 x 95 cm) containing Sepharose CL4B, equilibrated in 4M urea buffer. The chromatography was carried out and the peak (Ab 280 nm) corresponding to the fimbria was separated, concentrated by ultrafiltration followed by gel filtration on Sephacryl S-300.

Anti-pilus antibodies were induced by subcutaneous immunization of rabbits with the purified antigens. Each rabbit received 30 µg of pili emmulsified in Freund's Complete adjuvant. Similar boosters after 25 and 50 days the animals were reinoculated with equal amounts of antigen emulsified in 1 ml of Freund's incomplete adjuvant.

Since no purified antigen was available, antiserum against anti-CFAIV(CS4-CS6) was prepared as previously described (29), using a strain containing CS4-CS6 complex antigen. This strain was grown on CFA medium at 37°C for 18 h, resuspended in sterile saline, washed twice in saline and the final suspension made in saline containing 0,5% formaldehyde. The inoculation dosage was subsequently standardized according to Mac Farland's tube 3 scale. The rabbits were injected intravenously with five doses at 5-days intervals, with increasing volumes of the suspension. Blood was taken 10 days after the last injection and the rabbits were exsanguinated after checking the serum titre.

Before using, the sera were exhaustively absorbed with packed bacteria cells of homologous culture strains grown at 16°C. After absorption, the antisera were considered monospecific and ready for use when positive slide agglutination reactions were obtained only with strains grown at 37°C but not at 16°C. All sera showed titres above 1/1024 in agglutination reactions, but the usual dilutions used was 1/40, with appropriate controls.

Search of CFA-I, CFA-II and CFA-IV

Initially we used D-mannose haemagglutination tests looking for clumping of human (A type),

bovine and chicken red blood cells (RBC) as a presumptive indication of CFA-I; bovine and chicken for CFA-II and human (A type) and bovine for CFA-IV. All reactions were carried out at 4°C, with cultures grown on CFA medium. The 123 strains were examined using the haemagglutination test carried out essentially as described by Evans et alii (11) with the erythrocytes suspended in phosphate-buffered saline (PBS) pH 7,2, containing 1% D-mannose. After mixing the erythrocytes with the bacterial suspension, the glass slides were kept on ice for 5 min. and haemagglutination was examined by the naked eye. Serological confirmation of each colonization factor was carried out at room temperature by mixing one drop of specific antisera with bacterial suspension. Irrespective of the results of the haemagglutination tests, all strains were serologically examined. Positive reactions (haemagglutination or serological), were tested again with the culture growing at 16°C, for confirmation of absence of clumping at this temperature. In all reactions standard strains (CFAI+, CS2-CS3+, CS4-CS6+) were used as a reagent control.

RESULTS

After the tests, several haemagglutination patterns were identified in 27 strains (21,9%), 18 of these being toxigenic (20,1% of 87 ETEC) (Table II). Only two strains, 228-6 (LT/STa) and 273-2 (STa), showed serological positive results for CFAI. We could not identify positive serological reactions with the antiserum used in the absence of haemagglutination. The fact that many D-manose haemagglutinating strains were found and clumped one or more of the RBC tested should be further investigated.

DISCUSSION

ETEC strains are involved in many cases of acute diarrhea in both humans and animals. They have two pathogenic traits: adhesion to the small intestine and production of thermolabile and/or thermostable enterotoxins (1). Adhesion to the intestinal epithelium has been correlated with the production of specific antigens. The main adhesins

TABLE II - Results of haemagglutination and serological tests*

Enterotoxigenic phenotype	Origin	D-mannose haemagglutination			Serology			Number of strain
		Human	Bovine	Chicken	a-CFAI	a-CFAII	a-CFAIV	
LT	Paulinia	-	-	+	-	-	-	661
LT	Paulinia	+	+	-	-	-	-	5301-2
LT	Paulinia	+	-	-	-	-	-	5244-4
LT	Paulinia	+	-	-	-	-	-	5312-1
LT	O. Preto	+	+	-	-	-	-	312-2
LT	O. Preto	+	+	+	-	-	-	244-1
LT	O. Preto	+	+	+	-	-	-	275/1
LT	O. Preto	-	+	-	-	-	-	312-3
LT	O. Preto	-	-	+	-	-	-	39A
STa	Paulinia	+	-	-	-	-	-	5275-3
STa	Paulinia	+	+	-	-	-	-	5372-3
STa	Paulinia	+	+	-	-	-	-	5248-4
STa	O. Preto	+	+	+	+	-	-	273-2
LT/STa	Paulinia	-	+	+	-	-	-	523-4
LT/STa	Paulinia	-	-	+	-	-	-	5344-4
LT/STa	Paulinia	-	+	+	-	-	-	5234-2
LT/STa	O. Preto	-	+	+	-	-	-	33-1
LT/STa	O. Preto	+	+	-	+	-	-	228-6
Non-ETEC	Paulinia	+	-	-	-	-	-	5312-1
Non-ETEC	Paulinia	-	-	-	-	-	-	791
Non-ETEC	Paulinia	+	+	+	-	-	-	5602-1
Non-ETEC	Paulinia	+	+	+	-	-	-	5602-2
Non-ETEC	O. Preto	+	-	-	-	-	-	204-3
Non-ETEC	O. Preto	+	-	+	-	-	-	291-2
Non-ETEC	O. Preto	+	-	+	-	-	-	253-6
Non-ETEC	O. Preto	+	-	+	-	-	-	291-3
Non-ETEC	O. Preto	+	-	-	-	-	-	248-7

* The other strains were negative.

reported among human ETEC strains have been described as fimbrial and/or fibrillar structures, named generically colonization factors (CF). The first, CFAI, was described by Evans et alii (11) in 1975 in human ETEC strains, serotype 078:H11. Many other CFs were further described, such as CFAII (12), CFAIII (8,16), CFAIV (26,27), PCF 0159 (25), antigen 2230 (9), PCF 0166 (18), CS17 (19), PCF09 (14) and antigen 8786 (2). Several papers have been published on the incidence of CFAI and CFAII among ETEC strains (1,4,17,21), certainly because they were the first to be described. Conversely papers concerning CFAIV have been less frequent, where the frequency of this adhesin was observed to be around 5-6% among ETEC strains of human origin (3).

The low frequency of the most known CFs, found in epidemiological work with human ETEC from several parts of the world has stimulated several researchers to look for new adhesins and characterize them, whose fact explains the high number of morphological, biological and serological distinct adhesins so far described.

Among human ETEC strains isolated in Brazil only a few authors have studied the frequency of CFAI and CFAII (21) as well as the possibility of finding unknown CFs.

In the present work, surprisingly only one (273-2, Sta^+) out of 123 human *E. coli* strains has shown haemagglutination and serological characteristics of the CFAI standard strain. On the other hand, another ETEC strain (228-6, LT/STa^+) also was serologically agglutinated by the specific antiserum but haemagglutinated only human and bovine RBCs, but not that of chicken.

The low frequency of known CFs in our strains could be explained by the natural conditions of the communities studied. Both Paulínia and Ouro Preto are cities with populations of less than 100.000 inhabitants. In larger cities in Brazil, the possibility of finding known CFs could be different. On the other hand, as shown in Table II, there are several other *E. coli* strains with different patterns of haemagglutination. However, none of them showed serological positive reactions for the adhesins studied. In addition, the strains 275-1 and 244-1, both LT^+, were able to clump, in the presence of D-mannose, human, bovine, chicken as well as guinea-pig, sheep and horse RBCs (data not shown). It was also observed that this haemagglutination occurred with bacterial suspensions grown at 37°C, but not at 16°C, a behaviour characteristic of fimbrial CF (1). Therefore, the low frequency of known CFs among the 87 ETEC strains studied (2,3% positive), as well as the several D-mannose haemagglutinating patterns observed, suggest that is likely the possible presence of other adhesins, perhaps new ones not yet described as virulence factors of these strains.

RESUMO

Fatores de colonização entre amostras enterotoxigênicas de *Escherichia coli* (ETEC), isoladas de casos de diarréia em crianças nas cidades de Ouro Preto (Minas Gerais) e Paulínia (São Paulo), Brasil.

Foi pesquisada a presença dos fatores de colonização CFAI, CFAII (CS1-CS3) e CFAIV (CS4-CS6) em 87 amostras de *Escherichia coli* enterotoxigênicas ETEC e 36 não ETEC. Todas as amostras estudadas neste trabalho foram isoladas de casos de diarréia em crianças abaixo de 12 anos, nas regiões de Ouro Preto (MG) e Paulínia (SP). Apenas duas amostras de Ouro Preto (2,3% do total estudado), ambas capazes de produzir STa, apresentaram sorologia positiva para CFAI. Não foram encontradas amostras sorologicamente positivas para CFAII e CFAIV. Verificou-se a presença de várias amostras com características hemaglutinantes D-manose resistente para uma ou mais das seguintes hemácias; humanas, bovinas e de galinha.

Palavras-chave: fatores de colonização, ETEC, diarréia infantil.

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EFFECT OF (2,6-0-DIMETHYL) β-CYCLODEXTRIN (HEPTAKIS) ON CELL MASS, PERTUSSIS TOXIN AND FILAMENTOUS HEMAGGLUTININ PRODUCTION IN THREE *BORDETELLA PERTUSSIS* VACCINAL STRAINS

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ABSTRACT

We have studied the production of pertussis toxin (PT) and filamentous hemagglutinin (FHA) by three *Bordetella pertussis* vaccinal strains in culture supernatants and in supernatants of cells washed with high ionic strenght solutions. The strains were grown in Stainer-Scholte (SS) medium and in a modification of it that we called CL-basal (CL-b) and in these media with heptakis (2,6-0-Dimethyl β-cyclodextrin (MeβCD)). FHA and PT were estimated by total hemagglutination, differential hemagglutination (with cholesterol) and Dot-blot immunoassay. CL-b medium increase the cellular mass production and would also increase the stability and liberation of hemagglutinins. The addition of MeβCD to SS or CL-b medium would stimulate the production, exportation and stability of PT and FHA. These effects are greater for CL-b medium and more important for FHA than for PT. We obtained similar results with the addition of 0,5 or 1 g/l of MeβCD to CL-b. Culture supernatants contained FHA and PT but supernatants of cells washed with high ionic strength solutions contained basically FHA and would be used to improve its yield.

Key words: heptakis, pertussis toxin, filamentous hemagglutinin.

INTRODUCTION

The cellular vaccine against pertussis, composed of killed *Bordetella pertussis* cells, has been associated with many side effects. Some of these are mild, such redness and fever, but others are severe, such neurological damage and death (10). In response to problems of vaccine reactogenicity, there has been a great interest in *B. pertussis*, its compenents, their mechanisms of action, their role in pathogenesis and their immunoprotective effect in order to develop safer vaccines. Strains of *B. pertussis* undergo a form of variation, a change of phase, and only virulent or phase I bacteria synthesize toxins and other factors associated with pathogenicity. These factors include pertussis toxin (PT) also called leukocytosis promoting factor (LPF), filamentous hemagglutinin (FHA), adenylate cyclase, hemolysin, dermonecrotic toxin, pili, cytochrome d-629 and several outer membrane proteins such a 69 KDa protein. The expression of these virulent factors is coordinately regulated and

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requires the products of the *bvg* locus (*Bordetella* virulence gene). Alternative virulence states occur by two mechanisms, phase variation and phenotypic modulation. Phase variation is due to a low-frequency, very rarely reversible, alteration in the *bvg* locus (a frame shift mutation for Tohama III strain). Phenotypic modulation is a reversible response to environmental conditions. Growth at low temperature (28°C instead of 35°C) or in the presence of high concentrations of $MgSO_4$ or nicotinic acid results in an avirulent phenotype (2).

Many antigens of *B. pertussis* have been purified and tested in animal models, and only detoxified pertussis toxin (PT) gives a protection similar to that of the cellular vaccine. Other antigens that individually are not able to induce full protection in animal models, increase the efficacy of PT, i.e., FHA, an outer membrane protein (69 KDa) and the pili or fimbriae. A number of acellular vaccines have been proposed where detoxified PT has been used in animal models and in clinical trials alone or associated with FHA, with FHA plus a 69 KDa protein or with these antigens plus agglutinogen-2 (fimbriae) (9). Different acellular vaccines are made by laboratories of Canada, France, United States, Britain, Germany, Italy and Japan (5, 9). They are in different steps of development and only Japan adopted and licenced such products for both primary and booster doses. The japanese vaccine (14) contains basically PT and FHA, which are purified from culture supernatants of phase I *B. pertussis* cultivated in Stainer-Scholte medium (15). However, the production of PT in this medium is rather difficult, especially in shake cultures. One reason could be the susceptibility of *B. pertussis* to inhibitors present or produced in liquid medium, including sulfur, fatty acids and peroxides (16). To overcome growth inhibitors Imaizumi et al. have developed a medium with heptakis ((2,6-0-dimethyl) β-cyclodextrin(MeßCD)). This medium is a modification of SS medium and they designated it as cyclodextrin liquid (CL) medium (7). The CL medium stimulated cell growth and the production of PT and FHA in shake cultures of *B. pertussis* Tohama phase I (7, 8).

Phase I strains of *B. pertussis* produce two distinct hemagglutinins: PT and FHA, which can agglutinate chicken, goose and horse red blood cells (1, 13). The purified FHA has a specific hemagglutinating activity greater than ten times of PT (4). Only the hemagglutination of FHA is inhibited by micromolecular concentrations of cholesterol (13). This fact enable us to determine the relative contribution of each hemagglutinin to the total hemagglutinating activity.

This communication describes the effect of the addition of different concentrations of MeβCD to SS and CL-basal (CL medium without MeβCD) media, inoculated with three vaccinal strains of *B. pertussis*. We have evaluated stimulation of cell growth and enhancement of PT and FHA production. Since these hemagglutinins can be found in supernatant liquid cultures and can be extracted easily from young agar-grown cells with 2M sodium chloride (11) we have measured the production of these exportable antigens not only in culture supernatants but also in supernatants of cells washed with high ionic strength solutions in order to determine the residual activity associated with the cellular surface.

MATERIALS AND METHODS

Bacterial strains: *B. pertussis* Tohama phase I, was obtained from Yuji Sato (National Institute of Health, Tokio, Japan). Two other vaccinal strains, *B. pertussis* 10536 Kendrick Eldering (ATCC 10380) and 40103, were obtained from Laboratorio Central de Salud Pública de la Provincia de Buenos Aires, La Plata, Argentina.

Media: Solid medium: Bordet-Gengou agar (BGA), contained BGA base (Difco); glycerol: 1% (v/v); Proteose-peptone (Difco): 1% (w/v) and freshly citrated horse blood: 13% (v/v).

Liquid media: Stainer-Scholte (SS) and CL-basal (CL medium without MeβCD) and these media with 0.5 or 1 g/l of MeβCD. The compositions of the media are listed in Table 1.

TABLE 1 - Composition of SS and CL-basal media. The remarked figures indicate the differences between these media

Component	SS (g/l)	CL-basal (g/l)
Sodium L-glutamate	10,7	10,7
L-proline	0,24	0,24
NaCl	2,5	2,5
KCl	0,2	0,2
KPO_4H_2	0,5	0,5
$MgCl_2$. 6 H_2O	0,1	0,1
$CaCl_2$	0,02	0,02
Tris	6,1	6,1
L-cysteine(*)	0,04	0,04
$FeSO_4$. 7 H_2O (*)	0,01	0,01
Niacin (*)	0,004	0,004
Glutathione (reduced) (*)	0,10	0,15
Ascorbic acid (*)	0,02	0,40
Casamino Acids	-	10,00

Culture: strains stored frozen at -20°C with glycerol -15% (v/v) were grown up on plates of BGA for 72 h at 35°C. Cells were harvested into SS medium without supplements and used as seed suspension to inoculate the culture medium indicated in each experience (30 ml of medium in a 250 ml Erlenmeyer flask). The optical density (O.D.) was adjusted to about 0.250 at 530 nm (Metrolab 1700 spectrophotometer). Incubation was carried out in a shaker (Gyratory Bath Shaker G76, New Brunswick Scientific Co. Inc., Edison, N. J., USA) at 150 rpm, at 35°C and during the time indicated in each experience.

Culture supernatants (CS) and supernatants of cells washed with high ionic strength solutions (SCW): 1 ml sample of the corresponding medium was taken at different times of incubation and centrifuged at 13,500 g for 10 min to obtain the CS. The pellet was resuspended in 1 ml of 1 M sodium chloride 0.1 M phosphate buffer (pH 7.0), incubated 24 h at 4°C and centrifuged in the same condition to obtain the SCW. The samples were stored frozen at -20°C performing the different assays.

Hemagglutination assay: it was assessed in round-bottomed microtiter plates. Fifty microliters of the sample was serially twofold diluted in the microtiter plate in 50 nM mono- and disodium phosphate-buffered 0.85% sodium chloride, pH 7.5 (PBS). Then, an equal volume of a 0.7% (v/v) suspension of washed horse erythrocytes was added to each well. The suspensions were mixed thoroughly by tapping the plate and then allowed to stand for 1 to 2 h before reading. Endpoints were recorded as the last dilution to show disruption of erythrocytes settling.

Inhibition of hemagglutination due to FHA with cholesterol 125 µM: the differential assay was done as describe above, except that after the dilution of the sample with PBS, an equal volume of 250 µM cholesterol was added (a uniform suspension of cholesterol was made by dissolving it in 99% ethanol and then diluting the suspension to 250 µM in PBS at 50°C while vortexing). After 15 min at 23°C, 50 µl of erythrocytes was added. Endpoints were recorded as the last dilution to show disruption of erythrocytes settling.

Preparation of antibodies to FHA and PT: white rabbits weighing about 3 kg were immunized with FHA or PT (Sigma) detoxified by the method described by Munoz (12). Previously, the rabbits were bled to determine absence of total antibodies against *B. pertussis*. Immunization was performed with PT (10 µg/0.5 ml of a solution of 20 mM sodium phosphate, 0.5 M sodium chloride, 0.2M L-lysine, pH 7.6) or FHA (25 µg/0.5 ml of a solution of 20 mM sodium phosphate, 0.5 M sodium chloride, pH 7.6) with 0.5 ml of complete Freund adjuvant. The animals were injected subcutaneously. After 14 and 28 days, boosters of the antigens were injected and 20 days later the rabbits were bled twice a week during 3 months. The sera were titrated by ELISA. For Dot-blot assays we used the anti-FHA serum (17,857 UE/ml) obtained 62 days post immunization and diluted 800-fold in TS-BSA; and the anti-PT serum (32,780 UE/ml) obtained 21 days post immunization and diluted 2,000-fold in TS-BSA.

Dot-blot immunoassay: the nitrocellulose filters were placed in a solution of 50 mM Tris hydrochloride- 150 mM NaCl (pH 7.4) (TS) 30 min before beginning immunodetection procedures. A 96-well Filtration Manifold was assembled with the nitrocellulose filter and 100 µl of the sample or dilutions of it in TS were applied by vacuum, including 100 µl of TS as a control. The samples were diluted 1:50, 1:250, 1:1250, 1:6,250 and 1:31,250 to detect FHA; and 1:5, 1:10, 1:50 and 1:100 to detect PT. The wells were washed with 200 µl of TS and then blocked with 60 µl of TS with 3% (w/v) bovine serum albumin (TS-BSA) for 60 min. The wells were washed twice with 200 µl of TS and the filter was removed from the manifold. The filter was incubated with the primary antibody, anti-FHA or anti-PT, for 60 min, followed by two 30-min washes with TS. Then, the filter was incubated 60 min with biotinylated goat anti-rabbit Ig-G (BRL) diluted 1:1,000 in TS. After 3 washes with TS, the filter was incubated 60 min with streptavidin-horseradish peroxidase (BRL) diluted 1:1,000 in TS. Five 15-min washes with TS followed the incubation with the conjugate. The enzime reaction was started by the addition of TS containing 4-chloro-1-naphthol and H_2O_2. Positive reaction appeared as blue dots on the filter. When the reaction was complete, the filter was washed with distilled water (6).

RESULT AND DISCUSSION

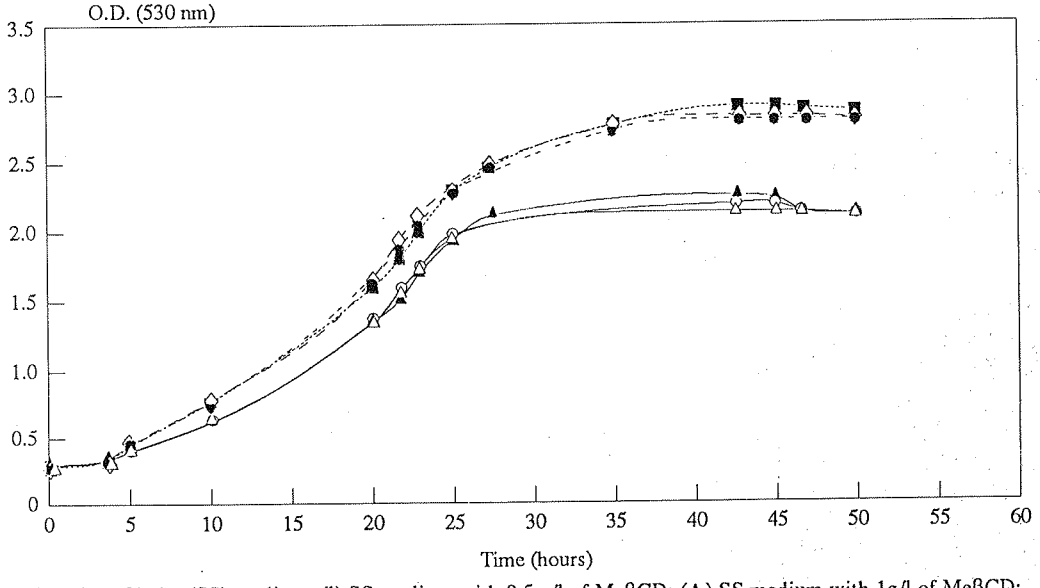
1) *B. pertussis* cultures in SS and CL-b media. Effect of the addition of MeβCD.

B. pertussis 10536 was grown in SS and CL-b media in these media with the addition of two concentrations of MeβCD: 1 g/l, which was recommended by Imaizumi (7-8), and 0.5 g/l. The bacte-

rial cell mass production was estimated by the O.D. of the cultures at 530 nm. The production of PT and FHA was estimated by hemagglutination, inhibition of hemagglutination due to FHA with cholesterol and Dot-blot immunoassay, in culture supernatants (CS) and in supernatants of cells washed with high ionic strength solutions (SCW). These parameters were evaluated at different times of incubation.

