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## Editorial / *Editorial*

Ao completar 25 anos de publicação ininterrupta, a Revista de Microbiologia, editada pela Sociedade Brasileira de Microbiologia, está passando por diversas modificações.

Visando a sua descentralização, a Revista de Microbiologia conta agora com 9 Editores Seccionais (nomes e endereços estão na página anterior). Os autores que pretendem publicar seus trabalhos devem encaminhá-los ao Editor Seccional mais relacionado com o tema dos mesmos. Cabe a cada Editor Seccional a recepção dos trabalhos e o gerenciamento de todo o processo de avaliação, feita por revisores por ele(a) designados. Uma vez aceitos para publicação, cabe à Diretoria da Revista o gerenciamento dos processos de revisão do inglês e de impressão desses trabalhos.

O Corpo Editorial foi modificado visando atender mais adequadamente as necessidades da Revista. Cabe ao Corpo Editorial a definição das políticas de publicação, bem como o julgamento final em caso de dúvidas.

A partir do volume 26, a Revista de Microbiologia passa a ser também denominada "Journal of the Brazilian Society for Microbiology".

O item "instruções aos autores" foi revisto e ampliado, de forma a fornecer informações mais completas e permitir melhor uniformização na preparação dos trabalhos que são encaminhados para publicação, principalmente no que diz respeito às referências bibliográficas.

Estas modificações são importantes para que os trabalhos desenvolvidos pelos pesquisadores brasileiros sejam reconhecidos internacionalmente e para que a Revista de Microbiologia e a Sociedade Brasileira de Microbiologia possam ser melhor divulgadas. A Diretoria acredita que essas alterações deverão contribuir muito para a modernização da Revista de Microbiologia e pede o apoio e a colaboração de toda a comunidade científica brasileira e internacional.

*Having completed 25 years of ininterrupted publication, the* Revista de Microbiologia, *edited by the Brazilian Society for Microbiology, is undergoing several modifications.*

*Aiming at its decentralization, the* Revista de Microbiologia *now comprises a body of Section Editors (their names and addresses are listed on the previous page). Authors should submit their manuscript to the Section Editor of the subject area closest to their own work. Section Editors are responsible for the receipt of manuscripts and handling of their assessment by referees that they designate. Once accepted for publication, manuscripts are sent to to the Board of Directors of the* Revista de Microbiologia, *which proceeds to the evaluation of the English language and printing of the text.*

*The Editorial Board was modified to provide a service of improved quality and also more directed to the needs of the* Revista de Microbiologia. *It is a function of the Editorial Board to define publishing policies as well as to reach the final decision on a manuscript in case of doubt.*

*Starting from Volume 26, the* Revista de Microbiologia *will also be named* "Journal of the Brazilian Society for Microbiology".

*The item "guidelines to authors" was reviewed and extended so as to provide more comprehensive information on the preparation of manuscripts submitted to publication.*

*The modifications introduced are important for the international recognition of Brazilian researchers and also for a more efficient circulation of the* Revista de Microbiologia *and acknowledgment of the Brazilian Society for Microbiology. The Board of Directors believes that these changes will contribute much to the modernization of the* Revista de Microbiologia *and expects support and collaboration of both the Brazilian and the International scientific community.*

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# ACUTE EFFECT OF AFLATOXIN $B_1$ ON INBRED STRAINS OF MICE - I

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## ABSTRACT

The acute effect of Aflatoxin $B_1$ ($AFB_1$) was evaluated on 30 days old C57Bl/6 male mice (average weight: 20 g), challenged with a single intraperitoneal dose of the mycotoxin. Three groups of 30 animals each were used: the intoxicated group and control groups I and II. Intoxicated mice were injected intraperitoneally with $AFB_1$ (60 mg/kg animal weight) dissolved in corn oil (0.01 mL/g), while control mice I received corn oil only by the same route. Lots of 10 animals were sacrificed from the intoxicated and control I groups 24, 72 and 168 hours after challenge. Control mice II remained untreated and were used as standards of normality for the biochemical (hepatic and renal function) and hematological evaluations. $AFB_1$ was detected in the livers of intoxicated mice 24 hours (1.46 ng/g), 72 hours (2.30 ng/g) and 168 hours (2.18 ng/g) after challenge. The most evident histologic lesions observed 168 hours after treatment with $AFB_1$ were vacuolization of hepatocytes and disruption of the architecture of the liver parenchyma. High serum levels of alkaline phosphatase were observed in the treated mice 24 and 72 hours after injection. The present study showed that the liver is the target organ for $AFB_1$ in C57Bl/6 mice, causing histopathologic lesions and biochemical changes which are probably related to a slower process of $AFB_1$ biotransformation and elimination, thus increasing the susceptibility of this mouse strain to the mycotoxin.

**Key words:** inbred mice, aflatoxins, acute aflatoxicosis, liver enzymes, histopathologic changes.

## INTRODUCTION

Aflatoxin $B_1$ ($AFB_1$) is a toxic metabolite produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* and is known for its hepatotoxic, hepatocarcinogenic and mutagenic effects on humans and several other animal species (8, 11).

Inside hepatocytes, $AFB_1$ is biotransformed by cytochrome P-450-linked microsomal monooxygenases and the reactions lead to the generation of aflatoxin $M_1$ ($AFM_1$), aflatoxin $Q_1$

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($AFQ_1$), aflatoxin $B_1$ - 2 , 3 - epoxide, aflatoxin $B_{2a}$ ($AFB_{2a}$), and other metabolites (3, 21, 24).

The diagnosis of acute aflatoxicosis is rather difficult since it depends on the observation of clinical signs of intoxication which could, however, be due to the ingestion of other hepatotoxins by the affected animal Therefore, it is important to use laboratory tests or a combination of tests that make possible the distinction between aflatoxicosis and other intoxications (17).

Vesselinouvitch *et al.* (19) investigated the hepatocarcinogenicity of $AFB_1$ in (C57Bl/6 x C3H) $F_1$ male and female mice using either newborn or young (4 to 7 days old) animals. These authors observed the development of malignant hepatomas in 50 to 100% of the young males and 22 to 55% of the newborn males but only in 7% of the young females, 82 weeks after treatment. Wogan and Newberne (22) described the presence of hepatic neoplasms in Swiss, C3HJB, HEN and C57Bl/6NB mice 70 weeks after being fed on rations which contained 1.0 ppm of $AFB_1$. Ueno *et al.* (18) observed that 7-days old (C57Bl/6 x C3H) $F_1$ mice inoculated with $AFB_1$ (6 mg/kg animal weight) by the intraperitoneal route developed hepatic carcinomas 12 months after treatment.

Gawai *et al.* (5) evaluated changes on microsomal and serum enzymes in rats, allogeneic mice, ducks and chickens. They found that, in rats and ducks, there was a reduction in the levels of hepatic microsomal enzymes and a rise in the serum concentrations of sorbitol dehydrogenase, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Increased levels of sorbitol dehydrogenase and AST were found in mice, and of ALT in chickens.

The aim of the present study was to evaluate the acute effect of $AFB_1$ on C57Bl/6 mice through a mycotoxicologic evaluation (detection of $AFB_1$ and its biotransformation metabolites) coupled to the analysis of histopathologic, biochemical (hepatic and renal) and hematological alterations.

## MATERIALS AND METHODS

### Animals

Ninenty 30-days old mice of the C57Bl/6 strain (average weight: 20 g) obtained from the Breeding Unit for Inbred Mice of the Instituto de Ciências Biomédicas (ICB), University of São Paulo (USP), were used in the experiments. The animals were grouped into lots of 10 each and housed inside polyethylene boxes on a bedding of wood shavings; water and a commercial ration previously tested for the presence of aflatoxins were administered *ad libitum*.

### Toxins

The solutions of $AFB_1$ injected into mice were produced at the Laboratory of Mycotoxins of the Microbiology Department, ICB-USP, using as source the $AFB_1$ secreting strain *Aspergillus flavus*, Northern Regional Laboratory (NRRL) 6513, obtained from the United States Department of Agriculture, Peoria, Illinois, USA. The solutions of aflatoxins $B_1$, $M_1$, $M_2$, $B_{2a}$ and aflatoxicol used as comparative standards for the mycotoxicological analysis were purchased from Sigma Chemical Company (St. Louis, Mo, USA).

### Experimental groups

a) Intoxicated mice: 30 mice were injected intraperitoneally with 60 mg/kg animal weight of AFB1 dissolved in corn oil (0.01 mL/g) (1, 13). Lots of 10 animals were killed for evaluation 24, 72 and 168 hours after challenge.

b) Control group I: mice were injected intraperitoneally with corn oil (0.01 mL/g). Lots of 10 animals were killed for evaluation 24, 72 and 168 hours after challenge.

c) Control group II: mice were left untreated, divided into lots of 10 animals each and used as source of blood and tissue samples to establish normal standards for the biochemical analysis of hepatic and renal function and for the hematological evaluations.

### Biochemical analysis of hepatic and renal functions

Mice were anesthetized with ethyl-ether and sectioned to expose the thoracic cavity; blood was collected from the coronary arteries with the aid of a Pasteur pipette then centrifuged at 1500 rpm for 5 minutes for the separation of serum from total blood. For each experimental group, 10 individually collected serum samples were pooled before use at each post-challenge time analyzed (24, 72 and 168 hours). Pooled sera were submitted to the following biochemical evaluations: total protein and albumin contents according to the Biuret and Green

Bromocresol methods, respectively, as described by Ritchie (14); total bilirubin and creatinine levels using appropriate commercial kits from Reactoclin; urea levels according to a modification of Croker's method (4); activity of the enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT) and alkaline phosphatase (AP) using appropriate commercial kits from Merck S/A Chemical Industries.

**Hematology**

Blood samples for hematological evaluation were derived from the same animals used to carry out the biochemical analyses on hepatic and renal function. An aliquot of blood was drawn from each mouse and placed in flasks containing 0.01 mL of 10% EDTA per ml of blood, so as to obtain a pool of blood (approximately 1 mL) from a total of 10 mice per group. Control group II was analyzed to establish normal conditions; sampling of the intoxicated and control I groups was performed at each post-treatment time established. Blood smears used for differential counting of erythrocytes were prepared with samples drawn from the tip of the animal's tail. Red Blood cell (RBC) counting and determination of Hemoglobin (Hb) content, Hematocrit (Hc) values and corpuscular values of erythrocytes, namely: Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Cospuscular Hemoglobin Concentration (MCHC), were done according to Birgel (2). The analysis of blood smears for differential counting of leukocytes was done according to Rosenfield as described in Birgel (2).

**Histopathology**

After blood collection, the abdominal cavity of each mouse was exposed to remove samples of liver and kidney. Again, tissue sampling was carried out at each post-challenge time analyzed, for both the intoxicated and control I groups. Pieces of the organs were fixed *in toto* in 10% formaldehyde and processed according to routine histological techniques. Sections were stained with hematoxylin-eosin as described by Luna (9).

**Mycotoxicologic analyses**

After removal of liver samples for histopathology, the remaining of the tissue was used to carry out toxin residue analyses. For all lots of experimental mice sacrificed, liver tissues were pooled before use. Detection of aflatoxin $B_1$ and its biotransformation products (aflatoxins $B_{2a}$, $M_1$, $M_2$ and aflatoxicol) was done by thin-layer chromatography (TCL) according to the method described by Trucksess and Stoloff (16) as modified by Sabino (15). Quantitative assessment of aflatoxin and metabolites was done using a photoelectric densitometer (Carl Zeiss ZK-6).

## RESULTS AND DISCUSSION

The results are presented in FIGURE 1 and TABLES 1, 2 and 3.