A) Bacterial cell mass: the growth rate and the baterial cell mass obtained in the stationary phase in CL-b medium were greater than that obtained in SS medium. The addition of 0.5 or 1 g/l of MeβCD to these media did not significatively modify the O.D. values obtained without this compound. (Fig. 1). CL medium, a modification of the synthetic medium SS, contains a higher concentration of ascorbic acid and glutathione and incorporates casamino acids and MeβCD. Imaizumi et al. (7) suggest that casamino acids and MeβCD improve cell growth. However, our results suggest that MeβCD would not be responsible of these improvement.

crease after the early stationary phase. In this medium the HA activity was greater in SCW than in CS (Table 2). These results are not due to the higher ionic strength of SCW since the presence of NaCl 1M did not modify the HA titers of CS. The ratio of specific HA activity of SCW to CS was even greater because the protein concentration was much lower in SCW than in CS (data not shown). The HA activity of SCW corresponds to the hemagglutinins associated to the cell surface and liberated by the high ionic strength solutions. The addition of MeβCD to SS medium enhanced the HA titers and the effect was greater with 1 g/l of MeβCD. This effect could be due to an improvement in hemagglutinins production and or exportation. The MeβCD also prevented the decrease in the HA titers the early stationary phase, Imaizumi et al. (7) suggest that MeβCD protects PT against degradation even after 72 h of incubation when partial cell lysis was observed. Another effect of MeβCD was a different response of cells washed with high strength ionic solutions, producing HA titers



(○) Stainer-Sholte (SS) medium; (l) SS medium with 0,5 g/l of MeβCD; (▲) SS medium with 1g/l of MeβCD; (■) CL-basal (CL-b) medium; (●) CL-b medium with 0,5 g/l of MeβCD; (◇) CL-medium with 1 g/l of MeβCD.

FIGURE 1 - Growing of *B. perfussis* 10536 in different media.

B) Total Hemagglutinating (HA) activity: The HA activity in the SS medium was found to increase throughout the logarithmic phase of growth, reach a maximum and then rapidly de-

of SCW rarely higher than those of the corresponding CS (Table 2). This result suggest that MeβCD would improve the liberation of hemagglutinins to the culture medium.

TABLE 2 - Hemagglutinating activity, with and without cholesterol 125 μM, of culture supernatants (CS) and supernatants of cells washed with high ionic strength solutions (SCW) of *Bordetella pertussis* 10536 grown in SS with MeßCD.

Incubation (hours)	CS						SCW					
	SS		SS+MeßCD (0,5 g/l)		SS+MeßCD (1,0 g/l)		SS		SS+MeßCD (0,5 g/l)		SS+MeßCD (1,0 g/l)	
	HA titer[a]						HA titer					
Chol.[b]	-	+	-	+	-	+	-	+	-	+	-	+
5	0	0	0	0	0	0	0	0	16	0	0	0
19	8	4	8	4	32	8	16	1	64	4	16	1
26	16	8	32	16	128	16	64	1	64	4	64	4
43	8	4	128	32	256	32	4	0	128	8	64	0
51	4	1	128	32	512	32	1	0	128	1	64	0

a= the results are expressed in hemagglutination units per 50 μl of sample.
b= samples treated without (-) or with (+) cholesterol.

TABLE 3 - Hemagglutinating activity, with and without cholesterol 125 μM, of culture supernatants (CS) and supernatants of cells washed with high ionic strength solutions (SCW) of *Bordetella pertussis* 10536 grown in CL-b medium and in CL-b with MeßCD.

Incubation (hours)	CS						SCW					
	CL-b		CL-b+MeßCD (0,5 g/l)		CL-b+MeßCD (1,0 g/l)		CL-b		CL-b+MeßCD (0,5 g/l)		CL-b+MeßCD (1,0 g/l)	
	HA titer[a]						HA titer					
Chol.[b]	-	+	-	+	-	+	-	+	-	+	-	+
5	0	0	2	0	0	0	0	0	0	0	1	0
19	8	1	128	16	64	16	8	1	256	2	64	1
26	16	8	256	64	512	32	16	1	256	4	256	4
43	32	8	512	64	512	64	16	1	512	4	128	2
51	32	8	1024	64	1024	64	8	0	256	2	128	2

a= the results are expressed in hemagglutination units per 50 μl of sample.
b= samples treated without (-) or with (+) cholesterol.

In contrast, the HA activity in CL-b medium was found to increase with the incubation time and to be highest in CS of stationary phase. This fact suggest that not only MeβCCD but also other components of CL medium contribute to the hemagglutinins stability and liberation. The addition of MeβCD to CL-b medium enhanced the HA titers and the effect was greater than that observed in SS medium (Tables 2 and 3). Imaizumi et al. (7) propose that MeβCD enhances the production of PT by absorbing some growth inhibitors and acting as a stabilizer to assist glutathione, an essential component for PT production. This second reason could explain the better effect of adding MeβCD to CL-b medium which contains 1.5 times the amount of glutathione contained in SS medium. The addition of 0.5 or 1 g/l of MeβCD to CL-b medium resulted in a similar effect (Table 3). The concentration of MeβCD in CL medium developed by Imaizumi et al. (7-8) is 1 g/l, the reduction of this component to 0.5 g/l could be important in the acellular vaccines production because of its high cost.

C) Inhibition of hemagglutination due to FHA with cholesterol: this technique was used to asses the contribution of FHA and PT to the total HA activity. The enhancement in the HA titers of CS observed when MeβCD was added to SS or CL-b medium was principally due to an increment in the FHA production and or exportation, as it will be noted comparing the HA titers with and without cholesterol (Tables 2 and 3). The diminution of the HA titers in presence of cholesterol was greater for SCW than for CS; indeed, the HA activity of SCW was basically due to FHA (Tables 2 and 3). FHA is a bacterial surface adhesine, that mediates binding of the bacteria to erythrocytes and other eucaryotic cells, and it is also an exportable protein. In CL-b medium and in media with MeβCD the HA titers of SCW were rarely higher than those of the corresponding CS. So that, the effect of liberation of hemag-

glutinins associated to the cell surface is mainly on FHA. However, the HA titers of SCW were high enough to propose the use of CS plus SCW to improve the FHA yield.

D) **Dot-blot immunoassay:** we obtained the same level of PT in samples of cultures incubated 43 hs in CL-b with 0.5 (CL-0.5) and 1 g/l of MeβCD (CL-1) and in SS with 0.5 g/l of MeβCD (SS-0.5) (Table 4). The level of PT in CS was greater than in SCW, instead the level of PT in SCW was very low. This is according to the fact that HA activity of SCW was basically due to FHA as shown by HA with cholesterol. On the other hand, we obtained the same level of FHA in CS and SCW of CL-0.5 and CL-1. Hence, SCW constitute an important source of FHA. The value of FHA was lower in SCW of SS-0.5 than in the corresponding CS but it was still important (Table 4).

TABLE 4 - Production of FHA and PT, evaluated by total and diferential hemagglutination and Dot-blot immunoassay, in culture supernatants (CS) and supernatants of cells washed with high ionic strength solutions (SCW) of *Bordetella pertussis* 10536 grown in different liquid media (43 hs of incubation).

Culture Medium	HA titer[a]				Dot-blot (PT)		Dot-blot (FHA)	
	CS		SCW		CS	SCW	CS	SCW
Chol.[b]	-	+	-	+	Numbers indicate the last dilution that shows positive reaction			
SS+0,5 g/l MeßCD	128	32	128	8	1/50	1	1/1.250	1/250
CL-b+0,5 g/l MeßCD	512	64	512	4	1/50	1	1/1.250	1/1.250
CL-b+1.0 g/l MeßCD	512	64	128	2	1/50	1	1/1.250	1/1.250

a = the results are expressed in hemagglutination units per 50 µl of sample.
b = samples treated without(-) or with (+) cholesterol 125µM.

2) Production of bacterial cell mass, PT and FHA by three vaccinal strains of *B. pertussis* in CL-0.5 and SS media.

The data shown in Fig. 1 and Tables 2, 3 and 4 led us to chose the CL-0.5 medium to increase the cell mass, PT and FHA production. We used SS medium as a reference. Cultures of *B. pertussis* Tohama phase I, 10536 and 40103 in these media were evaluated, at different times of incubation, for bacterial mass production (by the O.D. of the cultures at 530 nm) and for the production of PT and FHA (by total and differential hemagglutination, and Dot-blot immunoassay). These parameters were evaluated in CS and in SCW.

A) **Bacterial cell mass:** as we have shown with *B. pertussis* 10536 (Fig. 1), CL-0.5 medium increased the cell mass production for the three strains respect to SS medium (Table 5).

B) **Total HA activity:** accordingly with the data obtained with *B. pertussis* 10536 (Tables 2 and 3), the HA activity of the three *B. pertussis* vaccinal strains in SS medium was found to increase throughout the logarithmic phase and decrease after the early stationary phase of growth till be undetectable within 96 h of incubation (table 5). The HA activity in CL-0.5 medium increased throughout this time of incubation showing an important effect over the hemagglutinins stability.

TABLE 5 - Total and differential hemagglutinating activity of culture supernatants (CS) and supernatants of cells washed with high ionic strength solutions (SCW) of three *Bordetella pertussis* strains grown in SS and CL-0,5 media.

Strain	Incubation (hours)	O. D. (530 mm)		CS HA titer[a]				SCW HA titer			
		Chol.[b](-)		(-)		(+)		(-)		(+)	
		SS	CL-0,5	SS	CL-0,5	SS	CL-0,5	SS	CL-0,5	SS	CL-0,5
Tohama phase I	24	1.455	1.606	4	64	2	16	32	128	2	2
	48	1.843	2.363	1	1024	0	256	16	128	2	2
	96	1.890	2.552	0	2048	0	256	0	64	0	1
10536	24	1.323	1.436	16	128	8	16	64	128	2	2
	48	1.985	2.457	16	512	8	128	16	128	1	2
	96	2.079	2.646	0	1024	0	128	0	16	0	0
40103	24	1.606	1.985	32	64	16	16	4	64	1	1
	48	1.985	2.457	32	128	8	32	2	128	0	2
	96	1,985	2.457	0	128	0	32	0	0	0	0

a= the results are expressed in hemagglutination units per 50 µl of sample.
b= samples treated without (-) or with (+) cholesterol 125µl.

The CL-0.5 medium strongly enhanced the production and or exportation of hemagglutinins respect to SS medium. This effect was greater for *B. pertussis* Tohama phase I and 10536 than for *B. pertussis* 40103 (Table 5). The HA activity in SS medium was greater in SCW than in CS except for *B. pertussis* 40103. In contrast, in CL-0.5 medium the HA titers of SCW were rarely higher than those the corresponding CS. This fact confirms the effect of CL medium, specially MeβCD, in the liberation of antigens associated to the cell surface.

C) Inhibition of hemagglutination due to FHA with cholesterol: again, as we have shown in Table 3, the great increment in HA titers of CS in CL-0.5 was due principally to an increment in FHA production and or exportation; only 12.5 to 25% of the total HA activity was due to PT (Table 5). The proportion of FHA to PT in SCW was higher than in CS; for CL-0.5 medium the contribution of PT to the total HA activity in SCW was only 1.56% (Table 5). This was true for the three vaccinals strains of *B. pertussis* and confirms that the HA activity of SCW is basically due to FHA.

and the two procedures are not necessarily equivalent as was confirmed by Brown and Parker (3). This fact could explain some discordances as those observed in Tables 5 and 6 where at 96 h of incubation in SS medium no HA activity was detected whereas a FHA level was detected by immonoblot. The prolonged incubation could affect the HA activity but not the epitopes recognized by the antiserum.

The data shown in this study suggest that the improvement in cell mass observed in CL medium would be due to some modifications respect to SS medium, specially the addition of casamino acids, but not to the MeβCD. On the other hand, the improvement on PT and FHA production and or exportation would be mainly due to MeβCD. The enhancement in stability and liberation of PT and FHA, specially FHA liberation from the cell surface, would be due to modifications in CL medium including the addition of MeβCD.

CL medium improves the liberation of FHA from the cell surface but the use of high ionic strength solutions is still necessary to liberate

TABLE 6 - Production of FHA and PT, evaluated by Dot-blot immunoassy, in culture supernatants (CS) and supernatants of cells washed with high ionic strenght solution (SCW) of three *Bordetella pertussis* strains grown in SS and in CL-0,5 media.

Strain	Incubation (hours)	CS Dot-blot FHA[a]		CS Dot-blot PT[b]		SCW Dot-blot FHA		SCW Dot-blot PT	
		SS	CL-0,5	SS	CL-0,5	SS	CL-0,5	SS	CL-0,5
Tohama phase 1	24	1/5	1/1250	1/10	1/100	1/250	1/1250	1	1/5
	48	1/5	1/6250	1/10	1/100	1/250	1/6250	1	1
	96	1/250	1/6250	1/10	1/100	1/50	1/1250	1	1
10536	24	1/5	1/1250	1/10	1/100	1/50	1/250	1	1/5
	48	1/250	1/6250	1/10	1/100	1/50	1/1250	1	1
	96	1/250	1/6250	1/10	1/100	1/5	1/250	1	1
40103	24	1	1/1250	1/10	1/50	1	1/1250	1	1/5
	48	1/50	1/6250	1/10	1/50	1/5	1/1250	1	1/5
	96	1/250	1/6250	1/10	1/50	1/5	1/50	0	1

[a] and [b] = the Dot titers correspond to the last dilution of the sample that shows a positive reaction.

D) Dot-blot immunoassay: CL-0.5 medium enhanced the production and or exportation of FHA and PT respect to SS medium (Table 6). This effect was greater for FHA than for PT. The PT level was very low in SCW confirming that FHA is their principal component as it was shown by differential HA.

The HA assay measured activity, whereas immunoblots provided estimates of antigen levels, important amounts of this protein. Hence, we propose the use of CS plus SCW to increase the FHA yield.

Since we have observed similar results by adding 0.5 or 1 g/l of MeβCD to CL-b medium and because of the high cost of this cyclodextrin, we propose the use of CL-0.5 medium to improve cell mass, PT and FHA production in *B. pertussis*.

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MYXO AND PARAMYXOVIRUS SURFACE STRUCTURES: ANTIGENIC AND FUNCTIONAL STUDIES

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SUMMARY

The presence of specific carbohydrates on viral surface structures, developing an important role in their biological processes, was analysed using Newcastle Disease Viruses (SO-93, B_1 and La Sota strains) and Influenza virus (A/Influenza/PR/8/34 (H_1N_1) strain) as models.

Virus samples were concentrated by sedimentation process, purified by tartrate gradient, they were washed through another sedimentation step and evaluated by their protein content, hemagglutination titer and carbohydrate residues. This study developed with lectins and specific sugars could show carbohydrates as essential elements of virus surface structures, which were revealed for inhibition of precipitation lines among concanavalin A and Newcastle Disease Virus specific serum.

Key words: orthomyxovirus, paramyxovirus, influenza Newcastle disease virus.

INTRODUCTION

Envelope structures of RNA viruses as Influenza and Newcastle Disease Viruses (NDV) present glycoproteins that are responsible for biological activities such as those associated with adsorption, fusion and final cleavage process between virus structures and cells. Those activities are developed for HA (Hemagglutinin) and NA (Neuraminidase) structures of Influenzaviruses, responsible by the adsorption/fusion and cleavage (sialidase) functions, respectively. These same activities among Newcastle Disease Viruses are carry out for HN (Hemagglutinin-Neuraminidase) and F (Fusion protein) glycoproteic structures, which are inserted into their lipoproteic envelope (17).

Carbohydrates residues have important function as structural elements of those glycoproteic spikes described above with primordial role for their biological activities and during their stability and process of maturation. Direct correlation among extension of glycosilation process and biological activities of HA and NA spikes has already been reveled (12, 17, 19, 20). The sequences of aminoacids of those surface glycoproteic spikes determinate the specificity and localization of carbohydrate residues on their binding sites (5, 9, 26).

In the study on the character of those carbohydrates, many methodologies have been used. Lectin methodologies have already utilized as important tools for identification processes of carbohydrate residues (5, 6, 9, 13, 14, 16, 24, 27), including those on enveloped virus surfaces (5, 7,

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8, 22). Concanavalin A, a lectin that has specific affinity to manopyranoside, glycopyranoside or fructopyranoside residues have shown interaction with membranes of NDV infected cells (23).

HN and F Newcastle Disease Virus structures have been analysed by glycosilase activity and affinity chromatography technique showing presence of D-mannose, D-galactose, L-fucose and N-acetyl D-glycosamine residues in different percentages (9). Those carbohydrate residues on viral functional structures have been studied for their antigenic character, showing the carbohydrate residues as responsible for host system-Influenza virus cross antigenicity (28).

In this study, a more extensive analysis was projected to be developed about the character of carbohydrates present on surface structures of selected virus samples and its influence on the functional and antigenic activities of those virus. Methods for analysis of the interaction processes among viruses, cell receptors and antibodies using simple techniques and lectins were used as tools.

MATERIALS AND METHODS

1. Materials:

1.1 Virus samples:

1.1.1 Newcastle Disease Virus (NDV) - The following samples were studied:

- SO-93 velogenic sample that was provided by Dr. Ary Moreira de Souza, Dept. Virologia, Embrapa, RJ, Brazil;
- B_1 and La Sota (LS) lentogenic (vaccinal) samples.

1.1.2 Influenza A virus - Influenza A/PR/8/34 (H_1N_1) sample was studied.

1.2 Erythrocytes: goose erythrocytes were used after collection in Alsever solution, three washing steps (8) and adjusting to 0.5% concentration (PBS-pH 7,0).

1.3 Detergent (1): 0.2% Nonidet P-40 was used as detergent solution.

1.4 Lectins: different concentrations of Sigma's concanavalin/Con A (400, 200, 100 and 50 µg/ml in PBS-pH 7.0) and PNA (4 µg/ml in PBS-pH 7.0) were utilized. Arachis Hypogaea (peanuts lectin/PNA) was prepared as indicated for Lothan et al. (15).

1.5 Inhibitor Carbohydrate: 1.5 M alpha-methyl mannoside (Sigma) was used at 0.3 M final concentration as specific inhibitor to concanavalin A.

1.6 Serum: NDV immune serum against B1 sample was obtained from chicken, treated and titered by hemagglutination inhibition test (8).

2. General Methods:

2.1 Concentration and Purification Processes: virus samples were inoculated into allantoic cavity of 10-day-old embryonated chicken eggs and incubated for 48 hours at 37°C (8). Then, allantoic fluids were harvested and clarified by centrifugation at 7,500 x g for 30 minutes at 4°C. These clarified samples were 50 x concentrated by ultracentrifugation at 50,000 x g for 60 minutes (4°C), with final dilution of the pellet in Phosphate Buffer Solution (PBS) pH 7.0. The purification process was developed in potassium tartrate-glycerol (40% sucrose in 5% glycerol) density gradient by ultracentrifugation at 180,000 x g for 120 minutes at 4°C.

Virus bands were collected and after dilution (1:10 in 2 mM EDTA, PBS pH 7.0) they were spinned again at 50,000 x g for 60 minutes. The pellets were rediluted with PBS-EDTA and kept at 20°C until using (18).

2.2 Protein Dosage (3): Bradford method, using 0.1 ml of virus sample and 1.0 ml Coomassie Brilliant Blue was used.

2.3 Electron Microscopy: negative staining technique (10) and Phillips 301 Electron Microscope were used.

2.4 Hemagglutination Test: test samples (25 µl) were diluted serially in PBS 25 µl volumes and to each virus dilution was added of 25 µl volumes of 0.5% goose erythrocytes with 0.2% bovine albumine. The reactions were read after 2 hour incubations at 4°C, titers were considered as the reciprocal of the highest virus dilution responsible by total hemagglutination (8).

2.5 Preparation of slides for Double Immunodiffusion Test (4): slides were prepared using 1.2 ml volumes of melted 2.5% Noble Agar (Difco) at

42°C mixed with 1.8 ml volumes of heated 10 x PBS. This mixture was used to prepare the agar slides, which were cut with a 4 mm diameter model with 7 cavities, one of them in central position. The agar was aspirated from the cavities and the slides kept in humid chamber till using.

3. Specific Methods for Analysis:

3.1 By Hemagglutination Test (HA):

Quantitative Evaluation (21) :the best concentration concanavalin A for each virus preparation was evaluated - 0.2 ml of different lectin concentrations were mixed with 0.2 ml of each viral purified preparation and incubated for 1 hour at room temperature (25°C).

After incubation, those materials were centrifuged at 2,500 x g at 4°C for 20 minutes. After these procedures, 0.1 ml volumes of alpha-methyl mannoside were added at the supernatants and the pellets were eluted to original volumes with the same carbohydrate. The reaction presence was evaluated by hemagglutination test simultaneously with 3 series of controls of reaction:

1st. virus preparation + alpha-methyl mannoside;

2nd. virus preparation + Con-A + alpha-methyl mannoside;

3rd Con-A + alpha-methyl mannoside.

kinetics Evaluation: kinetics of that reaction was evaluated as above. Samples were collected after 5, 15, 30 and 60 minutes.

The test was developed as above, with centrifugation at 2,500 x g and addition of alpha-methyl mannoside at the supernatant and pellet to stop the reaction. This kinetics evaluation was developed with the A/PR/8/34 sample of Influenzavirus.

3.2 By observation of Agglutination in tubes: different viral dillutions (neat, 1:2, 1:4 and 1:8) in PBS-pH 7.0 were incubated with equal volumes (0.2 ml) of concanavalin A (100 µg/ml). The reactions were observed after 1 hour incubation at room temperature. A comparative study was developed with LS sample of NDV, using concanavalin A and PNA.

3.3 By Electron Microscopy: 1:2 dilution of La Sota sample was incubated with 100 µg/ml concanavalin A as above, for 1 hour at room temperature and the reaction was observed by electron microscopy.

3.4 By Immunodiffusion Test (25): in a first step, viral samples were treated with equal volumes of 3 types of detergent solutions already presented, 0.2% Nonidet P-40 was chosen as the best detergent solution to disrupt the virus structure (results not shown).

Concanavalin A at 400 µg/ml in PBS-pH 7.0 and NDV-serum with titer of 160 by Hemagglutination Inhibition Test were utilized in this Double Immunodiffusion Test. NDV serum and NDV virus antigen samples (15 µl) were distributed in the slides, as can be observed in the Figures 1 and 2 (Results), finally they were incubated for 24 hours at 4°C in humid chamber.

Then, the slides were washed, by overnight immersion (0.85% NaCl with 0.01% sodium azide) and the cavities were filled with 2.5% agar. They were covered with filter paper and dryed at 37°C. The dryed slides were stained with 0.1% Ponceau Solution for 30 minutes, destained with 0.2% acetic acid and observed (11).

RESULTS

1. Study of Virus-Lectin Interactions by Hemagglutination Test: the results of quantitative studies

TABLE 1 - Quantitative evaluation of Con. A-N and Con. A-Influenzavirus interactions by hemagglutination test.

Virus Samples	Virus Controls (HA titer/25 µl)	HA Titer/25 µl with different Con. A Concentrations (µg/ml) 200	100	50
A. NDV				
1. La Sota				
supernatant	512	8	64	128
pellet	ND	64	8	4
2. SO-93				
supernatant	256	32	64	128
pellet	ND	8	4	4
3. B_1				
supernatant	128	8	32	ND
pellet	ND	16	4	4
B. Influenza A/PR/8/34				
supernatant	1024	2	8	8
pellet	ND	256	256	ND
C. Negative Control Con. A+Methyl mannoside	-	<2	<2	<2

ND - not done

TABLE 2 - Kinetics evalutation of Con. A-Influenzavirus interactions by hemagglutination test.

Samples Tested	HA Titer/25 µl Time of reaction (minutes) 0	5	15	30	60
A/PR/8/34 + PBS	512	-	-	-	-
A/PR/8/34 + methyl mannoside	256	-	-	-	-
A/PR/8/34 + Cont. A					
supernatant	-	128	32	16	16
pellet	-	128	256	256	512

virus sample - 70 µg protein/ml
Con. A - 100 µg protein/ml
(-) negative

on the interaction process between concanavalin A, Influenza and NDV samples are showed in the Table 1. They are expressed by hemagglutination titer per 25 µl. Table 2 exhibits results from kinetics studies on the concanavalin A- virus particle interaction, after 5, 15, 30 and 60 minutes of incubation, using an influenzavirus sample (A/PR/8/34) as model.

2. Study of Virus-Lectin Interactions by Observations of Agglutination in Tubes - results of agglutination between lectin, Influenza A and NDV samples are showed in the Table 3. They are expressed as positive or negative reactions of hemagglutination, revealing presence or ausence of interaction among virus particles and lectins (concanavalin A and PNA).

3. Study of Virus Particle-Lectin Interactions by Electron Microscopy: the Figure 1 shows interaction between NDV (La Sota sample) particles and concanavalin A by electron microscopy technique.

TABLE 3 - Analysis on Con. A-Virus and PNA-Virus interactions by observation of agglutination in tubes.

Virus Samples	Lectin	Agglutination results Virus Dilutions Neat	1:2	1:4	1:8
A/PR/8/34	Con. A	+	+	+	ND
B_1 NDV	Con. A	+	+	-	ND
LS NDV	Con. A	+	+	+	ND
	PNA	+	+	+	ND
SO-93 NDV	Con. A	+	+	ND	-

(+) positive
(-) negative
ND - not done

Protein concentrations: A/PR/8/34 - 50 µg/ml
B_1 NDV - 135 µg/ml
SO-93 NDV - 124 µg/ml
LS NDV - 370 µg/ml

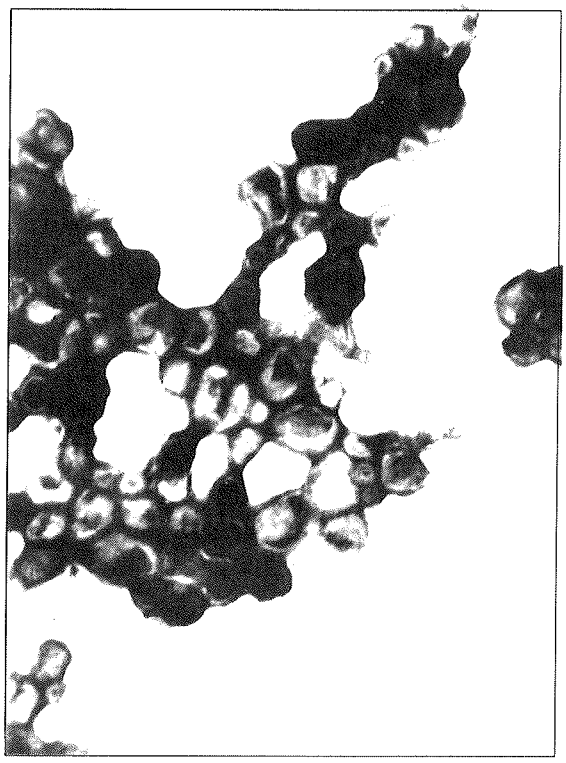
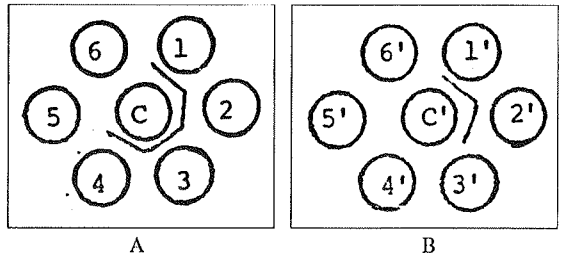


FIGURE 1 - Eletronic micrography showing La Sota sample of NDV and Con. A interactions (34,000 x - 1 cm = 294 mm).



A B

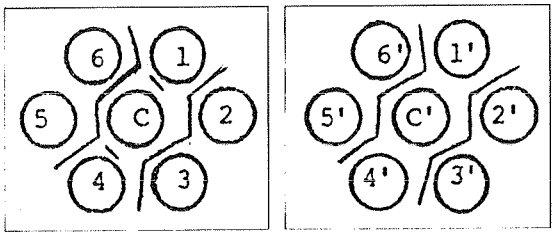
C B_1 sample + 0.1% Nonidet P-40
1 400 µg/ml Con.A
2 200 µg/ml Con.A
3 100 µg/ml Con.A
4 50 µg/ml Con.A
5 25 µg/ml Con.A
6 12.5 µg/ml Con.A

C' B_1 sample + 0.1% Nonidet P-40
1' neat NDV serum
2' 1:2 NDV serum
3' 1:4 NDV serum
4' 1:8 NDV serum
5' 1:16 NDV serum
6' 1:32 NDV serum

FIGURE 2 - Scheme of double immunodiffusium test - identity studies among B_1 sample of NDV and different Con. A and serum dilutions.

4. Study of Virus Particle-Lectin Interactions by Double Immunodiffusion Test with

NDV Policlonal Serum: the Figure 2 shows squeme of precipitation lines that expresses interactions among disrupted NDV virus-concanavalin A and NDV virus-specific antibodies. Those experiments were developed to find the best concanavalin A and serum dilutions to be used for identity studies. In the last step, the Figure 3 exhibits squeme of precipitation lines as results of NDV specific antibodies-lectin interactions, revealing identity.



A B

C NDV serum
1 100 µg/ml Con.A
2 B_1 sample
3 B_1 sample
4 100 µg/ml Con.A
5 B_1 sample
6 B_1 sample

C' NDV serum
1' 100 µg/ml Con.A
2' B_1 sample
3' B_1 sample
4' 100 µg/ml Con.A
5' B_1 sample
6' B_1 sample

A - without addition of alpha-methyl mannoside.
B - with addition of alpha-methyl mannoside.

FIGURE 3 - Scheme of the Double immunodiffusion test - identity studies among NDV serum, Con. A and B_1 sample of NDV, with and without addition of alpha-methyl mannoside.

DISCUSSION

The Table 1 and 2 show results of quantitative and kinetics studies of the lectin-virus interaction, by hemagglutination test. Titers decreased when we used concanavalin A and NDV or Influenzavirus samples, in adequated proportions. Quantitative studies showed that 100µg/ml concentration as the most efficient concanavalin A concentration and kinetics tests revealed 1 hour as the best incubation time as well. In those reactions, alpha-methyl mannoside was used as concanavalin A inhibitor.

Those kinetics and quantitative studies about virus-lectin interactions gave us data to follow the analysis. In fact, visible aggregates were observed at 100 µg/ml concentration of concanavalin A after 1 hour incubation. These aggregates were not developed if alpha-methyl mannoside, which has affinity for receptor sites of concanavalin A, was added.

The reactions revealed by agglutination in tubes were positive (Table 3) when concanavalin A (100 µg/ml) and NDV (B_1 and SO-93)/ Influenza (A/PR/8/34) samples or PNA (4 µg/ml) and La Sota strain of NDV were used. This positivity demonstrated by visible aggregates shows that the viral surface structures contain galactose and alpha-methyl mannoside residues, which are knowed as specific to PNA and Concanavalin A, respectively.

The Figure 1 demonstrates by electron microscopy the existence of receptor sites on virus surface structures with the revelation of virus-lectin complexes or aggregates, using samples obtained as above. The importance of carbohydrates as important elements on virus surface structures has been shown. The glycosilation process on endoplasmatic reticulum and Golgi apparatus has been exhibited as an essential step during the assembly and folding processes of virus surface glycoproteins (26).

Studies using enveloped (Sendai and Herpesvirus samples) and non enveloped (Poliovirus samples) viruses have showed that the treatment with concanavalin A inhibited only the infectivity of enveloped virus. The existence or ausence of carbohydrated structures (glycoproteins or glycolipids) on the virus surface, respectively among enveloped or nonenveloped viruses could explain it. That character was demonstrated by reversion of the inhibition of infectivity after addition of alpha-methyl mannoside (21).

The same results were obtained when were used different purified RNA viruses (Myxovirus, Arbovirus, Rhabdovirus, Poliovirus and SV40) or cells infected by them, aggregates were formed in both of those occasions. The same results were not observed with cells infected by Polioviruses and SV 40 purified particles. Addition of concanavalin A plus alpha-methyl mannoside prevented the developing of aggregates, while the contact of those virus particles with concanavalin A turned drastically lower the virus infectivity (2).

In the last step, analysis to study some test variables as diffusibility, specificity of detergents and the best concentrations of concanavalin A to be used in the immunodiffusion tests were developed. The results showed Nonidet P-40 as the detergent of best diffusibility and specificity, without induction of inespecific reactions with serum (results not shown). They also indicated the 100 µg/ml concentration and 1:2 NDV serum dilution to be used for the best conditions of reaction, as can be observed in Figure 2. The pre-

cipitation lines among virus, NDV serum and Con-A (Figure 3-A) show the existence of identity between lectin receptors and NDV surface antigens as already demonstrated by studies using Semliki viruses (22). The inhibition of precipitation lines by addition of alpha-methyl mannoside conffirms those data showed above, which permit to identify the chemical character of virus surface antigens as peptides exhibiting galactose and alpha-methyl mannoside residues (Figure 3-B).

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RESUMO

Estruturas de superfície de mixo e paramixovirus: estudos antigênicos e funcionais

Amostras de Vírus da Doença de Newcastle (cepas SO-93, B_1 e La Sota) e da Influenza (cepa A/PR/8/34 (H_1N_1) foram analisadas quanto ao caráter de suas estruturas de superfície, mais especificamente quanto à presença de carboidratos específicos, de comprovada importância no processamento das atividades biológicas virais.

As amostras virais foram concentradas por sedimentação, purificadas por gradiente, lavadas por sedimentação, avaliadas quanto ao seu teor protéico e título hemaglutinante e, então, estudadas quanto aos seus resíduos carboidratos.

O estudo realizado com uso de lectinas e açúcar específico (inibidor de Concanavalina A) revelou a importância e especificidade do caráter carboidratado de estruturas peptídicas de superfície viral, o que pode ser observado através da inibição do aparecimento das linhas de precipitação entre Concanavalina A e soro específico para Vírus da Doença de Newcastle.

Palavras chave: ortomixovirus, paramixovirus, influenza, vírus da doença de Newcastle.

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SINGLE CELL PROTEIN QUALITY PRODUCED FROM LIGNOCELLULOSIC MATERIALS BY THE ASCOMYCETE *CHRYSONILIA SITOPHILA* (TFB-27441 STRAIN)

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SUMMARY

Data are presented for amino acid contents of fifteen carbohydrates, lignocellulosic materials, including olive milling waste water. The selected substrate were: glucose, cellobiose, saccharose, microcrystalline cellulose, tannin, phlobaphene, bark, lignocellulose residue, sugar residue from organosolv process, rice hull, irradiated rice hull, orange bagasse, alpechin (5%), alpechin (10%) and irradiated alpechin (10%). Ratios of some amino acids of nutritional significance in conventional protein sources and those induced by different lignocellulosic substrate are presented. Also, a comparison of the ratios of individual amino acids to total essential amino acids (A/E ratio) and total essential amino acids to total amino acids recovered as nitrogen (E/T ratio), together with essential amino acid chemical scores, led us to compare the quality of each carbon source used. These analyses showed that orange bagasse, alpechin (10%) and bark were the most inexpensive materials and produced the highest protein qualities.

Key words: Single cell proteins, ascomycete, *Crysonilia sitophila*, lignocellulosic, bagasse, rice hull, olive milling.

INTRODUCTION

The lignocellulosic waste material accumulation in the world and their potential conversion to special products still remain unresolved (1,6,18,19). In Latin American countries there is still a good chance of efficiently utilizing our natural resources, especially the forest by-products of

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low commercial value obtained from the industrial processing of *Pinus radiata* in Chile (e.g. barks, tannins, phlobaphenes, carbohydrate residues from organosolv pulping), lignocellulosic waste materials in Brazil (rice hulls, orange bagasse) and olive milling wastewater (alpechin) in Spain. Many studies on single-cell protein (SCP) and enzyme production have been conducted, but further research with new lignocellulolytic microorganisms is still necessary (9,10).

The ascomycete *Chrysonilia sitophila* Mont von Arx (TFB-27441 strain) was isolated from a xylophagous insect (7,16). This strain, which exhibits high ligninolytic activity, was efficient in degrading several organic materials (12-14, 21, 22).

Since few lignocellulolytic microorganisms have been studied for direct SCP production from lignocellulosic materials (23), the *C. sitophila* (TFB-27441) stands as a potential microorganism for production of SCP because of its cellulolytic, proteolytic and ligninolytic enzymes using representative carbon source materials from Chile, Brazil and Spain.

MATERIALS AND METHODS

Samples: Samples of bark were taken separately from 30 year old *Pinus radiata* as published (3). Phlobaphene and tannins were extracted as described earlier (4). The insoluble material, after solvent extraction (bark residue), was further extracted with 1% NaOH to remove phenolic acids. Thereby rendering the lignocellulosic residue. Samples of rice hull from Goias State (Brazil) rice plantations were used. Olive milling wastewater, the by-product of olive oil technology, was obtained from Spain (used at 10% as carbon source). Brown, pelleted orange bagasse (São Paulo State), milled to 1 mm particle size was used as the sole carbohydrate source suspended in Czapek solution.

Sugar residues were obtained from organosolv pulping of *Pinus radiata* chips with formic acid treatment, followed by lignin precipitation and exhaustive formic-water solution evaporation (5). All the solid carbon sources were used at 1.5% concentration. The irradiation on rice hull and olive milling wastewater were carried out with a HQL-OSRAM mercury vapor lamp at a flux of 68 W/m^2 at >300 nm (using glass plates as the filter) (11).

Microoganism: *C. sitophila* (TFB-27441 strain) was selected and cultured in a Czapek or Fries medium (12,16). All the experiments were made by this method in triplicate. Amino acids from fungal mycelium were analyzed in either by a Beckman Model 119-CL or an Aminochrom II, Labotron System amino acid analyzer. All reported figures were averages from duplicate hydrolises, reproducible to within 3-5%.

RESULTS AND DISCUSSION

Evaluation of the single cell proteins produced by *C. sitophila* (TFB-27441 strain) with different carbon sources was performed in each cases. TABLE 1 shows the amino acid contents of common carbon sources, such as glucose, cellobiose, saccharose and micro-crystalline cellulose. Most of the amino acids of the total mycelial protein obtained from the substrate in TABLE 1 showed a distribution similar to or higher than those of the FAO standard requirement (TABLE 7), excepting the methionine content using saccharose, cellobiose or cellulose.

TABLE 1 - Amino acid distribution of *C. Sitophila* (TFB - 27441) mycelial protein (g of A. A./100 g of protein) derived from standard carbon sources.

A. A.	Glucose	Cellobiose	Saccharose	Micro-crystalline cellulose
Asp	11.29	12.47	10.60	12.47
Thr	5.01	5.19	5.15	5.19
Ser	5.18	5.60	4.61	5.60
Glu	13.88	10.63	14.18	10.63
Pro	7.76	5.80	7.73	5.80
Gly	6.14	5.23	4.85	5.23
Ala	8.81	7.47	9.03	7.47
Val	8.17	7.99	5.81	7.99
Met	2.12	0.99	1.19	0.99
Ile	3.91	9.40	4.39	9.40
Leu	12.93	9.36	5.80	9.36
Tyr	1.16	3.74	2.98	3.74
Phe	3.32	4.28	3.12	4.28
Lys	6.33	5.65	5.00	5.65
His	2.14	1.53	1.93	1.53
Arg	4.10	4.66	3.53	4.66
Cys	-	-	0.06	-

TABLE 2 shows the results which were obtained using forest products. Again, poor quality in the presence of phlobaphene and lignocellulose from *P. radiata* was obtained. Cystein was deficient protein derives from all the carbon sources used when compared with FAO values.

TABLE 3 shows the result with lignocellulosic materials from different sources and it was observed that methionine was defficient in rice hull,

TABLE 2 - Amino acid distribution of *C. Sitophila* (TFB - 27441) mycelial protein (g of A. A./100 g of protein) derived from forest products of *Pinus Radiata*.

A. A.	Tannin	Phlobaphene	Bark	Lignocellulose	Sugar Residue Organosolv
Asp	11.98	10.70	12.57	10.60	10.92
Thr	5.75	4.41	4.95	4.65	4.62
Ser	5.10	5.65	6.62	4.30	5.46
Glu	13.45	10.61	8.47	15.50	13.45
Pro	8.84	5.99	4.50	3.26	3.78
Gly	7.40	5.00	5.37	4.89	4.62
Ala	9.18	6.94	5.66	7.67	5.04
Val	8.07	6.12	6.58	5.50	5.04
Met	1.91	0.58	2.56	0.60	1.68
Ile	6.81	8.71	13.19	5.10	3.36
Leu	8.44	7.41	8.97	6.90	11.34
Tyr	0.52	2.72	3.16	2.81	3.78
Phe	3.81	3.13	3.04	3.22	4.62
Lys	1.12	4.86	4.69	5.52	5.88
His	2.01	1.81	1.24	2.25	2.52
Arg	4.14	8.05	2.67	5.01	6.72
Cys	0.07	0.42	0.01	0.06	1.68

TABLE 3 - Amino acid distribution of *C. Sitophila* (TFB - 27441) mycelial protein (g of A. A./100 g of protein) derived from lignocellulosic materials.

A. A.	Rice Hull Bagasse	Rice Hull Irradiated	Orange Bagasse
Asp	10.96	13.05	11.66
Thr	19.43	6.83	6.26
Ser	6.67	8.09	5.79
Glu	12.42	15.89	10.63
Pro	8.24	0.01	-
Gly	9.81	15.81	6.65
Ala	12.70	13.94	8.64
Val	0.01	0.01	8.25
Met	0.01	10.43	6.56
Ile	3.98	0.01	4.57
Leu	10.67	10.54	6.70
Tyr	0.01	0.01	0.08
Phe	0.01	0.01	3.67
Lys	3.71	5.48	7.08
His	0.01	0.01	2.63
Arg	2.00	0.01	1.64
Cys	-	-	-

but after irradiation a high content was found (10.43%). Both sources exhibited a tyrosine and phenylalanine defficiency. A significant methionine content was observed when orange bagasse was used (6.70%).

TABLE 4 shows the amino acid content induced by olive milling wastewater (alpechin). No large difference in using a 5% or 10% of the effluent in culture medium was observed. Also no effect after irradiation was noticed.

TABLE 4 - Amino acid distribution of *C. Sitophila* (TFB - 27441) mycelial protein (g of A. A./100 g of protein) derived from olive milling wastewater (Alpechin).

A. A.	Alpechin 5% Czapek	Alpechin (10% Czapek) Nonirradiated	Alpechin (10% Czapek) Irradiated
Asp	10.30	10.70	11.30
Thr	5.20	5.00	5.50
Ser	4.80	4.00	4.90
Glu	12.50	9.70	14.10
Pro	8.90	6.40	10.60
Gly	4.90	4.41	5.40
Ala	6.00	11.30	3.70
Val	6.90	12.40	6.15
Met	1.20	0.90	0.80
Ile	3.60	5.20	4.30
Leu	10.20	8.94	12.20
Tyr	3.60	2.90	2.30
Phe	5.10	4.50	7.80
Lys	9.10	7.00	7.60
His	2.00	2.10	1.60
Arg	5.80	4.70	1.90
Cys	-	0.90	-

In TABLE 5 we have compared the mycelial protein which exhibited the better qualities in relation to the FAO standard calculating the ratios of some essential amino acids of nutritional significance with known proteins.

Biological quality in a protein can be assessed not only by the absolute amount of each essential amino acid but also by their internal balance. Isoleucine, for example, should keep a molar ratio not lower than 1:3 (0.33) to leucine. Threonine is another essential amino acid found in short supply in vegetable proteins and encountered in high quality proteins in almost equimolar ratios with serine. TABLE 5 shows that low quality proteins, usually of vegetable origin, are rich in arginine and histidine, in relation to lysine. These data attest to the improvement of the internal amino acid balance throughout fermentation, with the exception of isoleucine/leucine which, neverless remains at acceptable values. The high lysine/arginine ratio of mycelial protein when orange bagasse was used as the inducer, was probably the result of the unusually low level of arginine found in *C. sitophila*. Likewise, the low content of tyrosine in the fungus was responsible for the extremely low tyrosine/phenylalanine ratios observed in sucrose culture. Low phenylalanine content in novel proteins would be desirable for phenylalanine intorelant individuals.

TABLE 6 shows the nutritionally significant amino acids for two known food proteins; casein and egg proteins. A comparison of the ratios of individual aminoacids to total essential amino acids

TABLE 5 - Ratios of some amino acids of nutritional significance in conventional and non-conventional protein sources (2).

Source	ILE/LEU	TYR/PHE	LYS/HIS	LYS/ ARG	THR/SER
Milk	0.65	1.05	2.95	2.12	0.78
Egg (whole)	0.75	0.74	2.66	2.66	0.59
Meat (bovine)	0.64	0.82	2.51	1.35	1.05
Rice	0.54	0.91	2.34	0.68	0.77
Maize	0.35	1.34	1.39	0.82	0.70
Sorghum	0.34	0.55	1.41	0.72	0.71
Wheat Flour	0.67	0.46	1.69	0.76	-
Peanuts	0.67	0.71	1.45	0.33	0.40
Yeast	0.57	0.95	2.58	1.70	1.04
Orange Waste	0.83	0.49	1.15	0.60	0.94 (a)
FAO	0.88	1.00	-	-	-
Soybean	0.75	0.75	2.34	0.93	0.93
Casein	0.54	1.10	3.95	2.03	0.73
SCP					
Induced by:					
Orange Waste	0.68	0.02	2.69	4.31	1.08
Saccharose	0.76	0.95	2.59	1.42	1.12
SugarResidue	0.30	0.82	2.33	0.88	0.85
Bark	1.47	1.02	3.78	1.78	0.75
Rice Hull	0.37	1.00	-	1.86	2.90
Rice Hull/hv	-	1.00	-	-	0.84
Alpechin	0.58	0.64	3.30	1.49	1.25
Alpechin/hv	0.35	0.29	4.75	4.00	1.12
Lignocel.Res.	0.73	0.87	2.45	1.10	1.08
Tannin	0.81	0.14	0.56	0.27	1.12
Phlobaphene	1.18	0.87	2.68	0.60	0.78

a) Ref. 22.