$AFB_1$ was found in the liver of intoxicated mice 24, 72 and 168 hours after treatment at concentrations of 1.46 ng/g, 2.30 ng/g and 2.18 ng/g, respectively (FIGURE 1, TABLE 1). However, other metabolites such as aflatoxins $M_1$, $M_2$, $B_{2a}$ and aflatoxicol were not detected (TABLE 1). The

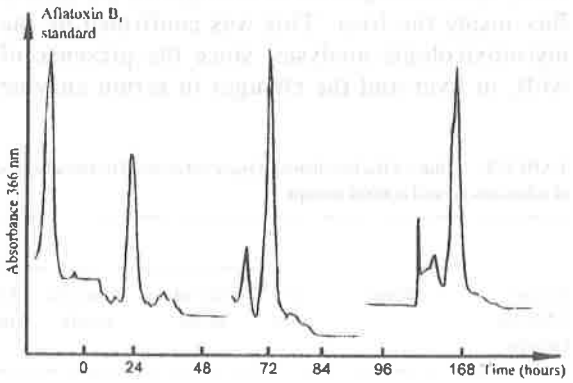


**FIGURE 1.** Spectrum of the standard reference $AFB_1$ and of $AFB_1$ residues present in liver extracts of C57Bl/6 mice 24, 72 and 168 hours after injection of the mycotoxin

**TABLE 1.** Mycotoxicological results for liver extracts of C57Bl/6 mice analysed 24, 72 and 168 hours after inoculation of the mycotoxin of aflatoxin $B_1$

| mice strain | Time after inoculation (hours) | Aflatoxins $B_1$ (ng/g) | $M_1$ | $M_2$ | $B_{2a}$ | Aflatoxicol |
|---|---|---|---|---|---|---|
| C57Bl/6 | 24 | 1,46 | ND | ND | ND | ND |
| | 72 | 2,30 | ND | ND | ND | ND |
| | 168 | 2,18 | ND | ND | ND | ND |

ND - Not Detected

data thus show that, even 7 days after inoculation of the mycotoxin, no biotransformation of $AFB_1$ took place. This fact is probably related to a slower rate of biotransformation and elimination of $AFB_1$ in C56Bl/6 mice. This feature should render the mice more susceptible to the effects of the toxin since, according to Wong and Hsieh (24) and Wei *et al.* (20), mouse resistance to the action of $AFB_1$ depends on convertion of the mycotoxin to other more polar and less toxic species (such as the aflatoxins $M_1$, $P_1$ or $Q_1$) and their fast elimination. Gurtoo *et al.* (6) showed that this bioconvertion process is regulated by genetic factors in strains C57Bl/6 and DBA.

On analyzing the biochemical parameters used to evaluated hepatic and renal function, it was found that serum levels of the enzyme alkaline phosphatase were much higher in intoxicated mice than in control I and II mice 24 and 72 hours after injection of $AFB_1$ (TABLE 2). The rise in concentration for this enzyme can be attributed to $AFB_1$ as a consequence of changes in the biliary flux inside the liver. This was confirmed by the mycotoxicologic analyses, since the presence of $AFB_1$ in liver and the changes in serum enzyme levels were detected during the same period (TABLES 1 and 2).

The hemograms obtained did not present striking alterations that should indicate a state of acute toxicity caused by $AFB_1$ (TABLE 3).

Histopathology of liver and kidney did not indicate the presence of renal lesions, demonstrating only changes in the hepatic parenchyma of intoxicated mice. The most evident hepatic lesions were observed 168 hours after the injection of $AFB_1$, with vacuolization of hepatocytes and disruption of the local tissue arquitecture.

The animals used in the present investigation received a high dose of aflatoxin $B_1$ (60 mg/kg animal weight) and yet did not develop hepatic carcinomas nor show any mortality. These findings are in agreement with the results described by Plantonow (12) and Wogan *et al.* (23) for allogeneic mice, which were treated with high doses for longer periods of time than the ones reported herein.

The $LD_{50}$ dose described in the literature for allogeneic mice is very variable, with values ranging from 9 to 60 mg/kg animal weight (7, 10). In the present investigation, despite using the highest value recorded (60 mg/kg animal weight), no

**TABLE 2** - Values of the biochemical parameters used to asses hepatic and renal function in C57Bl/6 mice 24, 72 and 168 hours after inoculation of aflatoxin $B_1$ and control groups

| Mouse C57Bl/6 Groups | Time | n *pool* | Parameters biochemical: Total protein (g/dl) | Albumin (g/dl) | Urea (mg/dl) | Creatinine (mg/dl) | ALT (U/l) | AST (U/l) | GGT (U/l) | AP (U/l) | Total Bilirubin (mg/dl) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | After $AFB_1$ inoculation (hours) | | | | | | | | | | |
| | 24 | 10 | 4.5 | 2.5 | 47.8 | 0.80 | 47.1 | 154.4 | 0.8 | 278.0 | 0.80 |
| Intoxicated | 72 | 10 | 4.5 | 2.7 | 49.0 | 0.44 | 55.7 | 222.4 | 1.5 | 409.0 | 0.50 |
| | 168 | 10 | 4.2 | 2.8 | 48.0 | 0.89 | 50.1 | 150.2 | 1.5 | 21.8 | 0.20 |
| | 24 | 10 | 4.0 | 2.7 | 47.0 | 0.40 | 62.2 | 174.8 | 0.8 | 9.1 | 0.20 |
| Control I | 72 | 10 | 4.1 | 2.7 | 47.0 | 0.37 | 64.5 | 183.9 | 1.7 | 31.4 | 0.04 |
| | 168 | 10 | 4.0 | 2.4 | 46.5 | 0.27 | 21.5 | 148.9 | 1.5 | 25.1 | 0.08 |
| | No treatment | | | | | | | | | | |
| | Sample 1 | 10 | 4.4 | 2.6 | 31.8 | 0.80 | 20.9 | 174.5 | 1.5 | 5.4 | 0.20 |
| Control II | Sample 2 | 10 | 3.9 | 2.6 | 49.9 | 0.18 | 25.0 | 89.0 | 12.1 | 12.7 | 0.93 |
| | Sample 3 | 10 | 4.1 | 2.7 | 42.8 | 0.30 | 24.1 | 97.0 | 9.0 | 5.4 | 0.72 |

n = Number of animals

**TABLE 3** - Hemogram values for C57Bl/6 mice inoculated with $AFB_1$ and control groups

| Mice C57Bl/6 groups | Time | *n pool | Erythrogram RBC ($mm^3 10^6$) | He (%) | Hb (g%) | MCV (u3) | MCH (δδ) | MCHC (%) | Leucogram Leucocytes ($mm^3$) | Neutrophils (%) | Eosinophils (%) | Lynphocytes (%) | Monocytes (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | After $AFB_1$ inoculation (hours) | | | | | | | | | | | | |
| | 24 | 10 | 5.6 | 24 | 8.5 | 42.8 | 15.2 | 35.2 | 5.200 | 18.0 | 0.0 | 71.0 | 11.0 |
| Intoxicated | 72 | 10 | 6.5 | 28 | 9.1 | 43.0 | 14.0 | 32.5 | 6.300 | 12.0 | 2.0 | 76.0 | 10.0 |
| | 168 | 10 | 5.5 | 25 | 8.7 | 44.9 | 15.6 | 34.8 | 6.100 | 22.0 | 5.0 | 60.0 | 13.0 |
| | 24 | 10 | 6.1 | 35 | 10.9 | 49.8 | 18.0 | 35.8 | 3.150 | 9.0 | 1.0 | 85.0 | 5.0 |
| Control I | 72 | 10 | 5.0 | 21 | 7.4 | 42.0 | 14.8 | 35.0 | 5.100 | 15.0 | 1.0 | 85.0 | 1.0 |
| | 168 | 10 | 5.7 | 28 | 9.2 | 48.6 | 16.0 | 32.8 | 8.550 | 11.0 | 5.0 | 80.0 | 4.0 |
| No treatment | | | | | | | | | | | | | |
| | Sample 1 | 10 | 6.2 | 32.0 | 9.6 | 51.1 | 15.3 | 30.0 | 6.250 | 35.0 | 3.0 | 53.0 | 9.0 |
| Control II | Sample 2 | 10 | 5.3 | 25.0 | 8.1 | 47.1 | 15.2 | 32.5 | 5.500 | 16.0 | 0.0 | 76.5 | 7.0 |
| | Sample 3 | 10 | 5.6 | 28.0 | 9.3 | 49.2 | 16.5 | 33.5 | 4.300 | 21.5 | 0.0 | 74.0 | 4.5 |

n = Number of animals per group
* = Same group of mice used for both hepatic and renal evaluation

mortality was observed among intoxicated mice. This observation may reflect the great genetic variability which exist among allogeneic mice compared to syngeneic mice. It is probable that the latter, for being genetically more homogeneous, are consistently resistant to high doses of $AFB_1$.

To resume, the results obtained in the present investigation show that $AFB_1$ acted essentially in the hepatic compartment of C57Bl/6 mice, since its presence in liver was detected for as long as 7 days post-treatment and caused elevation of alkaline phosphatase serum levels, as well as changes in the hepatic parenchyma. These alterations are probably related to a slower rate of biotransformation and elimination of the mycotoxin, which renders C57Bl/6 mice more susceptible to its effects.


## RESUMO

### Efeito agudo de aflatoxina $B_1$ em camundongos isogênicos - I

O efeito agudo da $AFB_1$ foi avaliado em camundongos C57Bl/6, machos, com 30 dias de idade e peso médio de 20g inoculados com dose única de $AFB_1$ (60mg/kg peso corpóreo). Os animais foram divididos em 3 grupos: grupo intoxicado (30 animais), grupo controle I (30 animais) e grupo controle II (30 animais). O grupo intoxicado foi inoculado por via intraperitoneal com $AFB_1$ (60mg/kg peso corpóreo) dissolvida em óleo de milho (0,01 mL/g) e o grupo controle II recebeu pela mesma via somente óleo de milho (0,01 mL/g). Lotes de 10 animais de ambos os grupos foram sacrificados após 24, 72 e 168 horas da inoculação. O grupo controle II não foi inoculado e serviu como padrão de normalidade para as análises bioquímicas (função hepática e renal) e hematológica. A $AFB_1$ foi detectada no fígado do grupo intoxicado após 24 horas (1,46 ng/g), 72 horas (2,30 ng/g) e 168 horas (2,18 ng/g) da inoculação. As lesões histológicas mais evidentes ocorreram após 168 horas da inoculação com $AFB_1$, com vacuolização dos hepátocitos e desarranjo da arquitetura do parênquima hepático. Os níveis séricos de fosfatase alcalina estavam elevados após 24 e 72 horas da inoculação no grupo intoxicado. Esse estudo indicou que o fígado foi o órgão alvo da $AFB_1$ nos camundongos C57Bl/6, provocando lesões

histopatológicas e alterações bioquímicas. Provavelmente essas alterações estão diretamente relacionadas ao processo mais lento de biotransformação e eliminação da $AFB_1$, que ocasionou maior susceptibilidade dos camundongos para os efeitos dessa micotoxina.

**Palavras-chave:** camundongos isogênicos, aflatoxina, aflatoxicose aguda, enzimas hepáticas, alterações histopatológicas.

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# COMPARISON OF TWO METHODS FOR THE DETERMINATION OF OCHRATOXIN A IN GREEN COFFEE BEANS

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## ABSTRACT

The laboratory of the Biological Chemistry Section of the Adolfo Lutz Institute has been asked to analyze ochratoxin A levels in green coffee beans exported to Greece and Lebanon since October 1989. The request follows the establishment of a maximum tolerance limit of 20mg/kg of OA for the imported products by these countries. The present study was undertaken to determine the most appropriate method for a routine OA analysis of green coffee beans at the Adolfo Lutz Institute. Two methods for ochratoxin A analysis by thin-layer chromatography were compared, namely AOAC (1990) and the Soares and Rodriguez-Amaya (1985) method as modified by Milanez and Sabino (1989). Accuracy (recovery of spiked samples), precision (day-to-day reproducibility) and practicality (applicability cost, speed, equipment and skill requirements, as well as exposure risk) were the parameters used for comparison.

The results showed that both methods are reliable. The coefficients of variation obtained were less than 30%, which are considered adequate for OA evaluation and demonstrate a good reproducibility. Recoveries from the spiked samples were over 70% and thus within levels acceptable for mycotoxin analysis. However, the practicality data indicated that the method of Soares and Rodriguez-Amaya (1985) as modified by Milanez and Sabino (1989) is more suitable for routine use.

**Key words:** ochratoxin A, green coffee beans, determination, thin-layer chromatography.

## INTRODUCTION

Ochratoxin A (OA) is a mycotoxin produced by several fungal species of the genera *Aspergillus* and *Penicillium*, mainly by *A. alutaceus* Berk. and Curt. (formerly *A. ochraceus* Wilh.) and *P. verrucosum* Dierckx (26). OA is primarily a nephrotoxin, but it also has potent teratogenic and mutagenic effects on rats and hamsters (12, 10, 14, 1, 7). Additionally, OA has been shown to be involved in the etiology of the disease known as "Balkan endemic nephropathy", a renal disorder appearing in 30 to 50 years old individuals of the Balkans (Bulgaria, Romania and Yugoslavia) that can slowly lead to death (2, 6, 8, 11).

OA has been detected in barley, beans, corn, green coffee beans, roasted coffee, and other grains (5, 13, 15, 16, 19, 20, 22, 25), and is quite stable to heat and resistant to autoclave sterilization in cereals (24). Nevertheless, considerable destruction of OA (83-89%) in contaminated green coffee beans was reported to occur during a heat treatment (190-227°C for 5-20 minutes) which simulated coffee beans roasting (13). Tsubouchi *et al.*(25)

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found OA in five out of 68 roasted coffee samples imported by Japan from Indonesia and Iemen. These samples presented 3.2 to 17µg/kg (ppb) of OA. The authors also analyzed nine samples from Brazil and none contained OA. However, Micco *et al.* (15) studied 29 samples of green coffee beans and roasted coffee from many countries, including Brazil, and their results showed 58% OA contamination, which ranged from 0.2 to 15 µg/kg (ppb). They also roasted naturally and artificially contaminated samples and found a percentage destruction of OA varying between 48-87% and 90- 100%, respectively.

Few are the countries that have established limits for OA contamination in grains, namely: Denmark, Rumenia, Czech Republic (4) and, more recently, Greece and Lebanon. These last two countries are demanding products with a maximum OA level of 20mg/kg (ppm) from the green coffee beans exporting countries. For this reason, the Adolfo Lutz Institute has been receiving samples of export grade green coffee beans for analysis.

This situation led to the main purpose of this work: to establish a rapid and selective method for the determination of OA in green coffee beans in order to efficiently handle the requests. Two methods were studied. One is from the official methods of analysis of the Association of Official Analytical Chemists (AOAC) (18) and is specific for green coffee beans. The other is the Soares and Rodriguez-Amaya method (21) as modified by Milanez and Sabino (17), which has been used for routine work of many products in our Biological Chemistry Section. Once selected, the method will evaluate OA levels in Brazilian green coffee beans intended for exportation.

## MATERIAL AND METHODS

**Material:** Green coffee beans sent by trading companies were ground to mesh 20 in a "Tecnal" Marconi mill model TE090. Samples with non-detectable OA were artificially contaminated with 30, 80 or 200 µg/kg (ppb) of a standard OA solution, which was spiked with a microseringe on the top of each sample. Five replicates were made for each level of spiking. No mixing was done, to prevent the standard solution from adhering to the walls of the flask. The samples were allowed to dry naturally overnight.

**Evaluation of natural contamination:** Four hundred and twenty one (421) samples of green coffee beans weighting approximately 1kg each were received for OA analysis at the Adolfo Lutz Institute between October 1989 and March 1992.

**Methods:** Two methods were compared:

method 1. - Described by Scott (18) in the AOAC Manual as a thin layer chromatographic method with a detection limit of 20µg/kg (ppb). The toxin was extracted from ground green coffee with chloroform. OA was entrapped on basic diatomaceous earth and the interferences were removed with hexane and chloroform. OA was eluted with benzene-acetic acid. Toxin levels were determined by fluorescent intensity on thin-layer chromatograms.

method 2. - The Soares and Rodriguez-Amaya procedure (21) as modified by Milanez and Sabino (17) is a TLC method with a detection limit of 10µg/kg (ppb). Thirty grams sample were blended with 180 mL of methanol and 20 mL of aq.4% KCl. The mixture was filtered through fluted qualitative filter paper; 100mL of the filtrate were transferred to a 400 mL beaker and mixed with 100 mL of a clarifying agent (10%$CuSO_4$/ 30% $(NH_4)_2SO_4$) and Hyflo Super Cel. The mixture was filtered again through fluted qualitative paper and a 100 mL aliquot was transferred to a separatory funnel with 100 mL of water. OA was extracted twice with 20 mL portions of chloroform. Chloroform extracts were combined and evaporated to almost dryness. The residue was redissolved and used for TLC, performed according to conventional techniques. Estimate levels of the toxin were determined by visually comparing the fluorescence intensity of tested samples with that of known standards.

## RESULTS AND DISCUSSION

TABLE 1 shows the recovery and reproducibility data for the two methods. Both techniques demonstrated recovery levels above 70%, with acceptable coefficients of variation of approximately 20% (less than 12% for method 1 and maximum of 26.2% for method 2). According to Horwitz *et al.* (9), coefficients of variation up to 32% are acceptable for any trace contaminant, at the level of µg/kg (ppb), for any chosen method.

TABLE 1 - Recovery and reproducibility data for the AOAC and modified Soares and Rodriguez-Amaya methods

| Method used | OA added (µg/kg) | % of Recovery* | Standard Deviation | C.V. (%) |
|---|---|---|---|---|
| Method 1 | 30 | 128,1 | 12,0 | 9,4 |
| | 80 | 78,6 | 9,0 | 11,5 |
| | 200 | 72,9 | 7,6 | 10,4 |
| Method 2 | 30 | 96,4 | 16,4 | 17,0 |
| | 80 | 100,0 | 26,2 | 26,2 |
| | 200 | 117,8 | 20,9 | 17,7 |

* - based on 5 determinations for each level

The second method (Soares and Rodriguez-Amaya modified) was originally developed for OA determination in corn, peanuts, beans, rice and cassava but not in green coffee beans. Two clarifying agents were tested: ammonium sulfate (30% aqueous solution) and cupric sulfate (10% aqueous solution). The latter did not perform well in the filtration step, as it retarded the process and formed a strong emulsion with chloroform. Absence of additional spots and elimination of streaking on the TLC plate were also taken into consideration to chose the best clarifying agent. It was concluded that ammonium sulfate performs better than cupric sulfate in samples of green coffee beans.

TABLE 2 shows the data on time consumption, cost and final volume of evaporated solvent. Method 1 required almost 6 hours while method 2 took 2 1/2 hours for completion of the whole procedure. Method 1 (AOAC) was lengthy mainly due to the column chromatography step, and also required the use of the vacuum pump for longer times.

Method 2 was 2.5 times less expensive than method 1 that used expensive reagents like the acid-washed diatomaceous earth, which is, moreover, also difficult to obtain.

Both methods proved to be exact and precise, but the practicality data (cost, speed, special equipment, skills needed and exposure risk) indicated that method 2 is the most appropriate for routine determination of OA in green coffee beans.

TABLE 2 - Evaluation of practicality data of the compared methods.

| Method | final volume of evap. solv. | time of analysis (h) | Cost (US$) |
|---|---|---|---|
| Method 1 | 100mL benzene | 6.0 | 15.00 |
| Method 2 | 20mL chloroform | 2.5 | 6.00 |

Applying method 2, 421 samples of green coffee beans were analyzed for OA contamination. Only one sample was positive, confirmed by derivatization procedures with the addition of 14% $BF_3$ in methanol. This was the only way to confirm the absence of OA in that sample; other steps taken for confirmation were not conclusive, such as change of the mobile phase and co-chromatography.

According to the literature, other methods for OA determination in coffee products have been developed mostly with HPLC. Terada *et al.* (23) established a method that includes extraction with methanol-1% sodium bicarbonate solution (1+1) and cleanup with SEP-PAK $C_{18}$ cartridge. The limit of detection was 2µg/kg (ppb), with a recovery of 81,5% and a coefficient of variation of 5,93. Cantafora *et al.* (3) also described a method that extracts OA with methanol and 5% sodium bicarbonate, but these authors used isooctane and the column recommended by AOAC for the cleanup procedure. OA quantitation was given by HPLC with a fluorescent detector, and the detection limit was 0,01 µg/kg (ppb). Recoveries ranged from 90 to 95%, with a standard deviation of 2,2 and a coefficient of variation of 2,4. These methods were really sensitive and precise; however, they are too sophisticated for routine analysis of OA in green coffee beans, where the detection limit required is 20mg/kg (ppm).

Although both methods studied in the present investigation were precise and exact, the differences in reproducibility and practicality obtained led us to recommend the second one, that is, Soares and Rodriguez-Amaya (21) as modified by Milanez and Sabino (17). It is a less expensive and faster technique, which employs routine materials and equipment and also involves lower exposure risks to toxic organic solvents for the analyst.

## RESUMO

**Comparação de dois métodos para determinação de ocratoxina A em café cru em grão**

O Laboratório da Seção de Química Biológica do Instituto Adolfo Lutz tem sido requisitado desde outubro de 1989 a emitir laudos de análise de

ocratoxina A (OA) em café cru em grão destinado à exportação para Grécia e Líbano, pois estes países impuseram o limite máximo aceitável de 20mg/kg de OA neste produto.

Foram comparados dois métodos de análise de ocratoxina A por cromatografia em camada delgada para café cru em grão. Um deles descrito no AOAC é específico para a determinação de OA neste produto e o outro foi desenvolvido por Soares and Rodriguez-Amaya (1985) e modificado por Milanez and Sabino (1989). Este último já é utilizado rotineiramente no laboratório nas análises de produtos como arroz, amendoim, mandioca, feijão, milho e seus derivados. Neste estudo foram levados em consideração os seguintes parâmetros: recuperação e reprodutibilidade do método, facilidade de operação, disponibilidade de materiais, exposição do analista a reagentes tóxicos, rapidez e custo para implantação na rotina de análises. Os resultados experimentais demonstraram que perante um método específico e consagrado, da AOAC, o método 2 mostrou ser bom e promissor. Escolheu-se a técnica de Soares and Rodriguez-Amaya modificada por Milanez and Sabino pois revelou possuir maior simplicidade de operação e menor custo.

**Palavras-chave:** ocratoxina A, café cru em grão, determinação, cromatografia em camada delgada.

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# SURVIVAL OF CULTURABLE *VIBRIO CHOLERAE* O1 AND NON O1 IN SEAWATER, FRESHWATER AND WASTEWATER AND EFFECT OF THE WATER ENVIRONMENT ON ENTEROTOXIN PRODUCTION

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## ABSTRACT

A study on the survival of *V. cholerae* O1 cholerae, *V. cholerae* O1 El Tor and *V. cholerae* non O1 in microcosms containing freshwater, saltwater and wastewater from the State of São Paulo, Brazil, was carried out. The effect of physicochemical environmental factors on the expression of vibrio enterotoxigenicity was also analyzed. The three *V. cholerae* strains tested lived better in filter-sterilized water, confirming that competition is a constraint for vibrios survival. Survival was greater at 10°C than at 25°C or 35°C, and it was also influenced by alkalinity, pH, chlorides, and sodium and potassium concentrations. Longer survival occurred in non-polluted seawater, confirming that these vibrios are autochthonous to marine environments. The toxigenicity of *V. cholerae* O1 cholerae and El Tor was not affected during the experimental period.

**Key words**: *V. cholerae* O1, *V. cholerae* non-O1, survival, cholera toxin, cholera.

## INTRODUCTION

Studies on the survival of pathogenic and potentially pathogenic vibrios in the aquatic environment are of major epidemiological concern, as in most cases water constitutes a very important route for the dissemination of cholera agent and other vibrios associated with gastrointestinal diseases.