(A/E ratio) and total essential amino acid to total amino acids (E/T ratio) for the various substrates (2) is also shown in TABLE 6.

The E/T ratios of all substrates compared rather well with that for the casein reference, while some substrates exhibited much higher values than of 2.02 for the FAO provisional reference protein (15).

TABLE 7 - Aminoacid distribution of several protein (g of A.A./100 g of protein) and other parameters.

A.A	Casein	Soybean	Peanuts	FAO (15)	Essential scoring (20) pattern
Asp	6.8	3.7	15.1	-	-
Thr	4.1	3.9	1.6	2.8	3.5
Ser	5.6	4.2	6.6	-	-
Glu	21.5	4.0	17.4	-	-
Pro	8.5	5.0	5.2	-	-
Gly	1.8	18.4	5.0	-	-
Ala	2.9	3.3	4.2	-	-
Val	6.4	5.3	4.4	4.2	4.8
Met	2.4	1.7	1.0	2.2	2.6
Ile	4.9	6.0	4.0	4.2	4.2
Leu	9.0	8.0	6.7	4.8	7.0
Tyr	5.5	4.0	4.4	2.8	7.3
Phe	5.0	5.3	5.1	2.8	7.3
Lys	7.9	6.8	3.0	4.2	5.1
His	2.0	2.9	2.1	-	1.7
Arg	3.9	7.3	10.6	-	-
Cys	0.3	1.9	1.6	1.2	2.6

TABLE 6 - Nutritionally significant amino acids (a,b).

A/E Ratio (c)	S.	B.	SR.	R.	Rhv.	AL.	ALhv.	L.	O.	T.	PHL.	C.	E.
Isoleucine	126	263	78	102	0.3	109	90	141	103	180	220	106	129
Leucine	167	186	261	273	304	187	254	189	150	223	187	194	172
Lysine	144	96	136	95	158	147	158	152	159	30	122	169	125
Total arom.	176	128	204	0.6	0.6	155	211	165	85	115	149	226	194
Phenylalanine	90	63	107	0.3	0.3	94	163	88	83	101	80	108	114
Tyrosine	86	66	97	0.3	0.3	61	48	77	2	14	69	118	81
Total sulfurcontaining aminoacids	36	53	78	0.6	301	38	17	19	148	53	26	57	107
Cysteine	2	-	39	-	-	19	-	2	-	2	11	6	46
Methionine	34	53	39	0.3	301	19	17	17	148	51	15	51	61
Threonine	148	103	107	495	197	105	115	128	140	152	111	88	99
Tryptophan	37	27	30	33	38	27	27	35	29	35	32	24	31
Valine	167	135	116	0.3	0.3	260	128	151	186	214	155	137	141
E/T Ratio (d)	2.4	3.2	2.7	2.4	2.2	3.1	3.0	2.5	2.8	2.4	2.6	2.9 (e)	3.2 (f)

a) S = Saccharose; B: bark; SR: Sugar residue; R: Rice hull; Rhv: Irradiated rice hull; AL: Alpechin; ALhv: Irradiated alpechin; L: Lignocellulosic residue; C: Casein; E: Eggs; O: Orange residue; T: Tannin, PHL: Phlobaphene;
b) Tryptophan taken as minimum of 1.3 g per 16 g N.;
c) Individual amino acid (mg) per g of total essential amino acids;
d) Total essential amino acids (g of amino acid) per g of total recovered nitrogen;
e) Calculated from data Ref. (8);
f) Ref. (17).

All substrates had A/E ratios equal to or greater than those of egg protein for leucine, isoleucine, threonine and valine.

Chemical scores were calculated using the National Academy of Sciences/National Research Council Scoring Pattern (20) (TABLE 7). The total sulphur-containing amino acids were the most limiting in the proteins obtained when saccharose, rice hull, alpechin/hv, tannin and phlobaphene were used as substrates (TABLE 8). Histidine in bark and lysine in tannin derived protein were deficient. The total aromatic amino acids were the second limiting factor with all the substrates. Chemical scores have inherent limitations and therefore are only an approximation of the nutritional quality (8).

do águas residuais de oliva (alpechin). Os substratos lignocelulósicos selecionados foram: glicose, celobiose, sacarose, celulose microcristalina, tanino, flobafeno, cortiça, resíduos lignocelulósicos, resíduos de açucares derivados do processo organosolv, casca de arroz, casca de arroz pré-irradiada, bagaço de laranja, alpechin (5%), alpechin (10%), e alpechin pré-irradiado, (10%). São apresentadas taxas de alguns aminoácidos de significado nutricional em fontes convencionais de proteínas e aqueles induzidos por diferentes materiais lignocelulósicos. Também são comparadas as taxas de aminoácidos individuais com aminoácidos essenciais (razão E/A) e aminoácidos essenciais totais recuperados como nitrogênio total (razão E/T), juntamente com a escore químico dos aminoácidos essenciais, o que permitiu avaliar a qualidade de cada fonte de carbono utilizada. Essas análises mostram que o bagaço de laranja, alpechin (10%) e cortiça foram os materiais mais econômicos e que resultaram em proteína de maior qualidade.

TABLE 8 - Chemical scores calculated limiting amino acids (a,b).

Amino acid	C.	S.	B.	SR	R.	Rhv.	AL.	ALhv.	L.	O.	T.	PHL.
Histidine	118	127	77	140	0.6	0.6	123	94	150	155	120	115
Isoleucine	116	117	333	80	95	0.2	124	102	128	109	164	222
Leucine	129	93	136	162	152	150	128	174	113	96	123	144
Lysine	154	109	98	115	73	107	137	149	123	139	22	102
Threonine	117	163	150	132	555	195	143	157	151	179	166	135
Trypyophan	100	118	118	118	118	118	118	118	118	118	118	118
Valine	133	134	145	105	0.2	0.2	258	128	131	172	170	137
Total sulphur containg a.a.	102	54	105	129	0.8	401	69	31	29	252	77	42
Total aromatic amino acids	144	93	90	115	0.3	0.3	101	138	95	51	60	86

a) Abbreviation as TABLE 2;
b) Calculated using the scoring in NAS (20) calculations based on total amino acids recovery. Chemical score (g aminoacid/16 g of test protein)/(g amino acid/16 g of N reference pattern) x 100.

In summary, considering the casein pattern, the ratio of amino acids of nutritional significance (E/T and A/E), together with chemical scores indicated that bark, orange bagasse and alpechin were the best carbon sources for good quality microbial proteins (SCP) from *Chrysonilia sitophila*.

ACKNOWLEDGEMENTS

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RESUMO

Qualidade de proteínas unicelulares produzidas de materiais lignocelulósicos pelo ascomiceto *Chrysonilia sitophila* (tfb-27441 strain)

Se apresentam dados do conteúdo de aminoácidos de 15 materiais lignocelulósicos, incluin-

Palavras-chave: Proteínas unicelulares, ascomycete, *Chrysonilia sitophila*, lignocelulósico, bagaço, arroz, azeitona.

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DISTRIBUTION OF ORAL YEASTS IN CONTROLLED AND UNCONTROLLED DIABETIC PATIENTS

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SUMMARY

The oral yeasts present in diabetic controlled and uncontrolled patients were studied. The methodology used permitted to conclude that a similar frequency of yeasts is observed in both groups. Carriers were observed in 21.4% (20.0% in the controlled and 22.8% in the uncontrolled group) while 14.2% presented clinical lesions. *Candida albicans* serotype A was the most frequent. The phospholipase was produced by 100.0% and 88.8% of the strains isolated from the controlled and uncontrolled group, respectively. Proteases were produced by 75.0% of the strains isolated from controlled group and 88.8% from uncontrolled group. *Rhodotorula rubra, Candida tropicalis, Candida krusei, Geotrichum candidum, Trichosporon beigelii, Cryptococcus laurentii* and *Candida glabrata* were the other species isolated. In this study we did not find significant differences between diabetic patient groups in relation to *Candida albicans* with a similar picture observed in healthy carriers.

Key Words: diabetic, yeasts, *C. albicans*, bioserotypes of *C. albicans*.

INTRODUCTION

Diabetes, an endocrine-metabolic disease, can present several repercussions in the mouth without characteristic signs or symptoms (10, 21). Based on case reports and very few well conducted clinical and laboratorial studies, diabetes has been considered for many years a predisponent factor for candidosis.

Peters *et al* (17) studied 400 diabetics, 200 controlled and 200 uncontrolled, beside 200 healthy control patients observing a similar frequence of *Candida albicans* in them although noticing the role of total prosthesis used by some patients of their casuistic.

The increase of glucose levels in blood and saliva, the greater adherence of *Candida* species to oral mucosae, as the major growth of *Candida albicans* on the stratum corneum of diabetic patients was well as the impaires function of polymorphonuclear leucocytes are some of the factors associated to oral candidosis (4, 15). It is important to refer that an increase of yeasts in the mouth cavity of either healthy or diabetic patients have been associated to the use of a total prosthesis (5, 20). The isolation of yeasts in healthy and diabetic individ-

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uals did not present significant differences although in these patients the infection can occur at a lower candidal load (7). Many others aspects are still unconclusive about oral candidosis and diabetes which induced us to investigate the presence of oral yeasts in controlled and uncontrolled diabetic patients determining also some biocharacteristics of *Candida albicans* to type the strains isolated from our casuistic.

MATERIALS AND METHODS

Casuistic

Seventy adult patients from both sexes, from 30 to 80 years old (mean age 55), were divided in two groups. The first group was composed by 35 controlled diabetic patients who used insuline, oral hypoglicemiants or an adequate diet. The second group was composed by 35 uncontrolled diabetic patients without dietary or therapeutical control. Their dental condition are presented on Figure 1. Any patient that was under antibiotic or corticotherapy was not utilized in the present study.

FIGURE 1 - Dental conditions of the diabetic patients.

Patients	N	Dentate	Edentulous	Total Prosthesis Wearers
Controlled	35	40.0%	60.0%	80.9%
Uncontrolled	35	34.2%	65.7%	82.6%

N = Number of patients

Sampling, culture and identification methods

The material was collected from the oral mucosae and sulcus beside all other areas clinically diagnosticated as candidosis either eritematous or pseudo-membranous. The hard palate and the dorsum of the tongue were avoided for sampling. Cotton swabs were utilized to obtain the material which was plated onto a Petri dish with Sabouraud dextrose agar (DIFCO), plus chloramphenicol (100 µg/ml) maintained at 25°C, until 15 days. All experiments were performed in duplicate. The different morphological colonies were selected and were sub-cultured on the same fresh medium in tubes.

Yeast identification was performed using the criteria of Kreger-van Rij (11) and Lodder (14).

Serological methods

Specific serotype A antiserum was prepared in New Zealand rabbits males according to Hanseclever & Mitchell (8, 9) by slide agglutination technique, being strains ICB 12 (A) and ICB 156 (B), tested together with the strains isolated from our patients.

Proteases and phospholipases production

A study of extracellular enzymes (proteases and phospholipases) was performed according to Odds (15) and Price *et al* (18).

RESULTS

The distribution of yeasts in controlled and uncontrolled diabetic patients associated to carriers and the presence of lesions is presented on Table 1. Controlled patients presented 34.2% of yeast positive cultures and uncontrolled patients 37.1%; carriers being represented by 20.0% and 22.8% respectively. Both groups had the same percentage of clinically detectable lesions (Table 2). *Candida albicans* was the most frequent yeast isolated either in controlled (22.8%) or uncontrolled patients (25.7%), with a percentage of carriers for *Candida albicans* of 57.1% and 62.5%, respectively. The presence of *Candida albicans* in the lesions observed in both groups reached 80.0% (Table 2).

TABLE 1 - Distribution of oral yeasts from controlled and uncontrolled diabetic patients.

Patients		Carriers		Patients with lesion		Total of positive cultures	
		N	%	N	%	N	%
Controlled	(N=35)	7	20.0	5	14.2	13	34.2
Uncontrolled	(N=35)	8	22.8	5	14.2	13	37.1
Total	(N=70)	15	21.4	10	14.2	25	35.7

N = Total number of patients

Other yeasts as *Rhodotorula rubra* and *Candida tropicalis* were isolated in both groups.

In controlled patients, *Candida krusei, Geotrichum candidum* and *Candida* sp[1] were observed and in the uncontrolled group *Trichosporon beigelii, Cryptococcus laurentii, Candida glabrata* and *Candida* sp[2].

TABLE 2 - Frequencies of oral yeasts in controlled and uncontrolled diabetic patients.

Species of yeast	Controlled patients						Uncontrolled patients					
	Carriers (N=7)		With lesion (N=5)		Total of patients (N=35)		Carriers (N=7)		With lesion (N=5)		Total of patients (N=35)	
	n	%	n	%	n	%	n	%	n	%	n	%
C. albicans	4	57.0	4	80.0	8	22.8	5	62.5	4	80.0	9	25.7
R. rubra	2	28.5	0	0	2	5.7	1	12.5	0	0	1	2.8
C. tropicalis	0	0	1*	20.0	2	5.7	0	0	1*	20.0	1	2.8
Candida sp2	1	14.2	0	0	1	2.8	0	0	0	0	0	0
Candida sp2	0	0	0	0	0	0	1	12.5	0	0	1	2.8
C. krusei	1	14.2	0	0	1	2.8	0	0	0	0	0	0
G. candidum	0	0	1*	20.0	1	2.8	0	0	0	0	0	0
T. beigelli	0	0	0	0	0	0	0	0	1*	20.0	1	2.8
C. laurentii	0	0	0	0	0	0	1	12.5	0	0	1	2.8
C. glabrata	0	0	0	0	0	0	1	12.5	0	0	1	2.8

n = occurrence of species
* = occurrence in association
N = total of patients

An association of yeasts related to clinical lesions was represented by *Candida tropicalis* and *Geotrichum candidum* and *Candida tropicalis* and *Trichosporon beigelii* (Table 2).

Serotype A was isolated from 87.5% of *Candida albicans* strains in the controlled patients and 100.0% in the uncontrolled one (Table 2).

Production of phospholipase was observed in 100.0% of the strains in controlled group and 88.8% in uncontrolled one (Table 3).

TABLE 3 - Bioserotypes of *Candida albicans* isolated from the oral mucosae of controlled and uncontrolled diabetic patients.

C. albicans	Serotype	Phospholipase	Protease	N%
Controlled	A	(+)	(+)	7/87.5
Patients	B	(+)	(-)	1/12.5
(N=8)				
Uncontrolled	A	(+)	(+)	7/77.7
Patients	A	(+)	(-)	1/11.1
(N=9)	A	(-)	(+)	1/11.1

* = Characteristics of Bioserotypes:serotype, phospholipase and protease production
N = Number of total patients with Candida albicans.
(+) = Positive
(-) = Negative

Proteases were produced in 75.0% of the strains from controlled group and 88.8% of uncontrolled group (Table 3).

Considering serotypes, phospholipases and proteases, four different biotypes of *Candida albicans* were obtained (Table 3).

DISCUSSION

The controvertial aspects of yeasts in the mouth of diabetic patients are based on different approaches to confirm the real increase in their number or correlation to clinical lesions.

Our results indicated that in relation to *Candida albicans* or even other yeasts, there were not relevant differences between carriers of both groups (Tables 1 and 2).

Candidosis was present in a small percentage (14.2%) in both groups (Table 2), differing these results from those obtained by Lamey *et al* (12). The range of these aspects seems to depend on the methodology utilized. Nevertheless, the influence of local factors as the use of complete dentures or the presence teeth is also important because even in healthy patients these factors are relevant. Hyposalivation or xerostomia, deficient hygienic habits, are the most important local factor which favours the adherence of fungi either to the prosthesis surface or epithelium (1, 2, 5, 6).

Candida albicans was the most frequent yeast isolated prevailing the serotype A as in previous research in patients with oral cancer (95.0%) or even in healthy individuals - 66.7% - (16, 19) as in AIDS patients (3) from mouth samples observed in our country, which does not explain the real factors which determinate the serotype (15).

Protease production showed similar results to those obtained in healthy individuals while a higher production of phospholipase was ob-

served in diabetics when compared to healthy ones (19). So, considering serotype and exoenzymes production, the isolates of *Candida albicans*, in our casuistic, can be individualized in four bioserotypes.

An important aspect to point out was the presence of different yeast species compared to other studies as those from Loiselle *et al* (13) and Tapper-Jones *et al* (20), since these studies were directed exclusively towards the identification of *Candida albicans*. It is interesting to remember that although *Candida tropicalis, Geotrichum candidum* and *Trichosporon beigelii* can be isolated from the normal flora, they were isolated from clinical lesions or associated to other yeasts, sinalizing always the possibility that under certain conditions they can also act as pathogens (15).

The general picture of yeasts obtained from our casuistic did not reveal significant relationship between diabetes and candidosis, and leads us to believe that the relation host-parasite has to be better defined in this field to obtain an adequate profile of the disease and the infection or host/parasite relationship.

Although other biotyping methods could be used in future research as the study of morphotyping, biotyping, DNA restriction fragment lenght polymorphism analysis and assay with killer toxins, which could characterize better these strains in the mouth of diabetics in epidemiological studies.

RESUMO

Leveduras da mucosa oral em pacientes diabéticos controlados e não controlados

Estudamos a presença de fungos na cavidade bucal de diabéticos controlados e não controlados. A metodologia utilizada permite concluir que a freqüência de fungos foi semelhante em ambos os grupos. Como portadores, observamos 21.4% (20.0% nos controlados e 22.8% no grupo não controlado), enquanto 14.2% apresentavam lesões clínicas. *Candida albicans* sorotipo A foi a mais freqüente, sendo fosfolipase produzida em 100.0% no grupo controlado e 88.8% nos não controlados. Protease foi produzida em 75.0% das amostras isoladas do grupo controlado e 88.8% dos não controlados. Foram isoladas outras espécies de leveduras como: *Rhodotorula rubra, Candida tropicalis, Candida krusei, Geotrichum candidum, Trichosporon beigelii, Cryptococcus laurentii* e Candida glabrata. Neste estudo, não observamos diferenças significativas nos dois grupos dos pacientes diabéticos, em relação a presença de *Candida albicans*, inclusive com um quadro semelhante observado em portadores saudáveis.

Palavras-chaves: Diabéticos, Leveduras, *C. albicans*, Biosorotipos de *C. albicans*.

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ACCUMULATION OF CADMIUM FROM MODERATELY CONCENTRATED CADMIUM SOLUTIONS BY CHLORELLA AND SCENEDESMUS STRAINS

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SUMMARY

Cadmium accumulation from different solutions was studied with the use of three different microalgal strains. From these living microalgae, *Chlorella homosphaera* showed higher sorption capacity compared to *Scenedesmus quadricauda* and *Chlorella vulgaris* cells. *Chlorella homosphaera* and *Scenedesmus* strains were significantly affected by the presence of the metal, being *Chlorella vulgaris* not affected by a higher concentration (50 mg/l).

Key words: Cadmium, green microalgae, accumulation.

INTRODUCTION

The binding of metals from contaminated solutions by biological sorbents can be achieved through a wide variety of mechanisms. Some of these are metabolically mediated and others involve simple adsorption mechanisms. Specific examples and a detailed description of those sorption mechanisms can be found in a detailed review edited by Rehm and Reed[8]. The toxic effect of cadmium on living green algae from the genus *Chlorella* and *Scenedesmus* has been studied[1,4,6], as well as its transport[3] and ionic state of adsorbed metal by living cells[7]. Recently the binding of heavy metals by particulate biomass derived from *Chlorella* and *Scenedesmus* was studied[2], using individual metals or combined solutions. The present work compares the ability of three different microalgal strains to accumulate cadmium from moderately concentrated solutions. Living cells, as used here, are subject to deleterious effects produced by the presence of the metal, a fact not detected in metabolism-independent metal recovery, named "biosorption".

MATERIALS AND METHODS

1. Materials - Three different living green microalgae were used in this work. *C. homosphaera* and *S. quadricauda* were obtained from Escola de Química da Universidade Federal do Rio de Janeiro. *C. vulgaris* strain was obtained from Faculdade de Ciências Farmacêuticas da Universidade de São Paulo.

2. Culture medium for cultivation of microalgae - Living green microalgae were grown in Difco medium, as previously described[5,6]. The salts were dissolved in distilled water and the final pH of the medium adjusted to 7.0. The medi-

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um was autoclaved at 110°C and 1 atm for 20 minutes, allowed to cool and 4 ml of micronutrients solution, previously sterilized by filtration using 0.27 μm Millipore membrane, were added to the solution to act as growth factors[5]. From agar slants, algal samples were asseptically transferred to the culture medium, placed in a rotary shaker at 200 rpm, for 72 hours, under continous illumination and at room temperature, for cell growth[6].

3. Cadmium uptake by living algal cell - Four different cadmium solutions were prepared by using cadmium sulphate: 10.0, 20.0, 30.0 and 50.0 mg/l. This restricted range of cadmium concentrations was selected because it was expected to be detected some deleterious effects on the cells. 200 ml of each solution were added to Erlenmeyer flasks and grown cells were then added to these solutions to reach a final concentration of 0.5 g/l. As previously reported, *Chlorella* cells grow up to 4.0 g/l, measured by an absorbance at 430 nm *versus* dry weight of cells curve[6]. *S. quadricauda* strains grows up to 8.0 g/l, in the same conditions. This way it is possible to predict the inoculum for a 0.5 g/l final concentration of cells, in the different cadmium solutions.

Two sets of experiments were designed at this stage: in the first one the biological material was contacted with cadmium solutions for 5 minutes and in the second one, for 6 hours, both in a rotary shaker under the same conditions previously described for cell growth. These experiments were designed to establish the relationship between metal accumulation in biomass and time. The flasks were then removed from the shaker, their content centrifuged at 3200 g, in a Fanen Centrifuge Excelsa Baby I, Model 206, and the supernatant analyzed for cadmium concentrations. Each metal concentration was tested three times. The four cadmium solutions were tested for their stability in solution, and it was observed that cadmium dissolution was complete and constant in the time-course of the experiments.

4. Analysis - Cadmium concentrations were determined using a Varian Techtron, flame atomic absorption spectrophotometer, Model AA6.