Investigations have been undertaken by many authors to determine vibrios survival in the environment. Felsenfeld (5) reported that survival was dependent on biotype, biotype El Tor being more resistant than biotype cholerae to environmental conditions. Pesingan (15) studied the survival of *V. cholerae* El Tor in food, fomites and water, utilizing raw and autoclaved seawater and freshwater at different temperatures. Miyaki *et al.* (13) analyzed the effect of pH, temperature and osmotic pressure on the survival of *Vibrio cholerae* El Tor in distinct aquatic conditions. Besides these factors, Felsenfeld (6) studied other requirements that could favor the persistence of vibrios in the environment, like the presence of nitrogen compounds, NaCl, availability of oxygen, absence of competition for nutrients, presence of protective factors like slimes and absence of toxic substances such as free chlorine, iodine or fluorides at high

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levels. Isaacson and Smit (8) investigated vibrios survival in tropical waters, evaluating the role of environmental determinants such as temperature, pH, alkalinity, NaCl and sunlight. Singleton *et al.* (17, 18) studied the influence of salinity, organic nutrients and temperature on the survival of a toxigenic strain of *V. cholerae* O1 isolated in Louisiana. Amako *et al.* (1), Huq *et al.* (7), Tamplin *et al.* (19), and Thom *et al.* (20) studied the association between *V. cholerae* and zooplankton with respect to vibrios survival. Colwell (3) observed that alkalinity could enhance the maintenance and growth of *V. cholerae* in water. Perez-Rosas and Hazen (14) compared the *in situ* survival of *V. cholerae* and *E. coli* in marine water inside diffusion chambers. These authors, however, observed different survival rates when comparing results obtained by Coulter counting with epifluorescence data, which shows that results on survival can be influenced by the methodology used for bacteria detection. Lowry *et al.* (9) reported that enterotoxigenic *V. cholerae* O1 persisted along the Gulf Coast for at least 13 years and could contaminate crabs, shrimps and oysters.

The impact of physicochemical stress on the toxigenicity of *V. cholerae* was investigated by Miller and co-workers (10-12), who evaluated whether environmental conditions could affect the selection of toxigenic strains of *V. cholerae*. Studies on vibrios survival in environmental waters from the Southern Hemisphere are scarce. However, as cholera still plagues many countries in Latin America, there is a demand for data on the behavior of these bacteria in our milieu.

The aim of this study was to evaluate, under laboratory conditions, the survival of *V. cholerae* in samples of freshwater, saltwater and wastewater collected in the cities of São Paulo and Santos, State of S. Paulo, Brazil. An additional goal was to analyze whether the expression of enterotoxigenicity was affected after maintenance of the vibrio strains in the various water samples.

## MATERIALS AND METHODS

**Strains**: The classical toxigenic *Vibrio cholerae* O1 biotype IAL 5540 was kindly supplied by Dr G.V.A. Pessoa, from the Instituto Adolfo Lutz, S. Paulo, Brazil. Toxigenic *V. cholerae* O1 biotype El Tor ATCC 14033 and *V. cholerae* non O1 2868N were a gift from Dr R.R. Colwell, University of Maryland.

**Water samples and microcosms**: Freshwater was obtained from two different raw water reservoirs that supply water treatment plants of the Great São Paulo Region: the Alto Cotia Reservoir (TCA) and the Rio Grande Reservoir (TRG). TCA receives water from the Cotia River located in a watershed within the ecological reserve of Morro Grande, City of Cotia. Water from this reservoir is usually of good quality. TRG is a reservoir that receives water from the Rio Grande river in a protected region of the Billings reservoir, city of São Bernardo. This water is normally of good quality according to routine monitoring for fecal coliforms levels, yet it presents cyanobacteria blooms during the summer. Saltwater was collected from the sampling station SL5-São Lourenço, a non fecal polluted region of Bertioga beach, situated on the coast of the State of S. Paulo, Brazil (Atlantic Ocean). Raw wastewater was derived from the sewage treatment plant of José Menino (STP-JM), located in the City of Santos, State of S. Paulo, which receives mainly domestic wastes submitted to primary treatment.

**Chemical analysis of water samples used in the microcosms**: The chemical quality of freshwater, saltwater and wastewater was characterized according the following parameters: alkalinity (bicarbonate), chlorides, conductivity, chemical oxygen demand, hardness, phosphorus (ortophosphates), nitrogen (ammonia), nitrogen (nitrate), nitrogen (nitrite), nitrogen (organic), dissolved oxygen, pH, potassium ($K^+$), sodium ($Na^+$), and turbidity. Chemical analyses were performed at the S. Paulo State Agency for Environmental Control (CETESB) following the APHA methodology (2).

**Microcosms preparation**: Triplicate 500 mL samples of each water category were distributed inside 1,000 mL Erlenmeyer flasks. Microcosms with raw water and filtered sterilized water were prepared. Filter sterilized water was obtained after passage under pressure through membrane filters with porosities of 0.45 µm and 0.22 µm and 142 mm diameter (Millipore Co). Each bottle with the water to be assayed for vibrio survival was inoculated with 1mL of a suspension containing approximately $10^9$ cells of each vibrio strain, from

an overnight culture in tryptone soy agar with 1% NaCl at 35°C. The incubation temperatures for all the experimental conditions studied were 10°C, 25°C and 35°C. Daily samples were collected from each microcosm for vibrios enumeration and enterotoxin detection.

**Bacteriological analysis and enterotoxigenicity assay**: Counting was performed by the membrane filter method using HAWG membranes (47 mm in diameter) and vacuum filtration (Millipore Co). The culture medium was TCBS agar (Difco); incubation was carried out at 35°C for 24 h. Yellow colonies with the typical aspect of *V. cholerae* were inoculated in Kligler medium (Difco) and further incubated at 35°C for 24h. Isolates with the characteristics of *V. cholerae* were tested for the presence of oxidase. Strains of *V. cholerae* O1 were confirmed by agglutination with anti O1 antiserum. Non O1 strains were confirmed by the following biochemical tests: oxidation/fermentation of glucose, growth in peptone with and without NaCl (3%, 7%, 10%), fermentation of inositol and mannitol, decarboxylation of ornithine and lysine, dehydrolysis of arginine, and acetoin production.

The production of enterotoxin was tested in the Y-1 mouse adrenal cell line as described by Sack and Sack (16). For the preparation of extracts, bacteria were grown in Craig's medium (casaminoacids: 30 g, yeast extract: 4.0 g, dipotassium hydrogen phosphate: 0.5 g, in 1000 mL of distilled water, pH 7.2. Ten mL of filter sterilized 20% glucose solution were added to the medium after its sterilization by autoclaving). Cultures were incubated in a shaker (150 rpm) at 35°C for 18h and then centrifuged ( 3000 rpm, 20 min., 5°C). The supernatant was filtered in a swinnex through HAWG membrane filters (0.45 µm pore diameter; Millipore Co) and assayed for enterotoxin production. Y-1 cells were cultured in microplates (Cooke) in F10 medium (Flow) supplemented with 15% fetal calf serum and 10 µg/L gentamicin; incubation was carried out at 35°C for 48h, in an atmosphere of air containing 5% $CO_2$. Finally, 0.025 mL aliquots of the bacterial extract were added to each well; the microplates were incubated for 6h and 24h and examined under an inverted microscope (Olympus Optical Co., Mod. CKC-B) for evaluation of cytotonic effect.

## RESULTS

The survival time (in days) of the 3 strains in the microcosms studied under laboratory conditions are presented in TABLE 1. On the whole, for the three different water categories, all strains lived better in filter-sterilized samples, confirming that competition is a constraint for vibrios survival. With respect to temperature, the best and least favourable conditions for survival of the three *Vibrio* strains were 10°C and 35°C, respectively. At 25°C and 35°C, survival patterns in both raw and sterile TCA freshwater were similar, unlike those recorded for the other water sources, where vibrios performed better in sterile samples as compared to raw samples. *V. cholerae* O1 biotype cholerae survived better in saltwater and at 10°C, however, presented at 25°C, similar survival in relation to the others sources tested (TRG, SLS and STP-JM). Considering the two freshwater sources TCA and TRG, better survival was observed with the latter.

TABLE 2 shows that TRG, SL5 and STP-JM water samples presented higher alkalinity and higher levels of chlorides, sodium and potassium than TCA samples. These results could explain the distinct survival times of vibrios in the various water categories.

**TABLE 1.** Survival, in days, of *V. cholerae* O1 biotype cholerae, *V. cholerae* O1 biotype El Tor and *V. cholerae* non O1, in raw (R) and sterile (S) freshwater, saltwater and wastewater, at 10°C, 25°C and 35°C.

| WATER CATEGORY | | Freshwater TCA | | Freshwater TRG | | Saltwater SL5 | | Wastewater STP-JM | |
|---|---|---|---|---|---|---|---|---|---|
| Strains | TEMP | R | S | R | S | R | S | R | S |
| *V. cholerae* O1 cholerae | 10°C | 7 | 18 | 8 | 20 | 20 | 69 | 6 | 7 |
| | 25°C | 3 | 3 | 5 | 24 | 6 | 20 | 7 | 28 |
| | 35°C | 1 | 1 | 2 | 8 | 3 | 34 | 2 | 20 |
| *V. cholerae* O1 El Tor | 10°C | 10 | 29 | 19 | 22 | 14 | 38 | 7 | 56 |
| | 25°C | 6 | 5 | 4 | 11 | 8 | 24 | 6 | 27 |
| | 35°C | 2 | 1 | 3 | 8 | 2 | 13 | 1 | 12 |
| *V. cholerae* non O1 | 10°C | 6 | 12 | 28 | 32 | 34 | 24 | 3 | 42 |
| | 25°C | 7 | 7 | 8 | 43 | 15 | 65 | 1 | 35 |
| | 35°C | 3 | 4 | 6 | 25 | 14 | 15 | 1 | 14 |

TEMP: temperature; TCA: Alto Cotia; TRG: Rio Grande; SL5: São Lourenco Beach; STP-JM: Sewage Treatment Plant José Menino

**TABLE 2.** Physicochemical characteristics of water samples used in the preparation of microcosms.

| Water source: | Freshwater | | | | Wastewater | | Saltwater | |
|---|---|---|---|---|---|---|---|---|
| | TRG | | TCA | | STP-JM | | SL5 | |
| Variables: | R | S | R | S | R | S | R | S |
| Alkalinity mg/L (bicarbonates) | 10 | 8 | 2 | 3 | 173 | 152 | 103 | 99 |
| Chlorides mg/L | 38.5 | 39.6 | 4.0 | 3.9 | 82.5 | 170.0 | 20500 | 20100 |
| Conductivity 25°C s/cm | 178 | 182 | 19 | 24 | 803 | 875 | 53400 | 55300 |
| C.O.D. mg/L | 14 | 12 | 15 | 20 | 386 | 153 | n.t | n.t |
| Hardness mg/L | 21.2 | 21.7 | 3.1 | 3.7 | 102 | 100 | 6600 | 6630 |
| Orthophosphate ug/L | 5 | 5 | 25 | 10 | 3000 | 2900 | 40 | 35 |
| $NH_3$-N mg/L | 0.35 | 0.13 | 0.10 | 0.06 | 43.0 | 35.0 | 0.14 | 0.12 |
| $NO_3^-$-N mg/L | 0.12 | 0.40 | 0.07 | 0.12 | 0.01 | 0.03 | 0.01 | 0.01 |
| $NO_2^-$-N mg/L | 0.01 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| N mg/L | 0.9 | 0.7 | 1.8 | 1.2 | 44.0 | 35.0 | 0.7 | 0.5 |
| D.O. mg/L | 6.0 | 8.0 | 7.0 | 7.5 | 1.6 | n.t. | 7.3 | 5.3 |
| pH | 6.5 | 6.6 | 7.0 | 6.1 | 7.0 | 7.2 | 8.2 | 8.1 |
| $K^+$ mg/L | 1.6 | 1.8 | 0.8 | 0.8 | 14.5 | 14.0 | 380 | 360 |
| $Na^+$ mg/L | 23.5 | 25.0 | 1.0 | 2.9 | 41.0 | 43.0 | 7500 | 6030 |
| Turbidity (UNT) | 1.5 | 0.37 | 8.2 | 0.9 | 65.0 | 0.8 | 3.0 | 1.0 |

R: Raw; S: sterilized; n.t.: not tested; TCA: Alto Cotia; TRC: Rio Grande; SL5: S. Lourenco Beach; STP-JM: Sewage Treatment Plant Jose Menino; COD: Chemical Oxygen Demand; $NH_3$-N: ammonia nitrogen; $NO_3^-$-N: nitrate nitrogen; $NO_2^-$-N: nitrite nitrogen; N: organic nitrogen; DO: dissolved oxygen.

*V. cholerae* O1 El Tor survived similarly to biotype cholerae at 25°C. However, in general terms, no expressive differences in survival times were detected between these two biotypes.

All the toxigenic strains tested had a cytotonic effect on Y-1 cells during the survival periods covered for each one.