RESULTS AND DISCUSSION

Figure 1 indicates cadmium accumulation by *S. quadricauda*, *C. vulgaris* and *C. homosphaera*, in the described conditions. The figure shows cadmium accumulation at 5 minutes and 6 hours of contact between the metal solution and the living microalgae. Each group of two bars represents the metal accumulation at 5 minutes and 6 hours, re-

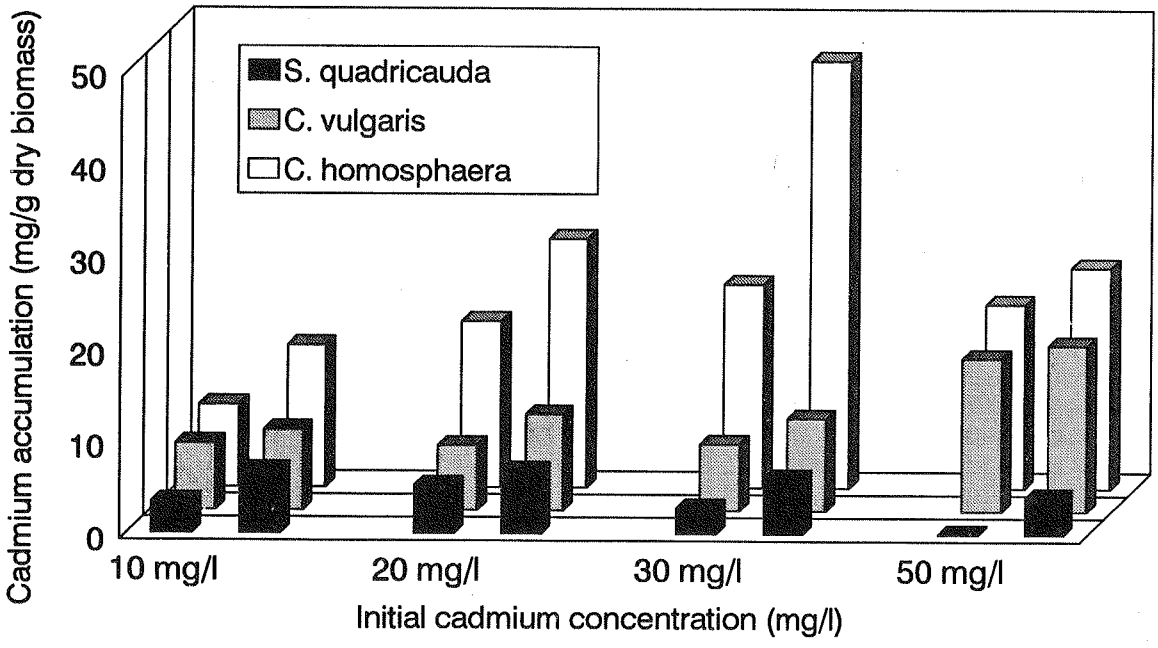

FIGURE 1 - Cadmium accumulation at 5 minutes and 6 hours of contact between the metal solution and the living microalgae.

spectively. The comparison between each set of bars illustrates a clear time dependence process. The amount of captured cadmium was much greater after the six-hour test than the amount captured in the process run during 5 minutes, for all tested solutions. The residual metal concentrations, at 6 hours, were smaller in all cases, confirming higher metal accumulation. This difference would indicate that the main sorption mechanism is not restricted to cell adsorption. Probably the metal is being intracellulary transported, a fact that demands confirmation. Anyway, the equilibrium between metals in solution and biomass particles was not achieved, even after 6 hours; this could be an indication of the presence of metabolic activity. Another point that must be emphasized is the fact that at a cadmium concentration of 50.0 mg/l the accumulation capacity of the cells decreases, in comparison with the 30.0 mg/l cadmium solution, irrespective of the contact time, exception made for *C. vulgaris* strain. After 5 minutes, the accumulation capacities for *C. homosphaera* cells, for solutions containing 30.0 and 50.0 mg/l were 22.0 and 20.0 mg cadmium/g dry cells, respectively; after 6 hours the sorption capacities of these cells, from the same solution, were 46.0 and 24.0 mg cadmium/ g dry cells, respectively. It can be noted a decrease in metal accumulation with the increase in the initial metal concentration, in both cases. This can be explained in terms of the toxic effect of cadmium on *Chlorella* cells, already reported by others authors[1,3]. This fact, as reported in the literature, is marked after longer periods of time. The time dependence in cadmium accumulation by *Chlorella* cells as well as an exclusion mechanism as heavy metals tolerance for this genus is also stated in the literature [3,7].

A slight difference in the sorption pattern could be detected for *S. quadricauda* cells, from the results obtained during 5 minutes and 6 hours of contact between biomass and the metal solution. The toxic effect of the metal could also be observed for this species. The decrese in metal accumulation for cadmium concentrations greater than 20.0 mg/l confirms this observation. The maximum sorption capacity for *S. quadricauda* cells was 5.2 mg cadmium/ g dry cells and 6.8 mg cadmium/g dry cells, at 5 minutes and 6 hours, respectively. It is interesting to not that these limiting results were obtained from a solution containing initially 20.0 mg/l. Concentrations greater than that negatively affect the sequestering of metals. Comparing such results with those obtained for *C. homospharea* cells, it can be extracted that *S. quadricauda* cells are considerably more sensitive to cadmium[4]. This is confirmed by the growth curves of both species in presence of cadmium at various concentrations, where *S. quadricauda* cells had their growth affected by the presence of the metal in a higher extent than *C. homosphaera* [4].

Analogously *C. vulgaris* showed a time dependence for cadmium accumulation. The maximum sorption capacity for this species was 16.6 mg cadmium/g dry cells at 5 minutes of process and 18.0 mg cadmium/g dry cells for a period of 6 hours. In this case it was not detected a decrease in the metal accumulation by the cells, in the four different cadmium solutions tested, but, although its low sequestering capacity compared to *C. homosphaera* cells, it can not be neglected in metal accumulation studies, as it is not a cadmium sensitive species.

In general, from these experiments conducted with living cells, *S. quadricauda* were markedly affected by the action of cadmium on its structure, being their ability to accumulate this metal inhibited by concentrations higher than 20.0 mg/l. *C. vulgaris*, not facing this problem, showed increasing accumulation for more concentrated solutions in the range of concentrations studied. However, it is not comparable to *C. homosphaera*, which presented a relatively higher sorption capacity, despite this property being affected by cadmium concentrations higher than 30.0 mg/l.

From the three green microalgae tested, *C. homosphaera* showed higher capacity to accumulate cadmium from moderately concentrated cadmium solutions. *S. quadricauda* was markedly affected by the presence of the metal, showing low cadmium sorption. *C. vulgaris* showed a growing patter of cadmium accumulation for the tested solutions, but with lower sorption potential compared to *C. homosphaera* cells. Among the living microalgae tested, the maximum sorption capacity was achieved by *C. homosphaera* cells, from a 30.0 mg/l cadmium solution, equal to 48.0 mg cadmium/g dry cells.

RESUMO

Acumulação de cadmio a partir de soluções moderadamente concentradas de cadmio por cepas de Chlorella e Scenedesmus

A acumulação de cádmio a partir de soluções contaminadas com o metal foi estudada através do emprego de três diferentes microalgas. Dentre as

espécies testadas a *Chlorella homosphaera* mostrou maior poder de acumulação comparada com células de *Scenedesmus quadricauda* e *Chlorella vulgaris*. As linhagens de *Chlorella homosphaera* e *Scenedesmus quadricauda* foram consideravelmente afetadas pela presença do metal, sendo a *Chlorella vulgaris* não afetada pela mais alta concentração (50 mg/l).

Palavras-chave: cadmio, microalgas verdes, acumulação

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MYCOFLORA AND AFLATOXIGENIC SPECIES OF ASPERGILLUS SPP ISOLATED FROM STORED MAIZE

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ABSTRACT

Mycoflora and aflatoxigenic species from the genus *Aspergillus* were studied in ninety samples of maize from storage silos from different regions of Brazil. *Aspergillus, Penicillium, Fusarium, Rhizopus, Acremonium, Cladosporium, Neurospora* and *Paecilomyces* were the genera isolated. *A. flavus* was the most frequently isolated among the *Aspergillus* species from samples with moisture content (MC) between 14% and 18%. 39.4% of the isolates were toxigenic and produced only B aflatoxins. *A. parasiticus* was the third most frequent species, isolated from samples with MC between 13% and 18%. All cultures were toxigenic and produced aflatoxins B_1, B_2, G_1 and G_2. The other species isolated were *A. alutaceus, (A. ochraceus), A. petrakii, A. chryselius, A. versicolor, A. sydowi, A. fumigatus* and *A. niger,* which occured only in samples with moisture content between 12% and 13%.

Key Words: Zea mays (L.), maize, fungi, mycoflora, aflatoxigenic fungi.

INTRODUCTION

Maize (Zea mays) is one of the most widely cultivated crop in Brazil, with nearly 13 million hectares planted (9). Most of the production is destined to animal feed either directly, or as part of feed-mix, making 63% part of poultry feed and 75% of hogs feed (13).

Maize such as other agricultural products, is subject to the natural contamination of vegetal and soil fungi and several of these are potential producers of mycotoxins (16).

The fungi that infect grains have been divided into two ecological groups, field and storage fungi (4,5). Field fungi are so called because are reputed to invade seeds before harvest, while the plants are growing in the field, or after cutting and swathing, but before the harvest is threshed (5). These fungi require high moisture to grow, which is obtained by relative humidity (RH) in excess of 90%, given a MC of 20-21% in cereal grains (5).

During storage RH falls below 90% and no free water is available, the field fungi become dormant and the storage fungi appear, mainly comprising of 10-15 species of *Aspergillus* and few species of *Penicillium* (5).

Maize, like other cereals, with low moisture can be stored for long periods of time. However, if

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the storage conditions are not adequate, those fungi from natural origin as well others acquired during the manipulation can grow and produce mycotoxins (18).

After peanut, maize is the most mentioned product in the world literature regarding contamination by aflatoxins (Hesseltine et alii, 1981).

In Brazil, in spite of its great consumption and the adequate climatic conditions for the development of aflatoxins producing fungi the existing publications refer only to the detection of mycotoxins in maize and byproducts (11).

Considering these aspects, the objectives of the present study was to identify the mycoflora in maize samples from storage silos and the occurrence of aflatoxigenic species of the *Aspergillus* genus.

MATERIALS AND METHODS

Ninety samples of maize grains were used. From various regions of Brazil. The moisture content (MC) of the grains was determined through and Elotest instrument, model 777.

Isolation and identification of the fungi (3) - The samples were ground so that whole of material passes through 14 mesh sieve. Ten grams of the ground samples were diluted in 90 ml of sterile distilled water and from this, sucessive dilutions until 10^{-4} were prepared. One ml of each dilution was transferred to a Petri dish and 15 ml of acidified Sabouraud-dextrose agar (DIFCO) at about 45°C was poured over it and mixed with the samples. After agar solidication the Petri dishes were incubated at 25°C for five days. Fungi were identified by using usual techniques (1,2,14).

Screening for aflatoxins - The *Aspergillus* spp. isolates was inoculated in 250 ml of 2% yeast extract plus 20% sucrose YES medium (6) in 1 litre Roux Flask was inoculated with spores, incubated at room temperature for 10 days, and with three 25 ml portions of $CHCl_3$, in a gyratory shaker. The pooled extracts were filtered and evaporated to dryness in a flash evaporator, the residue was cooled and redissolved in 5 ml of $CHCl_3$. The aflatoxins standards (B_1, B_2, G_1 and G_2) used were obtained from SIGMA Chemical Company, U.S.A..

Thin-layer chromatograms (TLC) were developed in benzene: ethyl-acetate: ethanol (60:38:2) and the results indicated that qualitative exams could be performed easily and accurately without further purification. With the positive qualitative analysis, the following confirmatory tests were performed: development of the plate with ethyl ether in spraying of sulphuric acid solution (50%) on the spots to observe the change of the blue fluorescence (aflatoxin B_1) to yellow, and two-dimensional TLC spotting pattern.

RESULTS

The following genera were isolated in decreasing order or frequency: *Aspergillus* (72.2%), *Penicillium* (67.7%), *Fusarium* (62.2%), *Rhizopus* (7.7%), *Acremonium* (2.2%), *Cladosporium* (1.1%), and non sporulating fungi (5.5%).

The species of *Aspergillus* isolated were: *A. flavus* (36.6%); *A. oryzae* (9.9%); *A. parasiticus* (6.6%); *A. alutaceus (A. ochraceus)* (6.6%); *A. petrakii* (6.6%); *A. chryselius* (5.5%); *A. chevalieri* (4.4%); *A. versicolor* (2.2%); *A. sydowi* (2.2%); *A. fumigatus* (2.2%); *A. niger* (1.1%) and *A. tonarii* (1.1%). It was observed that the species *A. alutaceus, A. petrakii,* (*A. ochraceus* group), *A. chrysellius* (*A. cremeus* group), *A. versicolor, A. sydowi* (*A. versicolor* group), *A. fumigatus* (*A. fumigatus* group) and *A. niger* (*A. niger* group) were present only in the samples with MC between 12% and 13%. *A. parasiticus* (*A. flavus* group) was isolated from samples with 13.2% to 17.8% MC and *A. flavus* (*A. flavus* group) in samples between 12.1% and 18.8%, but it was more frequent in samples with MC above 14%. The *Penicillium* and *Fusarium* species were isolated from samples between 12% and 18% of MC.

The *A. flavus* isolates (39%) were toxicogenic and produced aflatoxin B_1 or B_1 and B_2. All cultures of *A. parasiticus* tested produced aflatoxin B_1, B_2, G_1 and G_2.

DISCUSSION

Del Prado and Christensen (1952) have suggested the possibility of evaluation of the storage conditions of cereal grain, through the study of its mycoflora.

Christensen & Kaufman (1965) and Christensen and Sauer (1982), classified the fungi that contaminate grains according to their need of water and predominance in field or in storage. The authors noted that the "field fungi" invade grains with moisture of 20-21%. Whereas the storage fungi *Aspergillus* and *Penicillium* which predominate in grains 13% and 18% of MC.

TABLE 1 - Absolute (AF) and relative (%) frequency of *Aspergillus*, *Penicillium* and *Fusarium* isolated from 90 samples of maize from storage silos from different regions of Brazil, in relation to the moisture content of these samples.

Fungi	Moisture content in the maize samples (%)						
	12.0⊢13.0	13.0⊣14.0	14.0 15.0	15.0 16.0	16.0 17.0	17.0 18.0	18.0 19.0
A. flavus							
AF	4	1	8	8	3	5	4
%	11.4	11.0	53.3	72.7	50.0	50.0	10.0
A. oryzae							
AF	6	1	2	-	-	-	-
%	17.1	11.0	6.66	-	-	-	-
A. parasiticus							
AF	-	4	-	-	-	2	-
%	-	4.44	-	-	-	20.0	-
A. alutaceus							
AF	6	-	-	-	-	-	-
%	17.1	-	-	-	-	-	-
A. alliaceus							
AF	6	-	-	-	-	-	-
%	17.1	-	-	-	-	-	-
A. petrakii							
AF	6	-	-	-	-	-	-
%	17.1	-	-	-	-	-	-
A. chrysellius							
AF	5	-	-	-	-	-	-
%	14.3	-	-	-	-	-	-
A. chevalierii							
AF	4	-	-	-	-	-	-
%	11.4	-	-	-	-	-	-
A. versicolor							
AF	2	-	-	-	-	-	-
%	5.71	-	-	-	-	-	-
A. sydowi							
AF	2	-	-	-	-	-	-
%	5.71	-	-	-	-	-	-
A. fumigatus							
AF	2	-	-	-	-	-	-
%	5.71	-	-	-	-	-	-
A. niger							
AF	1	-	-	-	-	-	-
%	2.85	-	-	-	-	-	-
A. tamarii							
AF	-	-	-	-	-	-	1
%	-	-	-	-	-	-	25.0
Penicilliium spp							
AF	20	9	10	7	2	7	4
%	57.1	100.0	66.6	63.6	33.3	70.0	100.0
Fusarium spp							
AF	14	9	13	9	5	5	-
%	40.0	100.0	66.6	81.8	83.3	50.0	-
Nº of maize samples	35	9	15	11	6	10	4

(-) Absense of fungi

In the 90 samples analyzed in this experiment, the predominant genera were *Aspergillus*, *Penicillium* and *Fusarium* and the grains always presented MC varying from 12% to 18.8%. These results are in agreement with those observed by Christensen & Kaufman (1965).

According to these authors and McLean and Berjak (1987), the species of *Fusarium* that contaminated the grains in the field can remain for some months in the stored grains. This observation can explain the frequent occurence of the *Fusarium* in the samples analyzed in this study.

In the genus *Aspergillus*, the most frequently isolated species was *A. flavus* a fact that have been observed by other authors (11,12).

According to Christensen & Kaufman (1965), the species group from the *Aspergillus* genus which predominate in the stored cereal grains, are: *A. glaucus*, in grains between 13% and 14% MC, *A. candidus* and *A. alutaceus* in grains with 15% MC, and *A. flavus* in grains with 17.5% MC. Species of genus *Penicillium*, predominate in stored grain at low temperatures and MC over 16%.

Our results show that the fungi from the *A. glaucus* and *A. alutaceus* group were only isolated in grains with low MC (between 12% and 13%). The species *A. flavus*, in spite of isolated in grains with MC between 12% and 19%, was more frequent in the samples with levels of MC over 14%. The other fungi were isolated sporadically.

From the *A. flavus* culture, 39.4% were toxigenic and produced aflatoxin B_1 or B_1 and B_2. All the cultures of *A. parasiticus* produced aflatoxin B_1, B_2, G_1 and G_2. These results are in agreement with other authors (10). According to them all the strains of *A. parasiticus* are aflatoxin producers. Nevertheless, not all the strains of *A. flavus* have this capacity (17). These facts lead us to conclude that *A. flavus* and *A. parasiticus*, potential producers of aflatoxins, are part of the natural mycoflora of this type of product. Under favourable conditions, they may grow and produce aflatoxins.

Due to the impossibility of avoiding the natural fungic contamination of agricultural products in the field or during the manipulation after the harvest, it is necessary to maintain the adequate storage conditions in order to control the development of mycotoxins' producing fungi.

RESUMO

Microbiota fúngica e espécies aflatoxigênicas do gênero *Aspergillus* provenientes de milho armazenado

Pesquisou-se a microbiota fúngica e espécies aflatoxigênicas do gênero *Aspergillus* em 90 amostras milho provenientes de silos de armazenamento.

Foram isolados oito gêneros fúngicos: *Aspergillus, Penicillium, Fusarium, Rhizopus, Acrenonium, Cladosporium, Neurospora* e *Paecilomyces*. Do gênero *Aspergillus*, a espécie *Aspergillus flavus* foi a mais frequente, isolada de amostras com umidade entre 14 e 18%. De suas culturas, 39,4% eram toxigênicas e produziam somente aflatoxinas do grupo B. *Aspergillus parasiticus* foi aterceira espécie mais frequente, isolada de amostras com umidade entre 13 e 18%. Todos os isolados desta espécie foram toxigênicos e produziram aflatoxinas B_1, B_2, G_1 E G_2. As outras espécies do gênero *Aspergillus* foram: *A. alutaceus, (A. ochraceus), A. petrakii, A. chriselius, A. versicolor, A. sydowi, A. fumigatus* e *A. niger*, e ocorrem somente nas amostras com teores de umidade entre 12 e 13%.

Palavras-chave: milho, microbiota fúngica, aflatoxinas, fungos aflatoxigênicos.

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THE IONIC CHARACTER OF THE ENVIRONMENT IN THE FLOCCULATION OF PICHIA STIPITIS

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SUMMARY

Although cells of a flocculation strains of *Pichia stipitis* became completely dispersed by washes in distilled water, they reflocculated when suspended in solutions of individual medium components. Ionic strength was suspected to be the primary cause behind flocculation. The measurement of the electrical conductivity showed to be a practical way of evaluating the ionic strength of the environment. Accordingly, flocculation was more intense as the electrical conductivity of the aforementioned solutions increased. At higher values of electrical conductivity (> 1000 μS) the degree of flocculation was nearly constant, suggesting that charge reduction associated with conformational changes in cell wall proteins may be responsible for cell aggregation. Additionally, a decrease in electrical conductivity of the medium, through dialysis, reduced the ability of the cells to flocculate. The ionic character of the medium showed to be an important influencing factor in flocculation.

Key Words: *Pichia stipitis*, flocculation, ionic environment.

INTRODUCTION

Yeast flocculation describes an aggregation of cells, which usually develops during the late-exponential or stationary growth phase. There is a consensus that it involves a protein-carbohydrate (lectins) interaction between proteins on flocculating cell surfaces and mannans present in cell walls (7). The phenomenon is of interest in the development of industrial processes, in studies involving cell/cell interactions during fusion to produce zygotes and in sexual aggregation (e.g., a and α cells in haploid yeast cells).

Studies of yeast flocculation have been approached from various fields, which may be divided into three groups:

1. Effect of the environment on flocculation (2,4,8,13);
2. Genetics of flocculating yeasts (3,7,11);
3. Nature and structure of the cell wall of flocculating yeasts (9,12).

In this paper, the first of the above groups will be approached with one flocculating strain of *Pichia stipitis*, a D-xylose fermenting yeast.

There is no doubt that flocculation of pure cultures is a genetic characteristic, and several flocculation genes have already been identified in *Saccharomyces* species (3). Besides the genetic characteristics of the cells, other factors related to the environmental conditions can influence cell flocculation. The environment affects direcly the

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cell wall electrochemical properties (1). This is believed to be due to the neutralisation of anionic charges, primarily carboxyl groups, on the surfaces of the yeast cells (10). Therefore, expression of the ability to flocculate also depends on changes in the pH, temperature and the presence of a range of compounds in the medium which alter the ionic environment. Studies related to the ionic strength have been concentrated on the effect of cations. Very few works - the majority of them carried out with brewer's yeasts - analyse medium composition in itself on cell aggregation, mainly because of the complexity of the fermentation media used in beer production. There has been a great deal of work done on the effect of cations as floc-promoters in yeast - particulary calcium - however the explanations for this effect is still a subject of considerable controversy. What is known is that nitrogenous substances in the medium delay the point in the growth cycle at which brewers' yeasts become potentially flocculent (8) and that sugars are potent deflocculants for brewer's yeasts (2). The aim of this work is therefore to study the influence of the medium components, and the medium as a whole in the flocculation of *Pichia stipitis*.

MATERIALS AND METHODS

Microorganisms: The flocculating strain of *Pichia stipitis* 5774 was obtained from the Central Bureau voor Schimmelcultures (CBS) - The Netherlands.

Medium composition: Cells were propagated in a medium with the following composition: D-xylose, 20 g; urea, 1.25 g; KH_2PO_4, 1.10 g; yeast extract, 1.5 g; $MgSO_4.7aq$, 0.50 g; $CaCl_2.2aq$, 0.05 g; citric acid, 0.50 g; $FeSO_4.7aq$, 0.036; $MnSO_4.4aq$, 10.00 mg; $ZnSO_4.7aq$, 12.00 mg; $CuSO_4.5aq$, 1.00 mg; $CoCl_4.6aq$, 2.00 mg; $NaMoO_4.2aq$, 1.40 mg; H_3BO_3, 2.00 mg; KI, 0.36 mh; $Al_2(SO_4)_3$, 0.50 mg, in 1000 mL of distilled water. The initial pH was adjusted to 6.0 with 1M of HCl or NaOH, and D-xylose was sterilised separately from the other components.

Inoculum development: The inoculum was prepared by transferring a loopful of cells from the fresh slope medium to 500 mL-conical flasks containing 200 mL of medium. The inoculated flasks were incubated at 25°C on a rotatory shaker, at 180 rpm for 48 h.

Flocculation measurements: The degree of flocculation was evaluated by the percentage of cells remaining in suspension (R%), as described by Pereira Jr. & Bu'Lock (9). The R values were calculated by the following formula: $R\% = 100\ C_f/C_t\%$ where C_f is the free-cell concentration and C_t is the total cell concentration.

Electrical conductivity measurement: Different known concentrations of individual medium components, as well as fresh or cell-free spent medium (boiled or dialysed), and distilled water had their electrical conductivity measured by a 4010 Conductivity meter, manufactured by Jenway, U.K. The conductivity could be measured over five switched ranges from 0.01 µS to 200 mS. The apparatus cell constant presented a value of 1.03 µS. The pH of the solutions was adjusted to 6.0 with 0.1 M of HCl or NaOH, and their temperature was 25°C.

Experimental methodology: Concentrated solutions of individual medium components were prepared previously (25 g/L for urea, yeast extract, KH_2PO_4, and 200 g/L for D-xylose). Aliquots of them were added to a determined volume of a dispersed cell suspension and the volume made up to 100 mL with distilled water so as to obtain the intended concentration of each medium component in the cell suspension. Then, the whole content was tranfered to a 250 mL-conical flask, and the pH value was adjusted to 6.0, in the same way as it was for the electrical conductivity measurements. A 10 mL sample was removed from the well-mixed cell suspension for the determination of the total cell concentration and 50 mL placed in a measuring cylinder for the flocculation assay. The solution containing mineral salts and citric acid (SMS) was prepared 25-fold more concentrated than the actual concentrations of its components in the growth medium. Again, aliquots of this concentrated solution were added to a determined volume of a dispersed cell suspension and the volume was made up to 100 mL with distilled water in order to obtain the desired concentration in the test. The cells originated from the same cultural conditions, being centrifuged at 2000 g for 20 minutes and washed three times with distilled water in order to prepare a concentrated cell suspension (ca. 20 g dw/L) to be utilised in the experiments. Flocculation tests were also carried out either in fresh medium, containing all components together, or in cell-free spent medium, obtained after cell centrifugation and the supernatant filtered through a Millipore membrane (0.45 µm pore size, 47 mm diameter). The cell-free spent medium was also boiled for one hour and dialysed overnight, using celulose membrane dialysis tubing (Sigma). Again the same methodology was adopted for the flocculation assays.

RESULTS AND DISCUSSION

Deflocculation caused by washes in distilled water

Centrifuged cells were sequentially re-suspended in distilled water or in cell-free spent medium, both at pH 6.0. Upon each re-suspension the cells were homogenized by a stirring bar. So, a 'control' was set up to check whether shearing forces would cause any demage to the flocs, making them desaggregate (Fig. 1b), which did not take place. It was observed that when flocculating cells were washed with distilled water they lost their aggregation ability, becoming completely dispersed by the end of the second wash (Fig 1a). When those same cells were re-suspended in cell-free spent medium they regained their ability to flocculate. The cells that had been initially resuspended twice in cell-free spent medium (control), after centrifugation, also lost their ability to flocculate when later washed in distilled water. The findings led us to conclude that some component in the medium was inducing flocculation, therefore their isolated effect was examined.

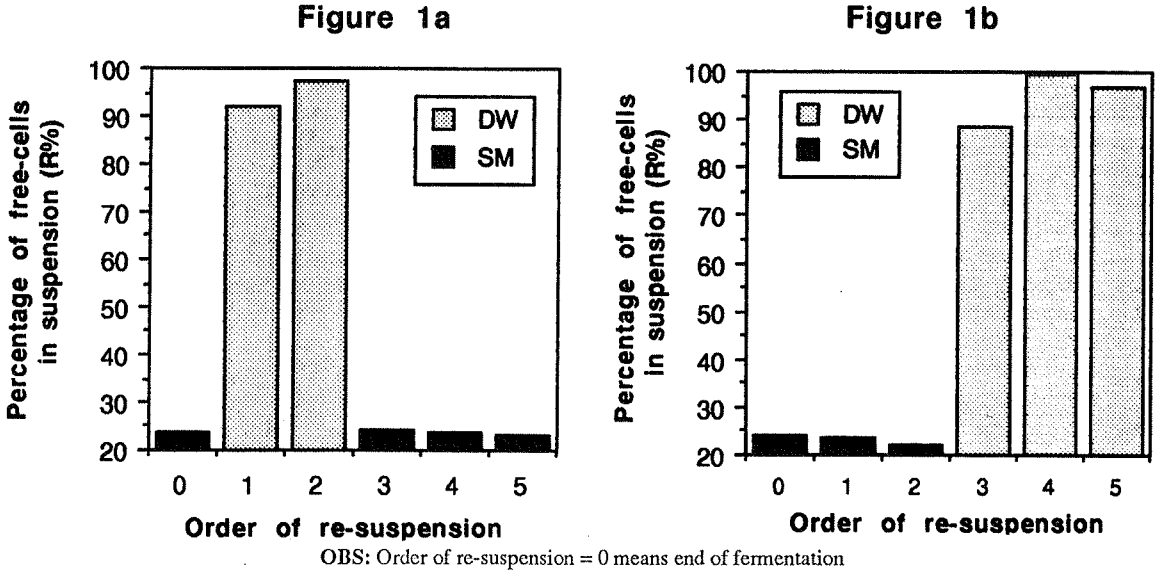

FIGURE 1 - Deflocculation caused by washes in distilled water (DW) and reflocculation by re-suspension in cell-free spent medium (SM). Cells of *Pechia stipitis* CBS 5774.

Isolated effect of the medium components on flocculation

Washed cells were re-suspended in different concentrations of individual medium components and, after pH adjustment, the flocculation test was immediately performed. It can be seen in the graph of Figures 2, that except for D-xylose all the other components can induce cell aggregation, however to different extents.

Considering the initial concentrations of the components in the medium one can conclude that

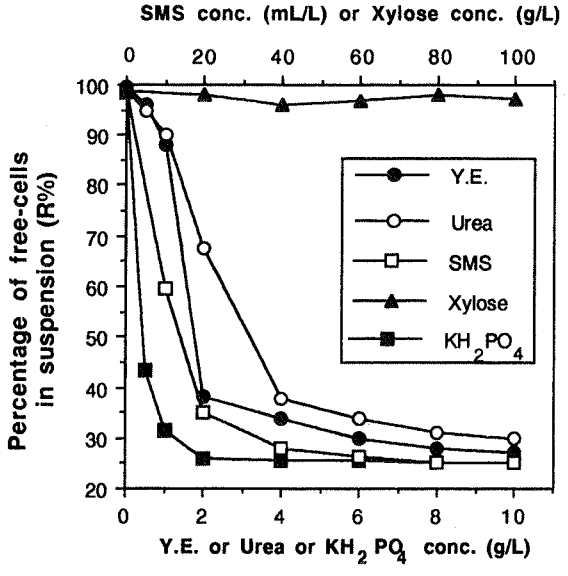

FIGURE 2 - Isolated influence of the medium components on flocculation of *P. stipitis* CBS 5774.

KH_2PO_4 and the solution of mineral salts are the ones which contribute more to the cell aggregation, although yeast extract and even urea can also induce flocculation in higher concentrations (up to 10 g/L). Nevertheless, all of them presented the same pattern, i.e. an increase in their concentrations favours flocculation. Glucose, mannose, maltose and fructose are reported to be potent defflocculants for brewer's yeast (2), and D-xylose shows not to be different in the flocculation of *Pichia stipitis*, even in concentrations as high as 100 g/L.

Taking these observations into account, it was suspected that the ionic strength might be the cause of the phenomenon observed. It seemed to us that a practical way to evaluate the ionic strength would be by measuring the electrical conductivity of the solutions of the medium components. Theoretically, the evaluation of the ionic strength (I.S. = $1/2 \sum c.z^2$), which takes into account the molar concentration of the ion (c) and its charge (z), would result in error. This would be due to the difficulty in considering the increase in the ionic strength caused by additions of HCl and NaOH, when adjusting the solutions' pH values. Moreover, electrical conductivity is directly proportional to ionic strength ($K = \sum z^2.F^2.u.c$, where 'F' is the Faraday constant and 'u' is the electrolytic mobility), and in dilute solutions it is a linear function of the concentrations of the components (5). The results of the electrical conductivity measurements of different concentrations of the medium components are plotted in the graphs of Fig. 3.

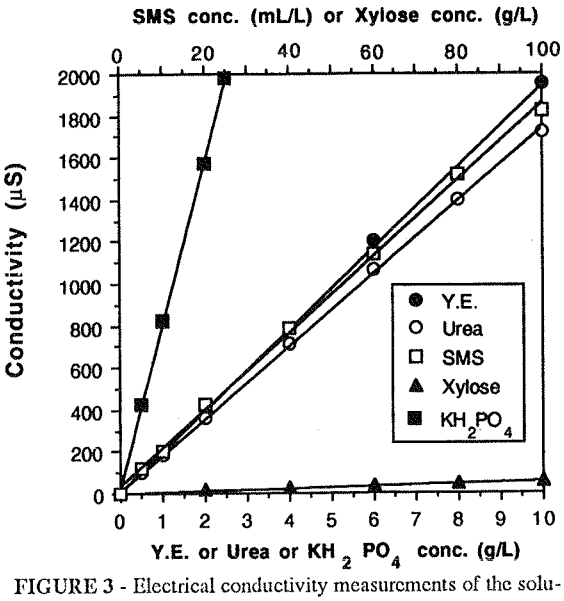

FIGURE 3 - Electrical conductivity measurements of the solutions of the individual medium components.

The percentage of cells remaining in suspension were plotted against the electrical conductivity measurements (Fig. 4). All medium components were taken into account, except for D-xylose, which exhibited no effect whatsoever on reflocculation of dispersed cells, even in concentrations as high as 100 g/L.

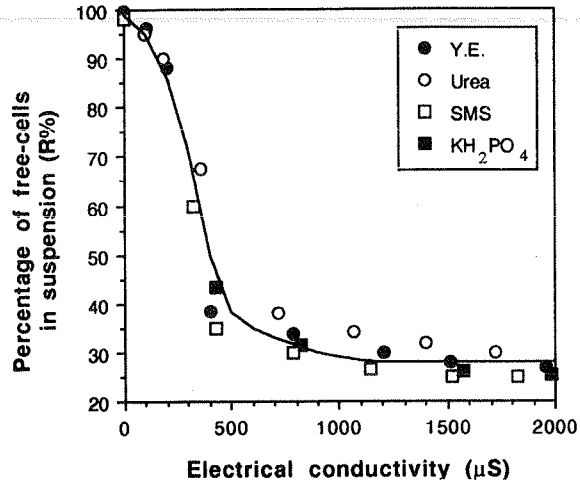

FIGURE 4 - Flocculation of *Pichia stipitis* as a function of the ionic character of the medium.

The pronounced effects of electrical conductivity, or in other words, of ionic strength, on the flocculation of *Pichia stipitis* strain CBS 5774 indicate that a reduction in the cell surface charge may have happened as the ionic strength of the solutions increased. Taylor & Orton (13) also found that flocculation intensity in *Saccharomyces cerevisiae* was dependent on the ionic strength of calcium solutions in Tris pH 7.6 buffer, and was maximum at about 0.01 ionic strength. But, then again their study was not done in the fermentation medium. Our previous work (9) showed the involvement of cell wall proteins in the phenomenon of flocculation, and like any other protein, they possess ionisable groups. They can therefore be made to exist in solution as electrically charged species, either as cations or anions. This effect is so marked in proteins that it serves as a basis for a considerable number of techniques, which permit their separation, and also the determination of their physical-chemical properties. It apperars that when the ionic strength of the medium is low, the cell wall protein assumes a conformational structure where one of its ionisable groups (probably

carboxyl) might prevail, and consequently cause the cells to repel from each other (10). It is also well known that the concentration of ions in the medium can significantly affect the surface properties of microorganisms (1). This is probably due to the fact that proteins can readily bind ions from the solution (6). In this way, as the ionic strength of the medium increases there might be conformational changes in cell wall proteins, associated with neutralization of repulsing charges. This would allow the forces responsible for aggregation to be operated. It is believed that hydrogen bonds between cell wall proteins and mannose residues on adjacent cell walls are the principal interactions that bring about flocculation in yeasts (7).

The combined effect of the medium components on flocculation

Four further measurements were carried out with known electrical conductivity of fresh and free-cell spent medium. The latter medium was either boiled for one hour or dialysed overnight. After the cells had been separated and washed twice, they were resuspended in such media, and the parameter R% determined immediately afterwards. Table 1 shows that a reduction in the electrical conductivity of the medium, through dialysis, diminishes the degree of flocculation, confirming that the ionic strength is a deciding factor in cell aggreggation. No tangible differences in the percentage of cells remaining in suspension were found when frash or spent medium (boiled or not) were used, which is certainly a reflexion of the higher electrical conductivity of these media.

TABLE 1 - Electrical conductivity of the medium and its relation to flocculation of *Pichia stipitis* CBS 5774 (CT~6.5 g/L); p = 6.0. The R values represent a mean of 3 measurements.

Medium	Elect. cond. (μS)	R (%)
Fresh medium	2680	23.4 +/- 1.6
Spent medium	1428	22.6 +/- 2.1
Boiled spent medium	1547	21.8 +/- 2.5
Dialysed spent medium	328	43.6 +/- 1.8

RESUMO

O caráter iônico no processo de floculação de *Pichia stipitis*

Embora células de uma linhagem floculante de *Pichia stipitis* tornaram-se completamente dispersas após lavagens com água destilada, essas mesmas células reflocularam quando suspensas em soluções de componentes individuais do meio. A força iônica de tais soluções foi suposta ser a causa primária do fenômeno. A medida da condutividade elétrica mostrou ser um modo prático de avaliar a força iônica do meio ambiente. A floculação foi mais intensa quanto maior o valor da condutividade elétrica das soluções, atingindo o seu ótimo e permanecendo praticamente constante para valores de condutividade elétrica acima de 1000 μS. Isto sugere que redução de cargas associada à mudanças conformacionais na estrutura das proteínas da parede celular tenham sido responsáveis pela agregação de células. Adicionalmente, uma diminuição da condutividade elétrica do meio, através de diálise, reduziu a habilidade das células de flocular. O carater iônico do meio mostrou ser um importante fator influenciador na floculação.

Palavras-chave: *Pichia stipis*, floculação, meio iônico.

ACKNOWLEDGEMENTS

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VIRULENCE OF *CANDIDA ALBICANS* "CLONES"

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SUMMARY

Lethal activity of blastospores and of intracellular and extra cellular extracts from yeast (Y) and micelial (M) growth forms, from five "clones" of different strains of *C. albicans*, selected on the basis of morpho-physiological and chromosomal characteristics, were studied in mice. The inoculation of $5x10^4$ blastospores from the different "clones" studied in mice did not kill the animals. When 10^6 blastospores from each "clone" was tested, the Ca 3.7 "clone" was the most virulent and Ca 5.5 the less virulent. The extracellular and intracellular extracts from the yeast (Y) phase of the Ca 3.7 "clone" killed all the animals just after inoculation. The extracellular extracts of the micelial (M) form of this "clone" were atoxic and the intracellular extract of the M form killed 67% of the animals. The intra or extracellular extracts from the Ca 5.5 "clone" was atoxic. SDS-PAGE analysis of the intra and extracellular extracts from the selected "clones" did not show a clear correlation with both virulence of the "clones" and toxicity of the extracts. When blastopores were added to mouse activated peritoneal macrophage cultures, the highest phagocytic index was observed with the Ca 5.5. The index of fungi killed by mouse peritonial macrophages was the same for Ca 3.7 and Ca 5.5 "clones".

Key Words: *Candida albicans;* "clones", virulence; cell extract, proteic fractions, toxicity.

INTRODUCTION

Among the species which cause candiosis *C. albicans* is considered the most virulent (26). The degree of virulence may vary from strain to strain (3) although some authors believe that such property may be unstable (11,27).

The biological determinants which confer virulence to *C. albicans* has been a matter of controversy. Adherence, dimorphism, toxin production, enzyme secretion, and certain cell wall components, all have been associated to virulence of the fungi (12,26). Nevertheless, the correlation between the pathogenicity of *C. albicans* strains and

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their cell and mollecular properties are not yet fully established (26).

Considering that strains of *C. albicans* may be constituted of heterogeneous populations (29), this work was undertaken in order to analyse the variations in the degree of blastospore virulence of selected "clones" of the parasite. A correlation between virulence and the intra and extracellular extract toxicity of the "clones" was demonstrated.

MATERIALS AND METHODS

Strains "Clones" Ca 1.2, Ca 2.4, Ca 3.7, Ca 4.10 and Ca 5.5, previously selected by morphophysiological and chromosomal characteristics from 5 different *C. albicans* strains (29,30), were used in this study.

Extracellular (EE) and intracellular (IE) extracts of the micelial (M) and yeast (Y) forms - A suspension of yeast cells (equivalent to nº 10 standard McFarland) were inoculated in 50 ml of Lee medium and incubated for 72 hs. The Y form was grown at 25°C and the M form at 37°C (21). Each culture was centrifuged and the cells respectively subcultured at 25°C and 37°C. To obtain the EE each "clone" culture sample was centrifuged for 15 min at 5,000 rpm. The supernatants were filtered by negative pressure (Seitz filter, 0.42 µm), concentrated under vacuum, dyalised and liophilized. The sediment of each sample was separated, washed twice with distilled water and used for the isolation of IE. The cells were suspended in 2 ml of water and transferred to a graal. In order to brake the cells, liquid nitrogen was added and the cells were broken with the use of a pistile. The resulting suspension was transferred to an Eppendorf tube and centrifuged at 10,000 rpm at 5°C for 30 min. and the supernatant liophilized.

Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis (SDS-PAGE) analysis of EE and EI - The proteic concentrations of M and Y from extracts were carried out by the Bradford method (8). An aliquote of each extract containing 20 µg of protein was diluted in sample buffer followed by the addition of 5% of B-mercaptoethanol (final concentration), and the solution warmed at 100°C for 3 min. The gel was prepared according to Laemmli (20) using as molecular mass standers, solutions of carbonic anidrase (29kDa), ovalbumin (45kDa) and serum albumin (66kDa). Gels were dryed and prepared according to Ansorge (5).

Animal Inoculation - Each "clone" was grown in Lee medium for 24-48h at 25°C, washed by centrifugation and suspended in saline at the concentrations of $5x10^4$ or 10^6 cells/300 µl. These cell concentrations, reported by Odds (26), respectively as the DL_{50} and DL for *C. albicans*, were inoculated (i.v.) in groups of 6 mice (NZW, 20-25g, with 30 days of age). Inoculated mice and control animals, were maintained under observation for 30 days.

Extract solutions (EE and EI from M and Y forms) with 25.5, 17, 8.5, 4.25 and 2.12 µg of protein/300 µl of saline were inoculated (i.v.) in 6 mice. Inoculated mice and the controls were maintained in observation for 30 days.

Phagocytosis - The ability of activated peritoneal macrophages to kill blastospores from the "clones" used was evaluated by the method of Kolotila et al. (19). Briefly, macrophages were obtained by inoculating i.p. 0.1 ml of 1% Concanavalin A (Sigma) solution. After 7 days, 3 ml of PBS were injected (i.v.) and the peritoneal cavity washed. The number of cells in the washing solution was adjusted to $9x10^5$ cells/ml. 30 µl of this cell suspension was placed on a round coverslips in a plastic plate of 12 wells. The cells were allowed to adhere to the glass for 15 min, the coverslips vigorously washed in PBS and maintained at 37°C in Eagle medium with 0.02 M Herpes buffer.

Yeasts were cultivated in YEPD for 24h at 25°C, the cells washed by centrifugation at 3,000 rpm for 10 min, and suspended in PBS. The cell suspension was adjusted to $2x10^6$ yeasts/ml. The yeasts were opsonized by mixing 1 ml of normal human serum, 1 ml of PBS and 1 ml of cell suspension. 0.1 ml of opsonized yeasts were added to the macrophage cultures and incubated at 37°C for 45 min. Then, 15 µl of neutral red solution (0.02 g/ml of PBS) was added to the plates and maintained at 37°C for 15 min. The coverslips were vigorously washed in PBS and mounted in the same solution for microscopic analysis. The phagocitic index was obtained by counting the percent of macrophages which phagocytised the yeast (25). The number of yeasts per macrophage and the number of stained yeasts (dead cells) were also evaluated.

RESULTS

Virulence of the "clones" - Inoculation of the yeast "clones" in mice resulted in different degrees of mortality of the animals, as shown in Ta-

TABLE 1 - Blastopores of "clones" arisen from different *C. albicans* strains, in NZW mice.

	$5x10^4$ cells/mice		10^6 cells/mice	
Clones	Death (nº)	Days (nº)	Death (nº)	Days (nº)
Ca 1.2	0/6	30	2/6	3
			1/6	5
Ca 2.4	0/6	30	2/6	5
			2/6	9
Ca 3.7	0/6	30	4/6	3
			2/6	4
Ca 4.10	0/6	30	2/6	7
			2/6	9
Ca 5.5	0/6	30	2/6	9

ble 1. The inoculation of $5x10^4$ blastospores per mouse did not kill the animals, but 10^6 resulted in the death of the animals in a different proportion in a period from 3 to 9 days. The "clone" Ca 3.7 was the most virulent killing all animals after 4 days of inoculation and the "clone" Ca 5.5 was the less virulent killing 2 out of 6 animals after 9 days of inoculation.

Proteic glycoproteic fractions of the extracts - In general, the number and the molecular mass of the proteic fractions varied according to the Y or M form of the "clone" and with the type of extract (Table 2). Although fractions with similar molecular masses were detected from the different extracts, the EI derived from any one of the "clones" presented a number of proteic fractions (21 to 34) superior to EE (5 to 8).

Extract toxicity - The EE derived from M forms of any of the "clones" did not cause death of the animals until 30 days after inoculation. For the other extracts, the results varied with the "clone" and its M or Y forms. The EE and EI from Y form of Ca 3.7 caused an anaphilatic type reaction and the death of all animals immediately or during the inoculation. The least toxic extract was obtained from the "clone" Ca 5.5 (Table 3).