## DISCUSSION

In this study, some of the principal ecological determinants were competition or other possible negative interactions between *V. cholerae* and the water microbiota. This could be demonstrated comparing the data obtained for raw and filter-sterilized water. Some examples can be observed in TABLE 1, where *V. cholerae* O1 cholerae survived 18-20 days in sterile freshwater and only 7-8 days in raw freshwater at 10°C and, likewise, 20 days in raw saltwater and 69 days in sterile saltwater at 10°C.

*V. cholerae* O1 El Tor presented lower survival. Felsenfeld (5) observed that biotype El Tor was more resistant than biotype cholerae to environmental conditions, reporting that *V.*

*cholerae* O1 cholerae survived for 7.5 +/- 1.9 days in well waters and El Tor for up to 19.3 +/- 5.1 days. In our study, *V. cholerae* biotype 01 cholerae presented a higher resistance in sterile saltwater than the El Tor biotype. This may be due to the use of a laboratory strain, confirming that, for survival studies, it might be advisable to use vibrios recently isolated from a clinical case, as reported by Singleton *et al.* (17). Alternatively, the finding may strongly support the idea that saltwater is the natural habitat of this microorganism, favouring the theory of Colwell that *V. cholerae* O1 is native to marine and estuarine waters (3).

Pesingan (15) studied the survival of *V. cholerae* El Tor in food, fomites and water, and observed that this biotype survived better in autoclaved water and in water maintained under refrigeration. Furthermore, it also presented better survival in seawater as compared to freshwater. This microorganism persisted in raw water from a deep well for 13 days at ambient temperature and for 18 days under refrigeration, and in autoclaved well water for 17 days at ambient temperature and 42 days under refrigeration. In seawater, this vibrio survived for 10-13 days at ambient temperature and for 58-60 days under refrigeration. Singleton *et al.* (17-18) reported that *Vibrio cholerae* lived for 42 days at 10°C in marine water with 25‰ salinity. In our study, no striking survival differences were observed between the cholerae and El Tor biotypes. Temperature variation led to some interesting results, better survival being observed at 10°C, though the water samples were obtained from a tropical region with 18°C-23°C average water temperature. As can be seen in TABLE 1, the survival time of *V. cholerae* O1 in sterile waters was also significant at 25°C. West and Lee (21), studying the *in situ* survival of *V. cholerae*, showed that serogroup O1 lived better at higher temperatures. Our data agree with the findings of Pesigan (15) and Felsenfeld (6), who reported a better survival at lower temperatures. *V. cholerae* non O1 presented a higher survival time than *V. cholerae* O1 in salt and fresh water, persisting poorly in wastewater. This could be explained by Colwell *et al.* (4) (findings of), who considered this vibrio to be native to estuarine and marine environments.

Other important ecological determinants considered in this study were some physicochemical aspects of the waters used for microcosm preparation, such as pH, alkalinity, dissolved oxygen, $Na^+$, $K^+$ and chloride ions, as well as nutrients like nitrogen compounds and phosphates. Miyaki *et al.* (13) studied the survival of *Vibrio cholerae* O1 El Tor in distinct aquatic sources and remarked that it depended mainly on pH, temperature and osmotic pressure. The bacteria did not support pH values lower than 5.0; under optimal pH condition, the osmotic pressure was 250 mOsm-700 mOsm. Felsenfeld (6) defined several requirements that favor the persistence of vibrios in the environment, namely: temperature (3°C); pH (6.0 - 9.8); osmolarity (200 mOsm - 700 mOsm), the presence of nutrients as nitrogen compounds, NaCl, availability of oxygen, absence of competition for nutrients, presence of protective factors like slimes and absence of toxic substances at high levels. Isaacson and Smit (8) studied vibrios survival in tropical waters and reported that temperature, pH, alkalinity, NaCl, and absence of sunlight favored the persistence and sometimes even the growth of these bacteria in the aqueous environment. Colwell (3) observed that alkalinity could enhance the maintenance and growth of *V. cholerae* in water, mentioning as examples the alkalinity of water and soil in the Ganges region, and alkaline characteristics of water supply reservoirs in Bangladesh.

Interesting findings can be derived from TABLE 2, which confirm the role of some environmental determinants on the survival of *V. cholerae*. The lower survival observed in TCA freshwater, as compared with TRG freshwater, could be explained by the lower levels of alkalinity, chlorides, hardness, nitrates, and sodium and potassium ions in the former. These variables are also relevant to seawater and sterile sewage, explaining also the better survival of *V. cholerae* O1 in microcosms prepared with these latter waters. The pH could also justify the improved survival of *V. cholerae* O1 in saltwater and its rapid decay in other water categories. However, the effects of isolated variables cannot be evaluated.

Considering the possible impact of chemical water quality on enterotoxigenicity to explain the findings reported by the WHO Scientific Working Group (22) about non toxigenic strains of *V. cholerae* in waters from non epidemic regions, the strains used in this study were daily tested for

cholera toxin production and no changes were detected. Miller *et al.* (10-12) studied the impact of physicochemical stress on the toxigenicity of *V. cholerae* O1 and also detected that exposure to various conditions of salinity, pH, cation composition and concentration did not select for hyper or hypo-toxigenic strains. In conclusion, viable and culturable *V. cholerae* O1 is able to survive in the aquatic milieu, being affected by variations in temperature, pH, alkalinity, chlorides, hardness, nitrates, and sodium and potassium levels. Longer survival occurred in non sewage polluted seawater, and as bacteria kept in this type of water were not affected in the expression of their toxigenic factor, the results confirms that these vibrios are native to marine environments and pose a risk to seafood contamination, which could be easily disseminated by such route.

## ACKNOWLEDGEMENTS

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## RESUMO

### Sobrevivência de *Vibrio cholerae* O1 e Não O1 em água do mar, água doce e águas de esgoto e efeito do ambiente aquático na produção de enterotoxina

Um estudo sobre a sobrevivência de *V.cholerae* O1 biotipo clássico, *V.cholerae* O1 El Tor e *V.cholerae* não-O1 em microcosmos contendo água doce, água do mar e águas de esgoto do Estado de São Paulo, Brasil, foi efetuado. O efeito dos fatores físico-químicos do meio sobre a expressão da enterotoxina dos vibrios foi analisado. As tres cepas de *V.cholerae* testadas apresentaram melhor sobrevivência em água previamente esterilizada por filtração confirmando que a competição é um fator interferente na sobrevivência dos vibrios. A sobrevivência foi superior quando o estudo foi efetuado na temperatura de 10°C em relação a 25°C ou 35°C, e foi tambem influenciada pela alcalinidade, pH e concentrações de cloreto, sódio e potássio. O maior tempo de sobrevivência foi observado em água do mar não poluída confirmando que estes vibrios são autóctones no ambiente marinho. A toxigenicidade de *V.chloreae* O1 clássico e El Tor não foi afetada durante o período de experimento.

**Palavras-chave:** *Vibrio cholerae* O1; *Vibrio cholerae* não-O1; sobrevivência; toxina da cólera ; cólera.

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# SPATIAL AND TEMPORAL DISTRIBUTION OF FECAL COLIFORMS, COLIPHAGES, MOULDS AND YEASTS IN FRESHWATER AT THE SEMI-ARID TROPIC NORTHEAST REGION IN BRAZIL (PARAÍBA STATE)

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## ABSTRACT

The distribution of fecal coliforms, coliphages, moulds and yeasts was evaluated during the dry (summer) and rainy (winter) seasons in three lakes and two streams presenting different levels of fecal pollution, located in a semi-arid region of the Northeast of Brazil (Paraíba state). Boqueirão lake waters were found to be suitable for bathing but not for unrestricted irrigation. Rain contributed to fecal pollution to a large extent. Fungal diversity in the lakes increased in parallel with fecal contamination. Moulds were present in all the samples but yeasts were consistently present in the high fecal pollution environments ($\chi^2o=69$; $\chi^2cr=9.21$; $\alpha=0.01$), where *Candida* exhibited the highest diversity (7 species). The incidence of NSF, *Candida* spp. and *C.albicans* was higher in the more polluted waters, showing statistically significant differences (NSF$\chi^2o= 26.2$; *Candida* spp.$\chi^2o= 12.96$; $\chi^2cr= 9.21$; $\alpha=0.01$). Both streams did not present any significant differences in the number of taxa and fecal concentrations. However, the incidence of NSF and *C.albicans* was associated with fecal coliform levels. The results suggested that NSF, *Candida* spp. and *C.albicans* are potential indicators of fecal contamination in tropical semi-arid freshwaters. Regional studies on substrate diversity could lead to a better understanding of the distribution and richness of geofungi.

**Key words**: coliphages, fecal coliforms, fungi, freshwater, tropic, semi-arid.

## INTRODUCTION

Rivers and lakes, traditionally used as sources of water, are complex ecosystems which have had their equilibrium altered by human activities. The negative impact is related to the risks associated with eutrophication and public health due to contamination with feces, domestic and industrial sewage and agricultural and pasture runoff (6)

Fecal coliforms are the most commonly employed indicators of fecal pollution in the aquatic environment. However, their use has been

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questioned by several authors, because in some instances these organisms were not isolated even when pathogenic organisms were constantly present. Furthermore, these coliforms are less tolerant to the effect of solar energy, and in tropical regions they may multiply in waters not classified as fecally polluted. Additionally, they are not considered good indicators of virus contamination (5, 9). More recently, coliphages have been proposed as suitable indicators of fecal and sewage contamination. Their advantages over fecal coliforms include a higher resistance to environmental factors and disinfection, ease of enumeration and rapid obtention of results, as well as their application as models for the presence and behaviour of enteroviruses in water and sewage (11). Positive correlations between coliphages and fecal coliforms have been demonstrated (4).

Various studies have shown the association between fecal indicators, the level of organic pollution and the presence of some genera and species of fungi (3, 8, 19). APHA (1) has emphasized the highly diversified nature of the micobiota, which reflects a high variety of substrates and also gives evidence of the stability of the community in the environment. The seasonal distribution of moulds and yeasts is influenced by both climatic changes and the introduction of exogenous substrates (16, 19). Martins *et al.* (12) studied the seasonal distribution of fungi in a Brazilian subtropical stream during the wet and dry seasons, in order to ascertain whether specific genera could be used as indicators of pollution during the summer and winter periods. Little or no information on this topic is available for the Northeast of Brazil.

The purpose of this research was to determine the distributions of fecal coliforms, coliphages, moulds and yeasts in lentic and lotic freshwater bodies in a semi-arid tropical region of Northeast Brazil, and to compare the relative incidence of isolation of fungi with the levels of fecal contamination.

## MATERIALS AND METHODS

**Sampling locations.** The five water bodies were located in the Paraiba State, Brazil. Samples were collected from three artificial lakes situated in Campina Grande City (Açude de Bodocongó and Açude Velho, 7°11'S 35°52'31"W, 550 m above the sea level) and in the Boqueirão municipality (Açude de Boqueirão, 7°29'20"S 36°17'03"W, 450 m above the sea level), and in two streams located in Campina Grande city (Riacho de Bodocongó) and within the Sapé municipality (Riacho São Salvador, 7°06'S 35°14'W, 85 m above the sea level). Morphometric details of the lakes are given below:

| Lake | Area (Ha) | Depth (m) mean | max | min | Volume ($m^3$) | Detention time (years) |
|---|---|---|---|---|---|---|
| Açude Velho | 17,7 | 2.5 | 4.51 | 2.5 | 515,863 | 4 |
| Açude de Bodocongó | 35 | 3.0 | 8.5 | -- | 1,020,000 | - |
| Açude de Boqueirão | 2678 | -- | 58.00 | -- | 577,260,000 | 3.5 |

Açude Velho and Açude de Bodocongó receive sewage and urban drainage from the city of Campina Grande. Açude de Boqueirão also receives agricultural run-off. Furthermore, the three lakes are used for fishing purposes whereas Boqueirão is used as a source of drinking water and as a bathing site. The communities living near this lake use the water for human drinking purposes without any treatment. São Salvador stream is nearly 18 km long, and receives along its course agricultural run-off, some effluents from fruit industries and the effluents from Sapé city's waste stabilization ponds. This source of water is used for domestic washing purposes, for the manufacture of bricks and as drinking water source for cattle. Bodocongó stream receives effluents from three industries (paper, leather, oil and soap) and also effluents from the local abbattoir. It flows through the west part of the city of Campina Grande, where it receives septic tank effluents; in the south of the city it also receives effluents of the city's sewage treatment plant.

**Climate characteristics.** The weather at Campina Grande and Boqueirão belongs to type BSh (Köeppen classification) and is called tropical semi-arid, with a hot and dry summer and a rainy winter concentrated in six and five months, respectively. The annual rainfall is about 600-800 mm in C.Grande and 150-200 mm in Boqueirão. The annual mean temperature is approximately 25° C, with a maximum of 34°C and a minimum of 18°C. Sapé climate belongs to Köeppen's type AS

(tropical-humid or tropical rainy wheather), with temperatures ranging from 20°C to 36°C and an annual rainfall of 1,500 mm, concentrated in six or seven months (10).

**Sampling periods.** Boqueirão lake sampling periods were: September 1989 - January 1990 (first dry season, S1); February - July 1990 (first rainy season, C1); August 1990 - January 1991 (second dry season, S2) and February - July 1991 (second rainy season, C2). For Bodocongó and Velho lakes the sampling periods were September 1989 - February 1990 (S1); March - August 1990 (C1); September 1990 - February 1991 (S2) and March - July 1991 (C2). Bodocongó stream sample collection was done during March - August 1991 (C2) and September 1991 - January 1992 (third dry season (S3). For São Salvador stream the monitoring periods were February - August 1991 (C2) and September 1991 - Janeiro 1992 (S3).

**Sample collection and processing.** Details of the sampling locations and their characteristics are given in TABLE 1. Samples were collected monthly and in some cases forthnigthly. Water samples were collected at 30 cm from the surface of the lakes and streams in sterile wide-mouthed flasks containing 3mL EDTA 3% (w/v), and were preserved in ice. Processing was usually done within 2 hours of collection.

**Enumeration of fecal coliforms.** Fecal coliforms were enumerated by the membrane filtration technique as recommended in APHA (1).

**Enumeration of somatic coliphages.** Coliphages were enumerated only on stream samples using the method recommended by APHA (1). Counts were made after 24 hours of incubation at 35°C.

**Identification of moulds and yeasts.** The spread plate technique was used for the isolation of fungi using Sabouraud- Dextrose Agar with 100 g/mL of chloramphenicol (15). Aliquots of 0.2 mL were spread on the surface of agar plates and incubated for 5 to 15 days at 25° C in the dark. Identification was done to the genus level and whenever possible to the species level (13).

## RESULTS AND DISCUSSION

### Lentic water bodies.

**Fecal coliforms.** FIGURES 1, 2 and 3 show the log distribution of the geometric means for fecal coliforms in the three artificial lakes, during two dry seasons and two wet seasons. Results from the Açude de Bodocongó for the first dry season were not included because only two samples were collected in this period. Fecal coliforms were obtained in increasing order as follows: Boqueirão,

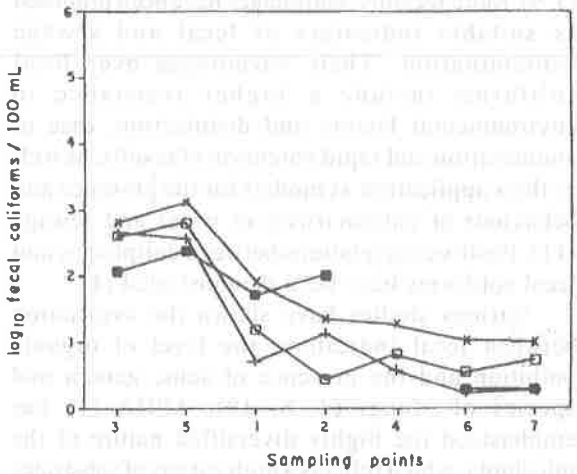


FIGURE 1 - Distribution of fecal coliforms in Boqueirão lake, Paraíba State, Brazil (geometric means; ■ :first dry period, Sept 1989 - Jan 1991, n= 18; + : first rainy period, Feb - Jul 1990, n= 49; ⊟ : second dry period, Aug - Jan 90, n = 42; ✳ : Second rainy period, Feb -Jul 1991, n = 41)

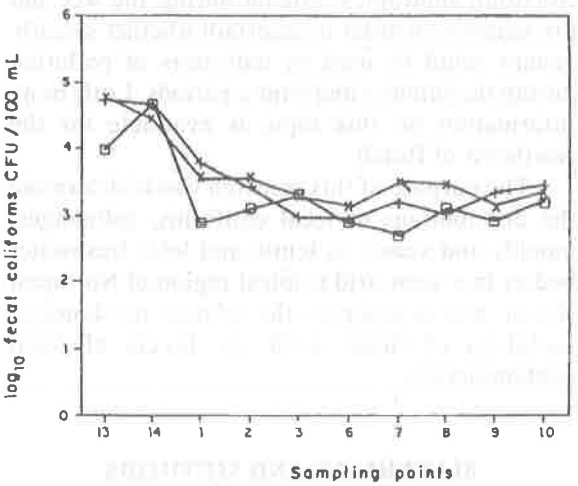


FIGURE 2. Distribution of fecal coliforms in Bodocongó lake, Paraíba State, Brazil (geometric means; + :first rainy period, March - Aug 1990, n= 104; ⊟ :second dry period, Sep 1990 - Feb 1991, n= 58; ✳ : second rainy period, March - Jul 1991, n = 59)

TABLE 1. Location and characteristics of sampling points

| Water body | Sampling point | Description |
|---|---|---|
| Açude de Boqueirão | BQ1 | At the bank and near the boarding point. |
| | BQ2 | At the bank and near to a hotel. |
| | BQ3 | At the shores; point of washing of clothes, water collection, bathing. |
| | BQ4 | At the centre, 4 km from BQ2. |
| | BQ5 | At the bank and with similar uses to BQ3. |
| | BQ6 | At the centre, 2 km from BQ5. |
| | BQ7 | At the centre, between BQ2 and BQ4. |
| Açude de Bodocongó | BD1 | At the northeast bank near to a sport field. |
| | BD2 | Effluent receiving point of the lake. |
| | BD3 | At the north bank near to the Faculty of Medicine. |
| | BD6 | At west central near to a mass of *E. crassipes*. |
| | BD7 | At the centre and right on line with BD3. |
| | BD8 | At the centre, 400m from BD7. |
| | BD9 | At the centre, 200m from BD8. |
| | BD10 | At the centre, 200m from BD9. |
| Açude Velho | AV3 | At the bank, receiving the effluent of the Piabas channel. |
| | AV4 | At the southeast bank; receives intermittent sewage discharge. |
| | AV5 | Stagnant water point at the northwest bank. |
| | AV8 | Discharge point of the lake. |
| | AV9 | At the north bank, near a continous discharge of urban drainage. |
| | AV13 | At the northwest bank; influenced by wind; accumulated detritus. |
| | AV14 | Extreme west of the bank; influenced by wind; macrophytes. |
| | AV7, AV11 & AV15. | Central equidistant points. |
| Bodocongó stream | BD4 | Near to the effluent of the açude, 85cm wide; receiving municipal abattoir and industrial effluents. |
| | BD5 | 150 m from BD4; 3.2m wide; rubbish and pigsties residues. |
| | BD11 | 1 km from BD5; 0.95m wide; abundance of organic residue. |
| | BD12 | Next to the National Health Foundation; 6.5 wide; sand and mud bottom. |
| | BD15 | Under the bridge of Floriano Peixoto Avenue; 3m wide. |
| | BD16 | Next to the Francisco Lopes Almeida bridge; 8.4m high. |
| | BD17 | Under the Alça Sudoeste bridge; 8.7m wide. |
| | TM | Under "Tres Irmãs" bridge at the Cruzeiro district; water damed and used for irrigation. |
| | RM | At the point of access to Catingueira district; 0.87m wide. |
| | C8 | After the confluence of Bodocongó and Depuradora streams with the effluents from the sewage works of Campina Grande city. |
| | FC | At a farm (Fazenda Caiçara); 18.5m wide; sand and mud bottom. |
| São Salvador Stream | SS1 | Under a fall, 200m before the discharge of point SS. |
| | SS | 20m after the discharge of stabilization pond effluent from Sapé city; 2.2m wide, sandy bottom. |
| | SS2 | 1.5km from SS; 4.5m wide place for washing clothes, mud and stones bottom. |
| | SS3 | 1km from the former; 4.2m wide, sandy bottom, next to a hand-made brick factory. |
| | SS5 | 2km from SS3, near a fall; 2m wide and stoned bottom. |
| | SS8 | Located 300m before the dam wall; 2m wide, mud bottom. |
| | SS9 | After the dam wall, next of the piped water exit; 5.7m wide |

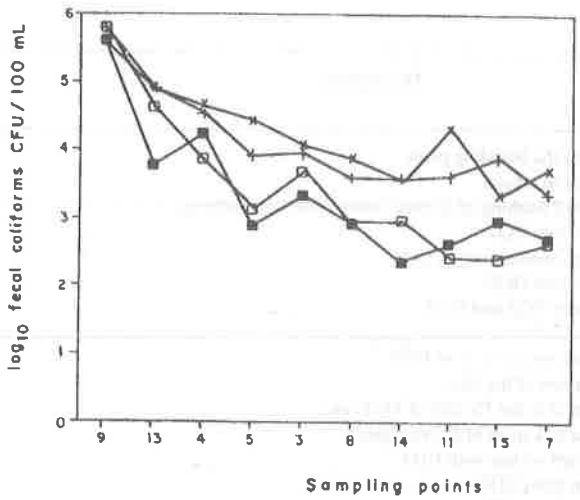


FIGURE 3 - Distribution of fecal coliforms in Açude Velho lake, Paraíba, State, Brazil (geometric means; -■- : first dry period, Sept 1989- Fev 1990, n= 98 ; + : first rainy period, March - Aug 1990, n = 95 ; -⊟- : second dry period, Sep 1990 - Feb 1991, n = 44; -✳- : second rainy period, March - Jul 1991, n = 47)

Bodocongó and Açude Velho. The majority of sample points showed a similar pattern of behaviour within the climatic periods. Highest counts occurred systematically near the shores in one or both rainy seasons. In Boqueirão, the highest values were obtained during the rainy seasons at stations BQ3 (6.56 X $10^2$ CFU/100 mL) and BQ5 (1.4x$10^3$ CFU/ 100 mL), where there was intense human activity. Relatively high concentrations at other points located on the shore observed during the second dry season could be attributed to occasional rains on the day of sample collection or 72 hours before. High counts of fecal coliforms were often associated with the introduction of fecal material from agricultural pasture run-off and superficial drainage from places with antropogenic activities (15, 18). BQ1 and BQ2 showed lower values in comparison to the other peripheral points (≥70 CFU/100 mL. Data from BQ1 showed statistically significant differences between the two rainy seasons and between the first dry and wet seasons (Student t-test, $\alpha$= 0.05). Data from BQ2 showed statistically significant differences throughout the four periods. All the central sampling stations had low fecal coliform counts which were not statistically different bewteen the four periods, except for BQ6 and BQ7 during the two rainy periods.

The highest values in Bodocongó lake during the wet seasons (4x$10^3$ CFU/100mL) were associated with the run-off from pasture lands, increased discharges from sewage outflow and urban drainage from the northwest part of Campina Grande city, which enters the lake from BD13 and BD14. Martins *et al.* (12) have pointed out that the increase in fecal coliform numbers during the rainy seasons is influenced not only by the entry of foreign material but also by the increased outflow of tributaries which reduce the retention time. The relatively small size and the shallow nature of this lake could substantially alter the velocity of water flux ,thereby decreasing the coliform die-off. There were no striking differences in the Bodocongó lake between the results from the inshore and offshore sampling stations, except for BD13 and BD14 points. The lowest fecal coliform numbers in BD6 (7x$10^2$-1.6x$10^3$ CFU/100 mL) and BD9 (8x$10^2$-2x$10^3$ CFU/100mL) could be attributed to the proximity of masses of *Eicchornia crassipes,* which could retain bacteria and organic matter coming from the margins and influent discharges, as observed by Rosa *et al.*(18) in the Lagoa Olhos D'Agua, Minas Gerais. In fact, macrophytes function like horizontal biological filters in aquatic ecosystems, which, in addition to clarifying the water, provide a support for bacterial fixation. Nearly all the sampling stations had similar fecal coliform counts in all the periods studied (Student t-test, $\alpha$=0.05). The results showed that fecal contamination was homogeneous and entered principally from BD13 and BD14. The median value for fecal coliforms between these two points was 30x$10^5$ CFU/100 mL, whereas for the rest of the points it was 1.2 x $10^3$ UFC/100 mL.