Blastospore phagocytosis and killing by peritoneal macrophages - The phagocytic index varied from "clone" to "clone". The highest index was obtained with yeasts from the "clone" Ca 5.5 and the lowest from the "clone" Ca 1.2. The phagocytic index for the "clone" Ca 3.7 was intermediary (Table 4). The susceptibility of yeasts from different "clones" to be killed by peritoneal macrophages is shown in Table 5. Cells from the "clone" Ca 5.5 were phagocytised in a larger number. This property did not correlate with the capacity of macrophages to kill the yeasts.

DISCUSSION

Our results confirm and extend previous observations concerning the heterogeneity of the populations of *C. albicans* strain (29).

In previous studies 4 different profiles of chromosomal DNA were traced for the 5 "clones" here analysed (30). Ca 3.7 and Ca 4.10, which presented similar DNA profiles, were serotype A, producers of germ tube and secreted proteinases. Ca 3.7 but not Ca 4.10 was able to produce phospholipases (29). As shown, these "clones" were notably different concerning their blastospore virulence to mice. On the other hand, the Ca 4.10 "clone" which differs from Ca 2.4 in their chromosomal profile (30) and serotype (29), originates blastospores with identical virulence as that of Ca 2.4 "clone". Nevertheless, the Ca 4.10 which has similar serotype and germ tube production to Ca 1.2, needed almost twice the time required by the blastospores of Ca 1.2 to kill one more animal. The "clone" Ca 5.5 also presented morpho-physiological characteristics similar to those of Ca 3.7, except for their serotype. Considering the results obtained with the blastospores inoculation, the Ca 5.5 can be considered the less virulent among the "clones", and Ca 3.7 as the most virulent.

Based on these results the degree of virulence of the "clones" could be directly related to the serotype, although it is largely reported in the literature that *C. albicans* virulence is associated to the serotype of the strain. Differences between strain serotypes A and B have been associated to virulence (1,2,3,6,13). However, comparing the serotypes of the "clones" here studied, it was not possible to correlate the serotypes and degree of virulence of the different "clones".

Other characteristic related to virulence is the exoenzymes. The "clones" Ca 1.2 and Ca 4.10, both serotype A as the "clone" Ca 3.7, had different patterns of exoenzymes. The "clone" Ca 1.2 did not secrete proteinases but secreted phospholipases. The "clone" Ca 4.10 secreted proteinases but not phospholipases (29). When these two "clones" were compared in view of their virulence, both were less virulent than the Ca 3.7 which secrets both proteinases and phospholipases. These results also show that also exoenzyme secretion can not be directly related to virulence of the "clones".

Another characteristic associated with virulence is the production of a germ tube (9,31), which seems to turn the yeast more invasive in the host tissue (4,14,18). Our results could not demonstrate a

TABLE 2 - Esquematic representation of the apparent molecular weight of the intra (IE) and extracellular extracts (EE) of the Y and M forms of *C. albicans* analysed by SDS-PAGE.

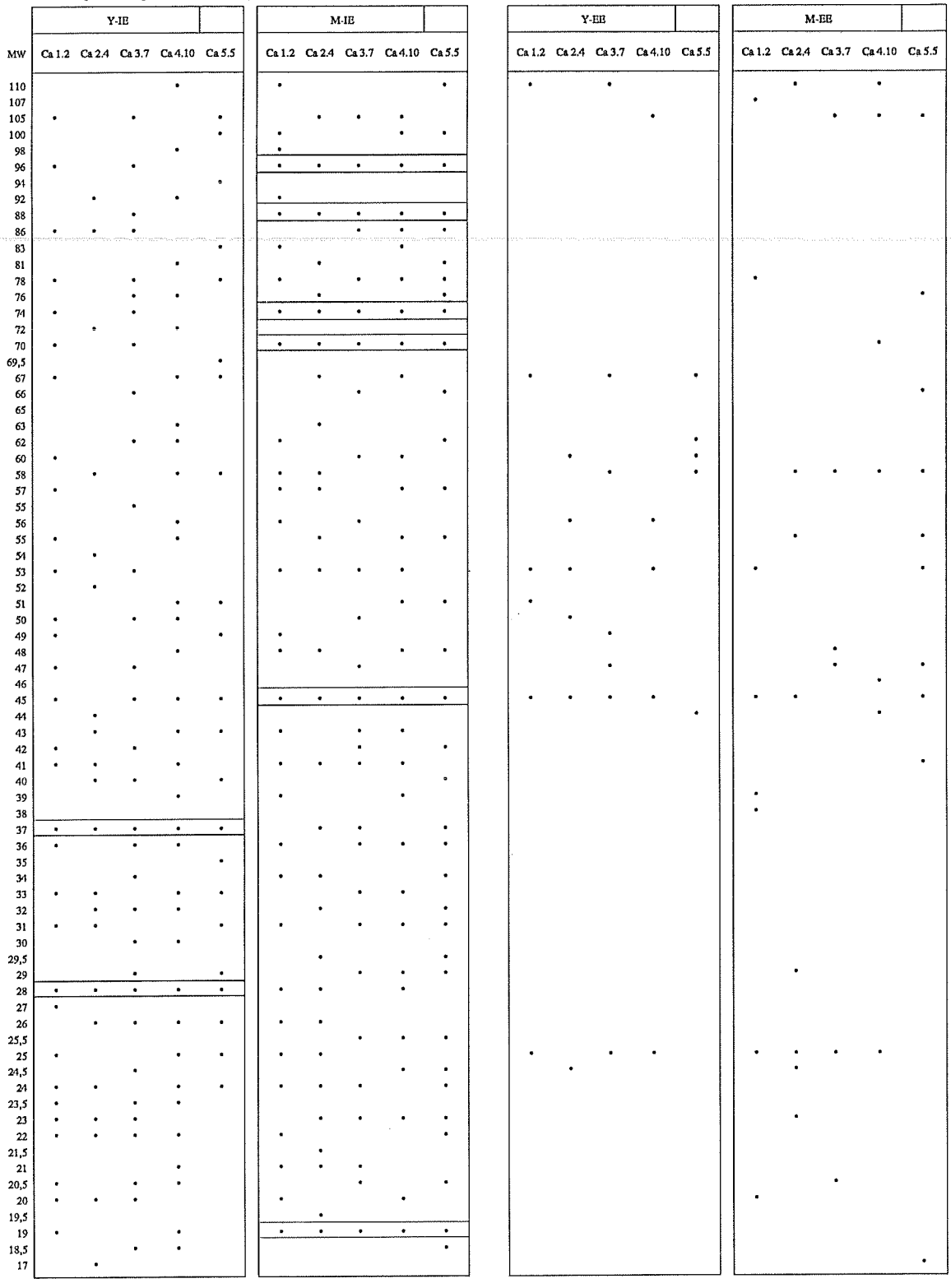

TABLE 3 - Extracellular (EF) and intracellular (EI) extracts inoculation of micelial (M) and yest (Y) forms of *C.albicans* "clones" in mice.

	EE				IE		
	M		Y		M		Y
Clones	Death (nº)	Time hours	Death (nº)	Time hours	Death (nº)	Time hours	Death (nº)
Ca 1.2	0/6	-	2/6	24	0/6	-	-
Ca 2.4	0/6	-	0/6	-	4/6	0	-
Ca 3.7	0/6	-	6/6	0	4/6	24	0
Ca 4.10	0/6	-	4/6	24	2/6	0	-
Ca 5.5	0/6	-	0/6	-	0/6	-	-

TABLE 4 - Phagocytic macrophage index to *C. albicans* "clones".

Clones	(%) Macrophages that phagocytose	Mean of yeast per macrophage	Phagocytic index
Ca 1.2	17.25	2.35	40.53
Ca 2.4	19.75	2.4	47.4
Ca 3.7	28.5	2.1	59.85
Ca 4.10	38.0	2.0	76.0
Ca 5.5	43.75	2.2	96.25

TABLE 5 - Phagocytosis of *C. albicans* "clones" in vitro.

Clones	(%) Macrophages that phagocytose	(%) Macrophages with live cells	(%) Macrophages with dead cells	(%) Macrophages with live/dead cells
Ca 1.2	17.25	3.75	8.5	5.0
Ca 2.4	19.75	3.5	10.75	5.5
Ca 3.7	28.5	9.75	6.75	12.0
Ca 4.10	38.0	11.0	7.75	19.25
Ca 5.5	43.75	13.0	6.75	24.0

correlation between germ tube formation and virulence since, even the less virulent "clone", the Ca 5.5, which did not kill mice produce germ tubes.

The virulence of *C. albicans* strains has also been associated to endotoxins, characterized as glicoproteins and acid proteins isolated from the fungi cytoplasm (16,17,28). Anaphilatic shock, among other type of reactions, has been described to occur in animals inoculated with intracelular extracts of the yeast or with purified toxins (10,15,16,22).

C. albicans exotoxins with low toxicity have also been reported (7,24,32). Our data corroborates these observations since the EE obtained from the M forms of the "clones" was unable to kill inoculated mice. Nevertheless, a high level of toxicity was detected when the EE obtained from the Y forms tested. EE from the Y forms were tested. EE from the Y forms of the "clones" Ca 1.2, Ca 3.7 and Ca 4.10 killed the mice. The EE extract from the Y form of the "clone" Ca 3.7, the most toxic, was able to kill all mice during or immediately after inoculation.

The IE of the M and Y forms of the "clones" showed different degrees of toxicity. The IEs from M forms of "clones" Ca 2.4, Ca 3.7 and Ca 4.10 showed a certain degree of toxicity. However, only the IE from the "clone" Ca 3.7 killed all the inoculated animals. These results show distinct characteristic of the "clone" Ca 3.7 which directly correlates with the high virulence of blastospores of this "clone" when compared with the other "clones".

Our results clearly demonstrate that different "clones" of *C. albicans* show different degrees of virulence although the different parameters related as virulence factors (12,23,26) were not able to explain these differences. It becomes clear that the "clone" Ca 3.7 must be reevaluated since it showed a strick correlation between blastospore virulence and IE toxicity.

The relationship between the fagocytic and killing indexes of *Candida* cells by activated peritoneal macrophages and fungi toxicity could not be established. Nevertheless, the less virulent "clone", the Ca 5.5, showed the highest index of phagocytosis but the lowest index of killing. The highest index of killing was observed with the "clone" Ca 2.4 which showed an intermediary degree of virulence "in vivo". The "clone" Ca 3.7, the most virulent "in vivo", showed an intermediary index of phagocytosis and killing of the *Candida* cells.

SDS-PAGE analyses of the intra and extracellular extracts showed marked differences. Intracellular extracts presented a higher number of components than those obtained with EEs. The analysis of these results did not allow the characterization of the degree of virulence of the selected clones.

Based on the results herein obtained, it is evident that further studies are needed in order to point out with security the factors associated to the different degrees of virulence of *Candida albicans*.

RESUMO

Virulência de "clones" de *Candida albicans*

Com base em características morfo-fisiológicas e cromossomais foram selecionados cinco "clones"

oriundos de diferentes cepas de *Candida albicans*. Por meio de inoculações em camundongos investigou-se a atividade letal de blastósporos e de extratos intra e extracelulares das fases leveduriforme (Y) e micelial (M) dos cinco "clones". A inoculação de $5x10^4$ blastósporos de qualquer dos "clones" estudados não foi letal para os animais. Por outro lado, a inoculação de 10^6 blastósporos mostrou que o "clone" Ca 3.7 era o mais virulento, enquanto que o Ca 5.5 era o menos virulento. Os extratos intracelular e extracelular da fase Y do "clone" Ca 3.7 foram letais para todos os animais, logo após a inoculação. Entretanto, o extrato extracelular da forma M deste "clone" não mostrou toxicidade, enquanto que, o extrato intracelular matou 67% dos animais. Os extratos oriundos do "clone" Ca 5.5 não apresentaram toxicidade. A análise dos extratos dos diferentes "clones" por SDS-PAGE não mostrou ocorrência de uma nítida associação entre virulência dos "clones" e a toxicidade dos extratos. A partir do estudo com macrófagos "in vitro" obteve-se para o "clone" Ca 5.5 o maior índice de fagocitose. Entretanto, a percentagem de macrófagos com células mortas foi a mesma para os "clones" Ca 3.7 e Ca 5.5.

Palavras-chave: *Candida albicans*, virulência, "clones", estratos celulares, frações protéicas, toxicidade.

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IN VITRO INHIBITION OF SPORES GERMINATION OF *ALTERNARIA SOLANI* BY THREE FUNGICIDES

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SUMMARY

In vitro inhibition of the of spores germination of *Alternaria solani* by iprodione, chlorothalonil, and anilazine at different dosages was studied. The highest concentration of active ingredient studied for each fungicide was equivalent to that recommended for the control of the early blight, under field conditions: 0.75; 1.80 and 1.44 g, respectively, of iprodione, chlorothalonil and anilazine per litre of water. A series of two-fold diluitions of each original concentration was studied in additional nine experiments. Eah of the three fungicides showed total in vitro spore inhibition at the highest rate, at six hours of incubation. At nine hours, only analazine mantained its full inhibition activity. The inhibition activity of iprodione decreased suddenly after 1/2 dilution, so that at the 1/8 dilution a total loss of inhibitory activity was observed. Chlorothalonil showed a progressive and slighter decrease of its activity as the dilution rate increased.

Analizine showed a high inhibitory activity at higher dilutions, without any loss up to 1/128 dilution. Even at 1/512 dilution, its activity was so high that only 20% of spore germination was observed at six or nine hours of incubation.

Key words: Alternaria solani,spore germination, fungicides, in vitro inhibition.

INTRODUCTION

The early blight caused by *Alternaria solani* (Ellis & Martin) Jones & Grout is one of the most common fungal diseases in tomato and potato crops. In tomato, early blight signals occur on leaves, stems and fruits, causing up to 46% of yield losses (4). The primary inoculum source is constituted by the crop debris, from where the conidia produced are wind disseminated to new tomato plants and potato crops. Under high moisture conditions, darkness, and temperatures between 24°C and 34°C, conidia germinate on the host surface and the fungus penetrates by means of an appressorium (8, 9).

Usually, the early blight is controlled preventively by applying one of the following chemicals; maneb, mancozeb, iprodione, chlorothalonil, or copper fungicides.

"In vitro" studies on inhibition of *A. solani* spores germination can be important in the previous chemical screening as fungicides for disease

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control. Specific culture media (5) and light conditions required for sporulation of *A. solani* make these in vitro studies. Satisfactory sporulation, however, can be obtained by the use of the method recently proposed, with alternated dark/light conditions, UV light use, and the addition of CaCO3 in the culture medium (7).

The objective of the present study is the determination of the in vitro inhibition activity of the *A. solani* spores germination by each on of the three fungicides: ipridione, chlorothalonil and anilazine.

MATERIALS AND METHODS

Ten different experiments were carried out to study the inhibitory effect of each one of the following chemicals: iprodine, chlorothalonil, and anilazine, on the *A. solani* conidial germination. Deionized water was used as the control treatment. In the first experiment, concentrations as active ingredients (a.i.) equivalent to those used for field control (3) of the disease 0.75g of iprodione; 1.80g of chlorothalonil and 1.44g of anilazine per litre of water were studied. In the subsequent nine experiments, rates were reduced to 1/2; 1/4; 1/16; 1/32; 1/64; 1/128; 1/256; 1/512.

One tenth (0.1ml) of a conidial suspension containing 50 to 100 conidia per microscopic field, were added to 1.0ml of each fungicidal water solution in hemolysis tube. The spore suspension was obtained by brushing the fungal growth in PDA-$CaCO_3$ medium in which micelia were submeted at alternated dark/V.V. light condition (5,7) after flooding the Petri dish surface with 3ml of deionized water. The *A. solani* isolate was obtained from tomato leaf lesions, in PDA the same used as stock culture medium.

In every experiment the randomized block experimental design was utilized with four treatments (three fungicides and one control) replicated three times. Spore germination was assessed at six and at nine hours of incubation at 27°C, by examination of five microscopic (100 x) fields on the slide for each replication. The percentage data obtained, were transformed in arc. sen $\sqrt{p100}$ for statistical analysis purpouses (1).

RESULTS AND DISCUSSION

At the highest rate, each of the three fungicides inhibited spore germination at six hours of incubation (Figure 1), but at nine hours, only anilazine determined total inhibition. At this time, 3%; 18% and 94% of germination were observed in iprodione, chlorothalonil, and control treatments (Figure 2).

Iprodione showed to be effective up to the 1/2 of the rate recommended for field control of the disease at six hours of incubation (Figure 1). Its inhibitory capacity suddenly decreased at dilutions tigher 1/2, so that at the 1/8 dilution a total loss of inhibition capacity occurred as well as at six and at nine hours of incubation (Figures 1 and 2).

Chlorothalonil showed a decrease in its spore germination inhibitory capacity from 1/2 dilution,

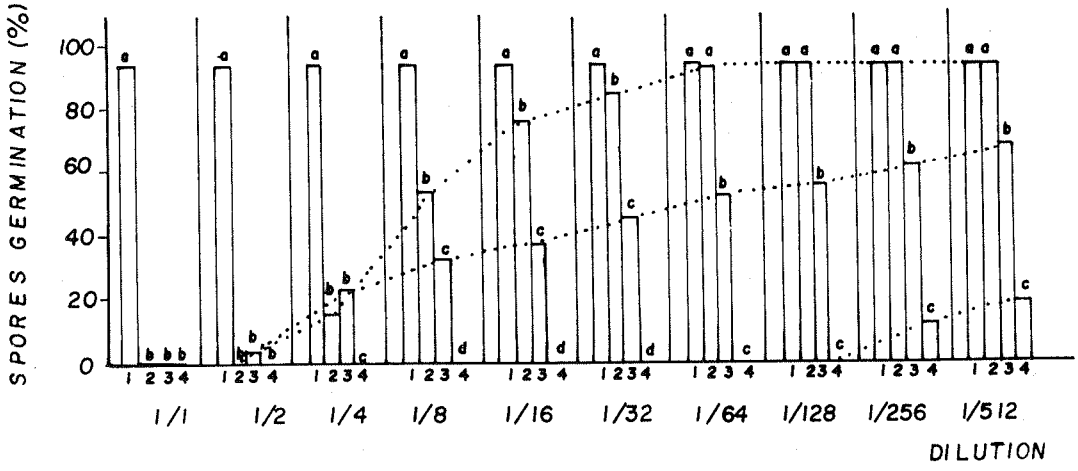

FIGURE 1- In vitro germination of the *Alternaria solani* spores in three fungicides water solution (1- control; 2- iprodione; 3- chlorothalonil and 4- anilazine) at differtent rates (1/1 equivalent to the rates for field control up to dilution 1/512), after six hours of incubation at 27°C. Bars indicated by the same letter do not differ by Tukey's test, 0.05.

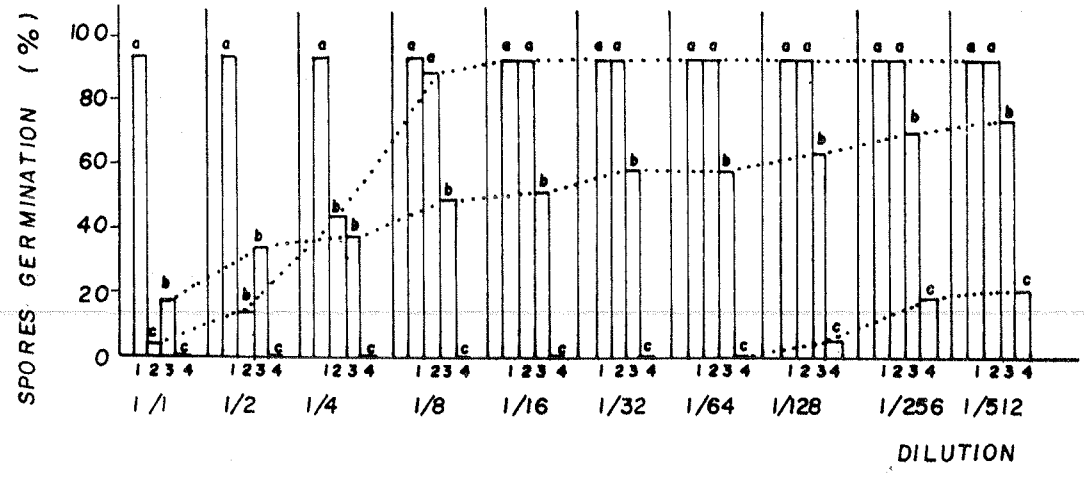

FIGURE 2 - "In vitro" germination of the *Alternaria solani* spores in three fungicides water solution (1- control; 2- iprodione; 3- chlorothalonil and 4- anilazine) at different rates (1/1 equivalent to the rates for field control up to dilution 1/512) after nine of incubation at 27°C. Bars indicated by the same letter do not differ by Tukey's test, 0.05.

but the loss of inhibition activity was progressive and in some instances slighter than that of iprodione (Figures 1 and 2).

Anilazine maitained its inhibitory capacity up to 1/28 dilution, at six hours of incubation. Even at the dilution of 1/256 or 1/512 its inhibitory activity was so high as only 20% of germination was observed, at six or nine hours of incubation (Figure 2).

Since anilazine showed a high inhibitory activity at higher dillutions, it has a high potential as a chemical for early blight control.

The results obtained show that the "in vitro" evaluation of the inhibition of the spores germination can be utilized only as an indication of the fungicide effect on the control of the tomato early blight. In field conditions other factors such as environmental conditions and application techniques can interfere on the fungicidal effect. So, in this paper, anilazine showed to be the most effective chemical "in vitro" conditions, but, in field conditions, according to Chupp & Sherf (2) iprodione, chlorothalonil and anilazine do not differ in the control of the potato early blight, and according to Fraire Mora (6), anilazine is less efficient than chlorothalonil.

RESUMO

Inibição "in vitro" da germinação de esporos de Alternaria solani por três fungicidas

Foram realizados 10 experimentos em condições de laboratório, utilizando-se isolados selvagens locais do fungo *Alternaria solani* obtidos de folhas de tomateiro. O primeiro experimento consistiu na inoculação de esporos em soluções dos fungicidas seguintes, cujas concentrações estão expressas em ingredientes ativos (i.a.): iprodione 0,75g/l; chlorothalonil 1,80g/l e anilazine 1,44g/l. Outros nove experimentos foram realizados, concomitantemente, mas reduzindo-se as concentrações para: 1/2, 1/4, 1/8, 1/32, 1/64, 1/128, 1/256 e 1/512. Essas diluições foram feitas em tubos de hemólise. Em todos os casos, foi incluído um tratamento testemunha, com água deionizada. Os cultivos experimentais foram mantidos em ambiente de 27°C. As avaliações consistiram na determinação da porcentagem dos esporos germinados em 5 campos microscópicos (aumento de 100 x) realizadas 6 horas e 9 horas após a inoculação dos esporos em cada solução de fungicida, sendo os dados transformados em arc. √sen p/100 para o tratamento estatístico.