Açude Velho lake showed the highest level of fecal contamination during the rainy seasons ($10^3$-$10^5$ CFU/ 100 mL), when urban drainage and sewage effluents entered from AV3, AV4 and AV9. ($10^3$-$10^5$ CFU/ 100 mL). FIGURE 3 shows the highest levels of fecal coliforms for both rainy seasons. It also shows the different level of fecal pollution in the lake as a consequence of wind action and water circulation. Winds blow the incoming waters towards stagnant margin points (AV5, AV13 and AV14), which could have more fecal coliforms than the entry points (AV3 and AV4). The central points also showed higher fecal pollution in the winter, with statistically significant differences during the dry season (Student t-test, $\alpha$= 0.05).

**The diversity and incidence of moulds and yeast.** TABLE 2 shows the incidence of moulds and yeasts in the positive samples. Moulds were present in 100% of all samples whereas yeasts were present in increasing order as follows: Boqueirão, Bodocongó and Açude Velho, in parallel with levels of fecal pollution. The statistical analysis showed that differences in percentage of yeast isolation between the three lakes were significant ($\chi^2o = 69$; $\chi^2cr = 9.21$; $\alpha = 0.01$). There were no significant differences between the streams.

TABLE 2. Frequency of isolation of moulds and yeasts in lentic and lotic freshwater bodies in the Northeast of Brazil, Paraíba. Sampling periods for lakes: September 1989 / July 1991; streams: February 1991 / February 1992

| Source | number of samples | percentage (%) moulds | yeasts |
|---|---|---|---|
| Açude de Boqueirão | 161 | 100 | 23 |
| Açude de Bodocongó | 225 | 100 | 47 |
| Açude Velho | 251 | 100 | 65 |
| São Salvador stream | 102 | 100 | 40 |
| Bodocongó stream | 177 | 100 | 36 |

TABLE 3 shows the incidence of moulds and yeasts and the total number of genera for each season and for all the periods in the three lakes. Fungi were more frequently isolated during the two rainy seasons. No specific genera of fungi were associated with particular seasonal cycles, but there was a great variety of distribution patterns. The predominant moulds in the three lakes were present in decreasing order as follows: *Penicillium* spp. NSF, *Curvularia* spp. *Cephalosporium* spp. *Cladosporium* spp. *Rhizopus* spp. and *Aspergillus* spp. In 637 samples examined, the incidence of NSF was significantly different ($\chi^2o = 22.1$; $\chi^2cr = 9.21$; $\alpha = 0.01$). The frequency of NSF was greater in the lakes with higher values of fecal coliforms. Consequently, the results suggest an association between pollution levels and the incidence of NSF. These results are similar to those of Purchio *et al.* (15) from the Baixada Santista beach in São Paulo, Brazil, and those of Renault *et al.* (17) from the Lagoa Olhos D'Agua (Minas Gerais, Brazil).

*Penicillium* spp. (incidence: 56-60%) and *Aspergillus* spp. (incidence: 12-22%) were constantly present in all the lakes but there was no obvious relationship with pollution levels. These results are different from those of Purchio *et al.* (15), who associated *Penicillium* spp. with higher levels of pollution and *Aspergillus* spp. with lower levels of pollution. In the present study, the incidence of *A.flavus, A.niger* and *A.terreus* was higher in Bodocongó and Açude Velho lakes, suggesting that these organisms have preference for a polluted environment. *Curvularia* spp. *Cephalosporium* spp. and *Cladosporium* spp. showed nearly the same incidence in the three lakes, but the predominant genus in the more polluted environment was *Rhizopus* spp. whereas in the less polluted lake it was *Epicoccum* spp., *Cunnigamela* spp. *Trichothecium spp. Syncephalastrum* spp. *Paecylomyces* spp. *Streptomyces* spp. and *Phoma* spp. appeared occasionally in the more contaminated lakes. A conclusion could not, therefore, be drawn. Furthermore, all of these organisms have an ubiquitous distribution.

*Rhodotorula* spp. *Sporobolomyces* spp. and *Candida* spp. were present in decreasing order in Boqueirão and Açude Velho lakes. In Bodocongó lake, the distribution pattern was: *Sporobolomyces* spp. *Rhodotorula* spp. and *Candida* spp. Whereas the frequency of isolation of *Rhodotorula* spp. was not statistically significant between results from both lakes, *Sporobolomyces* spp. and *Candida* spp. showed significant relationships with polluted freshwaters ($\chi^2o = 11.0$; $\chi^2cr = 9.21$; $\alpha = 0.01$). *Candida* spp. exhibited the highest diversity in the more polluted water bodies (6 and 7 species), which is associated with human detritus. During the rainy seasons, *Candida* spp. had the highest frequency of isolation and fecal coliforms were greater in numbers. Similar results were obtained by Martins *et al.* (12) for a sub-tropical stream. An increase in fecal coliform counts was invariably followed by a similar increase in *Candida* spp. and NSF incidence ($\chi^2o = 15.78$ $\chi^2cr = 9.2$; $\alpha = 0.01$).

The higher diversity of moulds and yeasts was obtained from samples collected by the periphery of the lakes and it was followed by increased numbers of fecal coliforms, being related to the variety of organic substrates from the influent streams, run-off from land and the activity from bathers. Various authors have shown that the diversity of fungal distribution is dependent on the nutritional content of the environment (17).

TABLE 3. Relative frequency of isolation of moulds and yeasts in the Boqueirão, Bodocongó and Velho lakes, Paraíba (Brazil), during the dry and rainy seasons

| source | Boqueirão lake | | | | | Bodocongó lake | | | | | Açude Velho lake | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| period | S1 | C1 | S2 | C2 | total | S1 | C1 | S2 | C2 | total | S1 | C1 | S2 | C2 | total |
| Number of samples | 21 | 49 | 42 | 49 | 161 | | 101 | 65 | 59 | 225 | 52 | 93 | 48 | 58 | 251 |
| MOULDS | | | | | | | | | | | | | | | |
| *A. achraceus* | 0 | 0 | 0 | 4 | 1 | ND | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. flavus* | 0 | 12 | 12 | 10 | 10 | ND | 14 | 11 | 32 | 18 | 4 | 14 | 6 | 5 | 8 |
| *A. niger* | 0 | 0 | 2 | 4 | 2 | ND | 6 | 3 | 25 | 10 | 0 | 4 | 8 | 14 | 6 |
| *A. terreus* | 0 | 8 | 5 | 6 | 6 | ND | 7 | 0 | 5 | 4 | 0 | 1 | 2 | 5 | 2 |
| *Absidia spp.* | 0 | 0 | 0 | 0 | 0 | ND | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Alternaria spp.* | 0 | 0 | 0 | 0 | 0 | ND | 0 | 5 | 0 | 1 | 2 | 1 | 0 | 2 | 1 |
| *Aspergillus spp.* | 33 | 16 | 0 | 14 | 14 | ND | 11 | 9 | 17 | 12 | 25 | 27 | 8 | 28 | 23 |
| *Cephalosporium sp.* | 14 | 20 | 12 | 24 | 19 | ND | 22 | 20 | 15 | 20 | 12 | 23 | 13 | 19 | 18 |
| *Cladosporium spp.* | 29 | 18 | 12 | 18 | 18 | ND | 20 | 14 | 39 | 23 | 15 | 26 | 17 | 28 | 22 |
| *Cunningamela spp.* | 0 | 0 | 0 | 0 | 0 | ND | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0.4 |
| *Curvularia spp.* | 14 | 31 | 12 | 29 | 23 | ND | 25 | 26 | 34 | 28 | 10 | 33 | 21 | 28 | 25 |
| *Epicoccum spp.* | 0 | 14 | 0 | 2 | 5 | ND | 0 | 2 | 0 | 0 | 0 | 4 | 6 | 0 | 3 |
| *Fusarium spp.* | 5 | 2 | 0 | 2 | 2 | ND | 6 | 5 | 20 | 9 | 4 | 8 | 4 | 16 | 8 |
| *Geotrichum spp.* | 0 | 4 | 2 | 6 | 4 | ND | 8 | 8 | 15 | 10 | 2 | 4 | 2 | 0 | 2 |
| *Gliocadium spp.* | 0 | 0 | 0 | 0 | 0 | ND | 0 | 0 | 3 | 1 | 0 | 0 | 0 | 0 | 0 |
| *Monilia sitophyla* | 5 | 4 | 5 | 10 | 6 | ND | 4 | 5 | 15 | 7 | 2 | 3 | 8 | 2 | 4 |
| *Mucor spp.* | 0 | 0 | 12 | 2 | 4 | ND | 4 | 11 | 10 | 8 | 2 | 1 | 2 | 9 | 3 |
| *NSF* | 10 | 37 | 52 | 14 | 30 | ND | 59 | 45 | 61 | 56 | 13 | 57 | 46 | 50 | 44 |
| *Paecilomyces spp.* | 0 | 0 | 0 | 0 | 0 | ND | 0 | 0 | 2 | 04 | 2 | 0 | 0 | 0 | 0.4 |
| *Penicillium spp.* | 38 | 57 | 57 | 73 | 60 | ND | 56 | 65 | 58 | 59 | 35 | 62 | 75 | 50 | 56 |
| *Phoma spp.* | 0 | 0 | 0 | 0 | 0 | ND | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0.4 |
| *Rhizopus spp.* | 14 | 4 | 33 | 14 | 16 | ND | 15 | 26 | 37 | 24 | 25 | 13 | 21 | 21 | 19 |
| *Syncephalastrum sp.* | 0 | 0 | 0 | 0 | 0 | ND | 4 | 0 | 3 | 3 | 2 | 0 | 0 | 0 | 0.4 |
| *Trichothecium spp.* | 0 | 0 | 0 | 0 | 0 | ND | 0 | 0 | 8 | 2 | 4 | 0 | 0 | 0 | 1 |
| *Ustilago spp.* | 0 | 0 | 5 | 0 | 1 | ND | 0 | 2 | 0 | 0 | 0 | 1 | 2 | 0 | 1 |
| No. of taxa | 9 | 13 | 13 | 16 | 17 | | 16 | 16 | 18 | 19 | 16 | 19 | 16 | 14 | 22 |
| YEASTS | | | | | | | | | | | | | | | |
| *C. albicans* | 0 | 0 | 0 | 2 | 1 | ND | 8 | 3 | 15 | 8 | 2 | 8 | 15 | 17 | 10 |
| *C. guilliermondi* | 0 | 2 | 2 | 0 | 1 | ND | 1 | 0 | 2 | 1 | 0 | 1 | 0 | 0 | 0.4 |
| *C. kefyr* | 0 | 0 | 0 | 0 | 0 | ND | 2 | 2 | 7 | 3 | 0 | 0 | 0 | 0 | 0 |
| *C. krusei* | 0 | 0 | 2 | 0 | 1 | ND | 4 | 2 | 7 | 4 | 0 | 2 | 4 | 9 | 4 |
| *C. pseudotropicalis* | 0 | 0 | 0 | 0 | 0 | ND | 3 | 5 | 5 | 4 | 0 | 1 | 0 | 3 | 1 |
| *C. tropicalis* | 0 | 0 | 0 | 0 | 0 | ND | 4 | 9 | 5 | 6 | 0 | 4 | 6 | 12 | 6 |
| *Candida parapsilosis* | 0 | 0 | 0 | 0 | 0 | ND | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 1 |
| *Candida spp.* | 5 | 8 | 2 | 4 | 5 | ND | 10 | 12 | 14 | 12 | 17 | 17 | 17 | 22 | 18 |
| *Candida zeylanoides* | 0 | 0 | 0 | 0 | 0 | ND | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 1 |
| *Cryptococcus spp.* | 0 | 0 | 2 | 0 | 1 | ND | 0 | 0 | 3 | 1 | 0 | 3 | 0 | 0 | 1 |
| *Rhodotorula spp.* | 10 | 8 | 43 | 16 | 20 | ND | 18 | 28 | 29 | 24 | 19 | 32 | 25 | 36 | 29 |
| *Saccharomyces spp.* | 0 | 0 | 0 | 0 | 0 | ND | 4 | 3 | 2 | 3 | 2 | 4 | 6 | 0 | 3 |
| *Sporobolomyces sp.* | 0 | 0 | 31 | 12 | 12 | ND | 28 | 20 | 22 | 24 | 0 | 20 | 25 | 29 | 19 |
| *Torulopsis spp.* | 0 | 0 | 0 | 0 | 0 | ND | 5 | 0 | 0 | 2 | 8 | 3 | 2 | 2 | 4 |
| *Trichosporon spp.* | 0 | 0 | 0 | 0 | 0 | ND | 1 | 0 | 0 | 0.4 | 0 | 0 | 0 | 0 | 0 |
| No. of taxa | 2 | 3 | 6 | 4 | 7 | | 12 | 9 | 11 | 13 | 5 | 13 | 8 | 8 | 13 |
| Total No. of taxa | 11 | 16 | 19 | 20 | 24 | | 28 | 25 | 29 | 32 | 21 | 32 | 24 | 22 | 35 |