Na maior concentração, todos os fungicidas inibiram a germinação de esporos de *A. solani*, nas avaliações realizadas 6 horas após a inoculação. Nas avaliações de 9 horas constatou-se que, apenas, o fungicida anilazine foi capaz de inibir completamente a germinação dos esporos, enquanto que nos demais tratamentos as porcentagens de germinação foram de 3%, 18% e 94% respectivamente para iprodione, chlorothalonil e água. Nos experimentos com menores concentrações dos fungicidas constatou-se significativos aumentos nas porcentagens de germinação de esporos, exceto para anilazine, que manteve ini-

bição até a diluição 1/64 (na avaliação de 9 horas após inoculação).

Palavras-chave: *Alternaria solani*, germinação de esporos, fungicidas, inibição "in vitro".

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PRODUCTION OF STAPHYLOCOCCAL ENTEROTOXIN A IN WHITE CHEESE

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ABSTRACT

White cheese has been involved in many staphylococcal food poisoning outbreaks in Minas Gerais State, Brazil. Experiments were conducted to determine at what stage in the making of white cheese is the enterotoxin produced that is the cause of staphylococcal food poisoning. Pasteurized milk was inoculated with 10^2 and 10^4 cfu/ml of an enterotoxin A-producing *Staphylococcus aureus* strain and incubated at 26°C-27°C for varying lengths of time before making the cheese. The milk, samples of the curd, and day-old cheese were used for determining staphylococcal count and the presence of enterotoxin. No enterotoxin was detectable in the cheese made from the milk inoculated with 10^2 cfu/ml. Enterotoxin was detectable in the cheese curd made from the milk inoculated with 10^4 cfu/ml after it had been held for 5 hours and was present in all cheese samples.

Key words: cheese, milk, staphylococci, enterotoxin, food poisoning.

INTRODUCTION

Staphylococcal food poisoning is a common foodborne disease that occurs in most countries of the world. The incidence of this disease is not known because it is relatively mild and most cases are not reported to health authorities. The vomiting and diarrhea that usually accompanies it lasts only a few hours to one day, hence, most people who are affected never seek medical attention (1). This type of food poisoning has come to the attention of public health authorities in Brazil in recent years and a number of outbreaks have been investigated. One of the most common items of food involved is white cheese, usually the home-made or small producer variety (3). Although the commercially manufactured Minas cheese could also be a source of staphylococcal food poisoning (8), it is less likely to be involved.

This research project was undertaken to demonstrate the probable conditions leading to the involvement of white cheese of the small producer variety in staphylococcal food poisoning.

MATERIALS AND METHODS

Staphylococcus aureus. The *Staphylococcus aureus* strain used in this study was FRI-100, a strain that had been isolated from a food poisoning

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outbreak in the United States of America. It produces enterotoxin A (SEA), the enterotoxin most commonly involved in staphylococcal food poisoning (10).

Preparation of inoculum-Strain FRI-100 was grown in brain heart infusion (BHI) broth at 37°C for 24 hours. The culture was used to inoculate slants of agar Lignières inoculated with the BHI broth culture were incubated at 37°C for 24 hours. One slant was used to inoculate two tryptic soy agar slants which were incubated at 37°C for 24 hours. Buffered peptone water (7 ml for each slant) was used to remove the cells from the slants. One ml of the cells suspension was diluted with 9 ml of the buffered peptone water and 1 ml was used to inoculate each of two plate count agar plates. Ten-fold dilutions from the first tube were made as necessary to record the cfu count.

Production of the white cheese-Each of 10 L of pasteurized milk was contaminated with 10^2 and 10^4 cfu/ml of *S. aureus* strain FRI-100. The milk was incubated at room temperature, 26° - 27°C. At time intervals of 0, 4, 7 and 10 hours for the milk inoculated with 10^2 cfu/ml and 0, 2.5, 5, and 7.5 hours for the milk inoculated with 10^4 cfu/ml, 2.5 L of milk was taken for making the cheese and a sample of milk was taken to be analyzed. The milk was warmed to 35°C in a double boiler followed by the addition of 0.8 ml of a 50% $CaCl_2$ solution, 25 ml of a lactic ferment, 12.5 g NaCl, and 110 mg of rennet powder. After 40-50 minutes of incubation at 35°C for coagulation, the coagulum was cut with stainless steel knives. The coagulum was allowed to stand for 5 minutes and then was mixed for 20 minutes at the same temperature. A sample of the coagulum was taken for analysis. After a second curd mixing for 5 minutes at 40°C, the curd was placed into round hoops of 500 g capacity for molding. After 20 minutes the cheese was salted with 15 g of NaCl. The cheese remained at room temperature 1 to 1.5 hours after which the cheese was put in a sterilized flask and put in the refrigerator at 6°C. A sample was taken for analysis on the following day.

Determination of staphylococcal count. Twenty-five grams of the cheese was suspended in 225 ml of buffered peptone water; 0.1 ml was placed on the Baird-Parker agar plates. Additional plates were prepared with 10-fold dilutions if necessary (9). The plates were incubated for 48 hours at 37°C.

Enterotoxin detection in the cheese: 50 g of cheese was homogenized in a Waring blender with 1-1.5 ml of 0.02 M $NaHPO_4$ in saline, pH 7.4, per gram of cheese(6). The homogenized cheese was adjusted to pH 4.5 and centrifuged. The pH of the supernatant fluid was readjusted to pH 7.4 and recentrifuged if any precipitate occurred. The extract was tested for the presence of enterotoxin by use of the ELISA ball kit obtained from Dr. Bomelli AG, Stationstrasse 12, CH-3097 Libbefeld-Bern, Switzerland (5). This method is sensitive to 0.5 ng/ml and is the most reliable of the sensitive methods available for checking foods for enterotoxin. One antibody-coated ball for enterotoxins A-D plus two balls coated with normal rabbit sera were placed in 20 ml of food extract and mildly shaken overnight. Each ball was removed from the extract and washed with the wash solution and each placed in a color coded tube for treatment with the conjugate. After 6 hours the conjugate was removed and the balls washed with the wash solution. One ml of the substrate was added and the color allowed to develop for 60 minutes. If any color developed, the extract was judged to contain the enterotoxins for which a color developed.

TABLE 1 - Staphylococcal growth and production of enterotoxin in white cheese produced with pasteurized milk inoculated with 10^2 cfu/ml.

	Time of milk incubation (hours)[a]							
	0		4:00		7:00		10:00	
Sample	cfu	SEA	cfu	SEA	cfu	SEA	cfu	SEA
Milk[b]	10^2	-	$9.7x10^2$	-	$1.0x10^4$	-	$9.3x10^4$	-
Curd[c]	$2.5x10^4$	-	$4.1x10^4$	-	$4.0x10^5$	-	$3.0x10^5$	-
Cheese[d]	$1.2x10^5$	-	$3.8x10^5$	-	$3.1x10^5$	-	-[e]	-

a - Time of incubation at 27°C after inoculation
b - Milk not analyzed for count after inoculation
c - Sample taken after first mixing of curd
d - Sample taken after cheese was stored overnight at 6°C
e - Count impaired by the presence of other organisms

RESULTS

Staphylococcal counts- The staphylococcal counts in the cheese are given in Table 1 and 2. In all cases where enterotoxin was detectable the count had reached approximately 10^6 cfu/g.

Presence of enterotoxin-. In almost all instances when the staphylococcal counts reached 10^6, SEA was detectable in the cheese (Table 2).

Change in pH during cheese making- The change in the pH during the cheese making is presented in Table 3.

TABLE 2 - Staphylococcal growth and production of enterotoxin in white cheese produced with pasteurized milk inoculated with 10^4 cfu/ml.

	Time of milk incubation (hours)[a]							
	0		2:30		5:00		7:30	
Sample	cfu	SEA	cfu	SEA	cfu	SEA	cfu	SEA
Milk	$4.1x10^4$	-	$5.0x10^5$	-	$1.3x10^5$	-	$2.2x10^5$	-
Curd[b]	$8.1x10^4$	-	$3.3x10^5$	-	$7.8x10^5$	+	$1.3x10^7$	+
Cheese[c]	$1.3x10^6$	+	$3.1x10^6$	+	$7.8x10^6$	+	$1.0x10^7$	+

a - Time of incubation at 27°C after inoculation
b - Sample taken after first mixing of curd
c - Sample taken after cheese was stored overnight at 6° C

TABLE 3 - Change in pH during the making of white cheese.

	Time of milk incubation (hours)[a]			
Sample	0	4:00	7:00	10:00
Milk[b]	-	6.5	6.3	5.7
Curd[c]	6.2	6.0	5.6	5.2
Cheese[d]	5.2	5.4	5.4	5.2

a - Time of incubation at 27°C after inoculation
b - Milk inoculated with 10^2 cfu/ml
c - Sample taken after first mixing of curd
d - Sample taken after cheese was stored overnight at 6° C

DISCUSSION

Two possibilities exists for the presence of enterotoxin in white cheese. One is that it is present in the milk used to produce the white cheese. Staphylococci was shown to be quite common in raw milk used for cheese making in Minas Gerais (7), hence, for enterotoxin to be present the staphylococci would have to grow in the raw milk. This is unlikely because unless the staphylococcal count is very high, the staphylococci will not compete with the other organisms present. The other alternative is that the milk was pasteurized and recontaminated before the cheese making, although apparently not all pasteurized milk is free of staphylococcal count (7). The fact that SEA, produced by human strains, is the enterotoxin responsible for most of the food poisoning outbreaks indicates human contamination. In one outbreak, three individual cheeses produced on successive days were all contaminated with staphylococci that produced SEA, and the presence of SEA in the cheese indicated human contamination during the production of the cheese (4).

If the contamination occurred immediately before the addition of the starter culture, it is unlikely that sufficient growth could take place to produce enterotoxin before growth was inhibited by the starter culture. Growth would occur only if the starter culture was not working properly, which could be a contributing factor (8). The fact that the pH is gradually lowered during the cheese making (Table 3) is inhibitory to staphylococcal growth. The other possibility is that the milk was allowed to stand at room temperature for several hours before the cheese was produced. In this case, as was demonstrated, sufficient growth occurred in the milk that only small additional growth was necessary during the early stages of the cheese making for enterotoxin to be produced. This is the most likely sequence of events leading to the production of enterotoxin and food poisoning for those consuming the cheese.

There is always the possibility that milk from a mastitic animal would contain sufficient staphylococci to overcome the presence of other contaminating organisms to grow and produce enterotoxin. In this case even if the milk were pasteurized, the enterotoxin is quite heat stable and survives pasteurizing. However, in the case of contamination from the cow, the enterotoxin produced would be either enterotoxins C or D, not A.

The milk used in the production of the white cheese should be kept refrigerated at all times, if

this is possible, except when the cheese is being produced. Keeping the milk refrigerated prevents the staphylococci from growing, hence, insufficient growth to produce enterotoxin would take place after the beginning of the cheese production. Essentially all staphylococcal food poisoning outbreaks are a result of human contamination during the preparation of the food. Many people are colonized with enterotoxigenic staphylococci and many infections on the hands and arms are caused by staphylococci (2). It is quite difficult to prevent the contamination of food with staphylococci. The best control of this type of food poisoning is refrigeration as staphylococci do not grow well in the cold 1. The major problem is that the average food handler does not realize that foods that can support the growth of staphylococci should be refrigerated at all times except when being prepared and served.

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RESUMO

Produção de enteroxina estafilocócicas em queijo branco

Queijo Minas tem sido um alimento envolvido em muitos surtos de toxinfecção alimentar em Minas Gerais. Foram conduzidos experimentos para determinar em qual estágio da fabricação do queijo Minas frescal a enterotoxina que cause este tipo de intoxicação é produzida.

Leite pasteurizado utilizado na fabricação do queijo foi inoculado com 10^2 e 10^4 U.F.C./ml de uma linhagem de *Staphylococcus aureus* produtora de enterotoxina A e incubado a 26 - 27°C por variados períodos de tempo, antes do preparo dos queijos. Amostras do leite do coágulo e do queijo foram utilizadas para determinar a contagem de *S. aureus* e a presença de enterotoxina A. A enterotoxina não foi detectada no queijo preparado com leite que tinha sido inoculado com 10^4 U.F.C./ml após ter sido mantido por 5 horas a 27°C e estava presente em todas amostras de queijo.

Palavras-chave: queijo, leite, estafilococos, enterotoxina, toxinfecção alimentar.

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EVALUATION OF DIFFERENT MEAT PEPTONES AS CULTURE MEDIA FOR BRUCELLA ABORTUS 19 VACCINE PRODUCTION

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Ernani Tadeu Ramos
Maria Kristine Giannoulakis

SUMMARY

Four different commercial brands of peptones were used for the culture of *Brucella abortus* Strain 19. A comparative trial with peptones of four different commercial brands using fermentation procedures revealed that there was significant variation in the growth of *Brucella abortus*. One of the four brands tested gave rise to a enhanced bacterial growth.

Key Words: *Brucella abortus* 19; vaccine production; culture media

INTRODUCTION

Live vaccines incorporating *Brucella abortus* Strain 19 are widely used throughout the world to control clinical brucellosis in cattle. Because the vaccine contains high numbers of organisms WHO, 1970 (9), industrial vaccine production requires the growth of large quantities of *Brucella abortus* Strain 19.

Meat peptones (enzyme hydrolyzed proteins of animal tissues) are incorporated in most culture media as the main source of protein nitrogen.

The use of dehydrated peptones has greatly facilitated the growth of *Brucella* sp Gerhardt,1958 (6). However, some authors found that some dehydrated peptones could be toxic to *Brucella* sp Schuhardt et al, 1949 (8). Others concluded that some types of peptones may adversely affect the agglutination of the bacteria Carrière et al, 1951 (3).

Peptones are one of the major components in the cost of production of *B. abortus* S19 vaccine. Most of the laboratories producting this vaccine in Brazil use imported dehydrated peptones which greatly increase the costs of production. When Martinez et al (1983) (7) compared media incorporating imported peptones with those containing peptones produced locally for the growth of *Clostridium perfringens*, they found that the local products gave the best yields.

In order to ascertain the most appropriate conditions to grow *B. abortus* S19 for vaccine production, an evaluation of meat peptones of four different sources, two imported and two locally produced, was carrid out. This paper describes the results of these trials.

MATERIAL AND METHODS

Culture medium - The medium was prepared with the following formula: meat peptone (5.41g), casein peptone (16.25g), yeast extract (8.0g), bacto dextrose (24.0g), monosodium phosphate (3.16g), disodium phosphate (1.06g), distilled water (1 litre).

The original formulation used two peptones, one obtained by enzymatic digestion of animal tis-

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sue (meat peptone) and other from casein. For this study only the former varied. Four industrially produced peptones of animal origin were compared. They were: Primatone (Sheffield, Norwich-USA), Bacto Peptone (Difco, Detroit-USA), Peptona de Carne (Biobrás, Montes Claros-Brazil) and Peptona Bacteriológica (Geyer, Porto Alegre-Brazil). For the purpose of this study the following identification were used for each brand of peptone: Sheffield = HS, Difco = US, Biobrás = MC, and Geyer = PA.

Three different batches of each product were tested with four repetitions and used in the production of four lots of vaccine. Media containing one of the peptones under test was prepared in sufficient quantity for preparing the four lots of vaccine. Each week one lot of media was seeded with *B. abortus* S19 and the cultures were evaluated as described below. The following month the process was repeated another randomly selected brand of peptone. Altogether 48 lots of culture media were prepared.

Vaccine production - The vaccines were produced in a New Brunswick Microferm fermentor (New Brunswick, N.J. - USA), containing 11 litres of media. The inoculum was prepared from ampoules of freeze dried *B. abortus* S19, kindly supplied by the Panamerican Zoonoses Centre (PAHO - Argentine).

Briefly, each week seven tubes of Tryptose Agar (Difco, USA) were seeded with *B. abortus* S19. After incubation, the cultures were suspended in sterile saline to obtain the inoculum with which the fermentor was seeded. Every suspension was tested for purity and smoothness.

Fermentation procedures were those described by Carrillo and Arce (1973) (4) for the production of *Brucella melitensis* Rev 1 vaccine, except that an incubation period of 52 hours was used.

Samples were collected aseptically at 0,4,8,24,32,48 and 52 hours of culture and subjected to the following tests: counts for number of colony forming units per mililitre (nCFU), optical density (OD), percentage of packed cell volume (PCV), pH, bacterial dissociation and purity, according to Alton et al 1988 (1).

The optical density was measured using a Coleman Jr. spectrophotometer, model 6/20 (USA) at 550 nm. The samples were diluted in 10% fromaldehyde and the readings multiplied by a factor dependent on the stage of incubation. The total OD was then corrected using the diluting factor. Results were expressed on an absorbance scale.

Statistical analysis - Mean values for nCFU, OD, PCV and pH were calculated for each stage of incubation from the results of the twelve cultures produced with each brand of peptone.

The significance of the differences of the means for nCFU was tested by the method of Newman-Keuls (Elliot, 1988) (5). Analysis of linear regression b1etween the nCFU and OD was carried out according to Ayres & Ayres Jr. (1987) (2).

RESULTS AND DISCUSSION

Means for OD, PCV, nCFU for each brand of peptone are shown in Table 1. Increasing the fermentation period from 48 to 52 hours did not significantly increase the number of cells obtained and when peptones US and PA were used, there was a decrease in the nCFU during this period.

There were not significant differences in the nCFU in the first 24 hours of fermentation among media prepared with different peptones. At 28,32,48 and 52 hours there were significantly higher counts in media prepared with peptone MC than in the other three (Table 2). These gave similar results except at

TABLE 1 - Mean for optical density, packed cell volume and number of colony forming units at various intervals during culture of *Brucella abortus* S19 in medium with peptones HS, US, MC and PA.

Hours	Optical density (absorbance)				Packed cell volume (%)				Colony forming units ($x10^9$/ml)			
Incubation	HS[a]	US[b]	MC[c]	PA[d]	HS	US	MC	PA	HS	US	MC	PA
24	1.4	1.6	1.6	1.5	0.6	0.7	0.8	0.7	14.9	19.3	20.4	16.8
28	2.3	2.5	2.7	2.4	1.1	1.2	1.4	1.1	31.3	35.6	42.4	32.3
32	3.2	3.4	3.6	3.3	1.7	2.1	2.1	1.7	48.7	50.9	61.8	52.0
48	6.2	5.7	6.6	5.7	3.2	3.2	4.3	2.9	84.3	79.8	103.3	80.2
52	6.4	5.7	6.8	5.9	3.6	3.5	4.4	3.2	87.0	77.4	106.6	79.7

a - Humko Sheffield
b - Difco
c - Biobrás
d - Geyer

TABLE 2 - Number of viable *Brucella abortus* S19 organisms at various incubation periods in culture media prepared with four different brands of peptone.

Hours of incubation	Number of colony forming units x10^9 ± standard deviation			
	Peptones			
	HS[a]	US[b]	MC[c]	PA[d]
24	14.90±4.50	19.38±6.03	20.42±4.19	16.82±5.48
28	31.34±7.55	35.60±11.1	42.38±5.82(1)	32.32±7.09
32	48.67±11.2	50.86±14.9	61.78±8.37(1)	51.98±8.37
48	84.27±8.70	79.85±10.4	103.3±18.2(1)	80.25±8.80
52	86.98±8.15	77.38±8.05	106.6±14.9(1)	79.75±6.71

(1) - Statistically different ($P<0.01$) at that incubation period.
a - Humko Sheffield
b - Difco
c - Biobrás
d - Geyer

32 hours when media prepared with HS showed significantly lower nCFU than the other two.

Both OD and PCV showed strong correlation with nCFU (Table 1). In addition the results of OD and nCFU followed the equation of linear regression ($y = -1.573+16.12\ x$) with a coeficient of determination of 0.996 and a coeficient of correlation of 0.998, from which it can be concluded that OD may substitute for the nCFU with a confidence level of more than 99%. The method saves a considerable time when compared to bacterial counts, however, although the nCFU decreased using peptones US and PA between 48 and 52 hours, this was not reflected in either the OD or PCV results and the latter actually increased, as would be expected because these methods do not distinguish between dead and live cells.

Very few (less than 1%) rough colonies were detected by the dissociation tests and there was apparent differences among the four media.

All media tended to become alkaline during the 52 hours of incubation but this was less marked with peptone MC. It is possible that the increased alkalinity reflected the presence of cellular breakdown products and was therefore more marked in those media where there were fewer viable bacteria.

The use of peptone MC will not only result in higher yields but being locally produced will offer cost benefits to the laboratories involved in *B. abortus* S19 vaccine production in Brazil. Although the other locally produced peptone (PA) was less effective than MC it gave similar results when compared to the two imported products and being cheaper, it offers an useful alternative.

From the results of this study it can be concluded that different brands of meat peptones can give different yields of bacterial growth. Therefore each laboratory should test meat peptones available on the market before starting *B. abortus* S19 vaccine production.

RESUMO

Avaliação de diferentes peptonas de carne como meio de cultura para a produção de vacinas com *Brucella abortus* amostra 19.

Quatro marcas comerciais de peptonas foram usadas para o cultivo de *Brucella abortus* amostra 19. Um estudo comparativo entre as quatro diferentes peptonas, usando-se procedimentos de fermentação, revelou que houve uma significativa variação no crescimento da *Brucella abortus*. Uma das quatro marcas testadas apresentou resultado superior às demais.

Palavras-chave: *Brucella abortus*, produção de vacina, meios de cultura.

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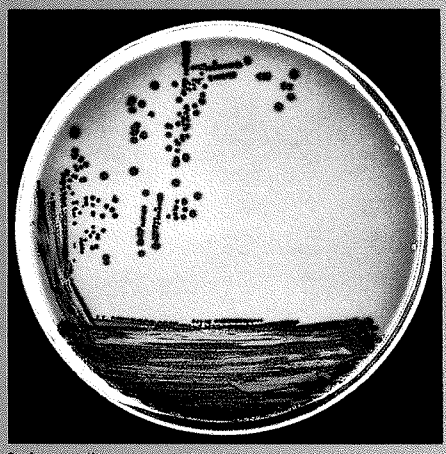

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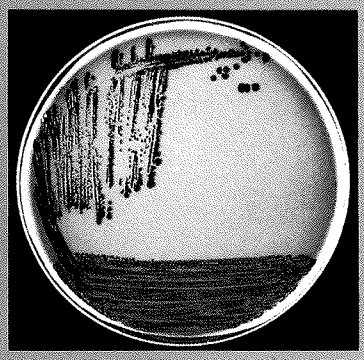

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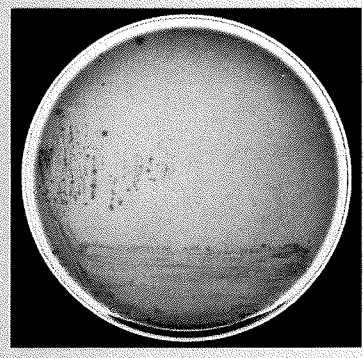

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