ND: not done

In Boqueirão, the diversity of yeast varied between 2 and 7 taxa at BQ4 and BQ3, respectively, both sites being shore sampling stations. In Bodocongó, median values of fecal coliforms between $2x10^4$ and $8x10^4$ CFU/100mL correlated with 15 and 22 fungal genera; $10^3$-$9.8x10^3$ CFU/100mL were associated with 7-17 taxa whereas fecal coliform numbers between $5x10^2$ and $8.2x10^2$ CFU/100mL were always associated with a diversity of 6-12 taxa. BD14, which constantly presented high fecal coliform numbers ($3.5x10^4$ CFU/100mL) due to the continuos fecal discharges into the lake through this point, showed a higher diversity during the winter or rainy season as compared to the dry period. Hagler and Ahearn (7) noted that the number and diversity of moulds and yeasts reflected upon the organic nutrients, which in turn depended on the eutrophication level. Açude Velho lake exhibited a higher diversity of moulds and yeasts in the rainy season at four shore points, with high fecal coliform counts and organic pollution characterized by low circulation patterns (AV3, AV5, AV13, AV14). A pattern similar to that observed in Bodocongó was evident in the results from Açude Velho. Diversities of 8 - 23 taxa were found in points with $1.2x10^3$ - $8.4x10^3$ CFU/100mL, and of 1 to 11 genera in association with counts ranging from $2.4x10^2$ to $9.6x10^2$ CFU/100mL. The large number of genera in AV5 (up to 23) was attributed to a high variety of substrates: decomposing aquatic plants, inflow of sewage and suspended organic solids. The only source of organic pollution at AV9 was from sewage, and this resulted in high fecal coliform counts but in a richness of only 12 taxa. These data suggest that fungal diversity is associated with fecal coliform levels only where there is high substrate diversity.

**Lotic water bodies**

**Fecal coliforms and coliphages**. FIGURES 4 and 5 show the distribution of fecal coliforms and coliphages in Bodocongó stream and in São Salvador stream. The highest fecal coliform and coliphage numbers were obtained from Bodocongó stream ($1x10^6$ CFU/100 mL and $1x10^5$ PFU/100mL, respectively) at all the sampling stations during both wet seasons. During the winter periods, their numbers increased by nearly 16%. Septic tank effluents were probably responsible for this increased pollution. Furthermore, effluents from the abattoir enter the stream at BD4 and BD5

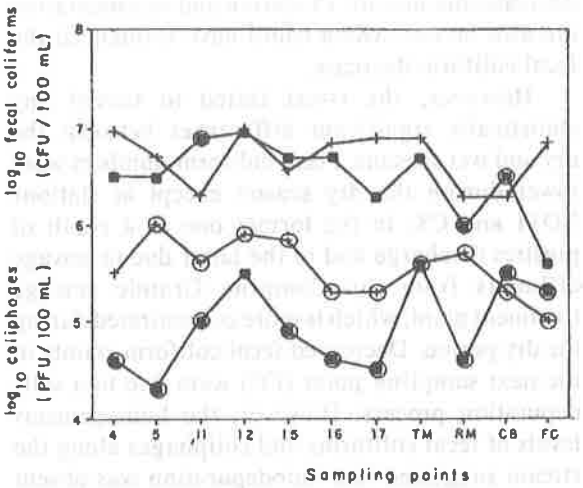


FIGURE 4 - Distribution of fecal coliforms (FC) and coliphages (Cfg) in the Bodocongó Stream in Paraíba State, Brazil. Geometric means. Rainy season: March-Aug. 1991; n=79. Dry season: Sep 1991 - Feb. 1992; n=76, (+ :FC, rainy season; ■ : FC, dry season; ⊕ : Cfg, rainy season; ● : Cfg, dry season)

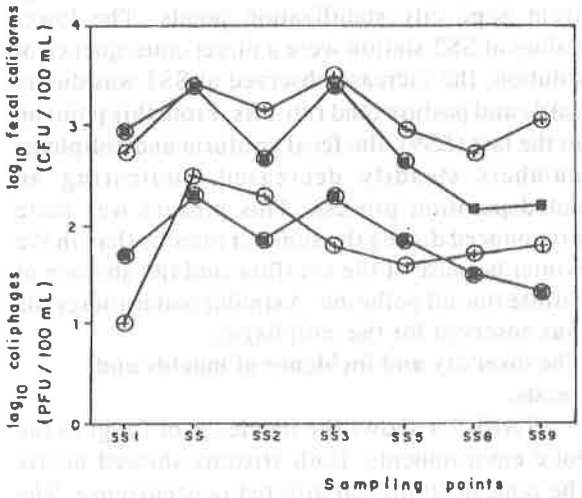


FIGURE 5 - Distribution of fecal coliforms (FC) and coliphages (Cfg) in São Salvador Stream in Paraíba State, Brazil. Geometric means. Rainy Season: Feb - August 1991 ; n = 64. Dry Season: Sep. 1991 - Jan. 1992 ; n=34 (+ : FC, rainy season; ■ : FC, dry season; ⊕ : Cfg, rainy season; ● : Cfg, dry season)

whereas the washing residues from pigsties are discharged at BD11. The lower concentration of fecal coliforms and coliphages at the RM station during summer was due to the repression effect at TM point, where irrigation by flooding was practiced. Consequently, the longer retention time and the macrophyte filter effect between the two points stimulated the fecal coliform and coliphage

decrease and die-off. Predation and sedimentation are also factors which could have influenced the fecal coliform decrease.

However, the t-test failed to reveal any statistically significant differences between the dry and wet seasons. Fecal coliform numbers were lower during the dry season except at stations BD11 and C8; in the former one as a result of pigsties discharge and in the latter due to sewage effluents from the Campina Grande sewage treatment plant, which is more concentrated during the dry period. Decreased fecal coliform counts in the next sampling point (FC) were due to a self-depuration process. However, the homogeneous levels of fecal coliforms and coliphages along the stream suggested that autodepuration was absent in nearly all this water body.

São Salvador stream presented the lowest coliform and coliphage values, particularly during the dry season. The higher values obtained at the SS sample point were due to the effluent discharge from Sapé city stabilization ponds. The lower values at SS2 station were a direct consequence of dilution; the increase observed at SS3 was due to cattle and pasture land run-offs. From this point up to the last (SS9), the fecal coliform and coliphage numbers steadily decreased, indicating an autodepuration process. This process was more pronounced during the summer months than in the winter because of the overflow and the absence of diffuse run-off pollution. A similar pattern of results was observed for the coliphages.

**The diversity and incidence of moulds and yeasts.**

TABLE 4 shows the incidence of fungi in the lotic environments. Both streams showed nearly the same diversity but differed in occurrence. The decreasing incidence of moulds in São Salvador stream was: *Penicillium* spp. *NSF*, *Rhizopus* spp. *Cladosporium* spp. *Aspergillus* spp. *A. niger*, *Mucor* spp. *Curvularia* spp. *Fusarium* spp. and *Cephalosporium* spp. which has a universal distribution in soil and water (12, 14, 16, 17). The yeasts, in decreasing order of frequencies, included: *Rhodotorula* spp. *Sporobolomyces* spp. *Candida* spp. *C. tropicalis*, *Saccharomyces* spp. *C. krusei* and *C. kefyr*. *C. albicans* was absent in this stream, whereas *Rhodotorula* spp. and *Sporobolomyces* spp. were present at most of the sampling points. *Candida* spp. were isolated only from stations receiving sewage or fecal contamination, indicating an association with organic contamination. There was no evidence of any genus specificity for the wet or dry season. NSF and *Penicillium* spp. had the highest incidence, followed by *Cephalosporium* spp. *Cladosporium* spp. *Rhizopus* in Bodocongó stream. Between the two streams, the frequency of incidence of NSF showed statistically significant differences (NSF: $\chi^2o = 21.77$; $\chi^2cr = 10.83$; $\alpha = 0,01$), suggesting its association with fecal pollution.

The yeasts isolated from Bodocongó stream were: *Candida* spp. *C. albicans*, *Rhodotorula* spp.

TABLE 4. Relative frequency of appearance of moulds and yeasts in the São Salvador and Bodocongó streams in Paraíba, Brazil, during the dry and rainy seasons

| water body | São Salvador stream | | | Bodocongó stream | | |
|---|---|---|---|---|---|---|
| period | C2 | S3 | total | C2 | S3 | total |
| No. of samples | (55) | (47) | (102) | (97) | (80) | (177) |
| MOULDS | | | | | | |
| *A. achraceus* | 9 | 4 | 7 | 5 | 3 | 4 |
| *A. flavus* | 2 | 19 | 10 | 9 | 19 | 14 |
| *A. niger* | 20 | 13 | 17 | 9 | 16 | 12 |
| *A. terreus* | 13 | 11 | 12 | 5 | 5 | 5 |
| *Absidia spp.* | 2 | 0 | 1 | 0 | 0 | 0 |
| *Aspergillus spp.* | 11 | 26 | 18 | 11 | 10 | 11 |
| *Cephalosporium spp.* | 13 | 13 | 13 | 18 | 26 | 21 |
| *Cladosporium spp.* | 18 | 23 | 21 | 22 | 14 | 18 |
| *Curvularia spp.* | 20 | 9 | 15 | 10 | 5 | 8 |
| *Epicoccum spp.* | 2 | 0 | 1 | 0 | 0 | 0 |
| *Fusarium spp.* | 16 | 11 | 14 | 4 | 5 | 5 |
| *Geotrichum spp.* | 16 | 13 | 13 | 4 | 4 | 4 |
| *Monilia sitophyla* | 11 | 15 | 13 | 6 | 3 | 5 |
| *Mucor spp.* | 18 | 11 | 15 | 19 | 3 | 11 |
| *NSF* | 27 | 21 | 25 | 65 | 41 | 54 |
| *Penicillium spp.* | 36 | 53 | 41 | 54 | 56 | 55 |
| *Rhizopus spp.* | 15 | 30 | 22 | 21 | 39 | 29 |
| *Syncephalastrum spp.* | 4 | 0 | 2 | 0 | 0 | 0 |
| No. of taxa | 18 | 15 | 18 | 15 | 15 | 15 |
| YEASTS | | | | | | |
| *C. albicans* | 0 | 0 | 0 | 16 | 9 | 13 |
| *C. krusei* | 2 | 0 | 1 | 0 | 1 | 0.6 |
| *C. kefyr* | 0 | 2 | 1 | 0 | 0 | 0 |
| *C. tropicalis* | 9 | 2 | 6 | 5 | 6 | 6 |
| *Candida spp.* | 7 | 4 | 6 | 9 | 18 | 13 |
| *Rhodotorula spp.* | 20 | 15 | 17 | 5 | 8 | 6 |
| *Saccharomyces spp.* | 2 | 2 | 2 | 3 | 3 | 3 |
| *Sporobolomyces spp.* | 15 | 11 | 13 | 6 | 10 | 8 |
| *Torulopsis spp.* | 0 | 0 | 0 | 1 | 0 | 0.6 |
| No. of taxa | 6 | 6 | 7 | 8 | 8 | 9 |
| Total number of taxa | 24 | 21 | 25 | 23 | 23 | 24 |

*Sporobolomyces* spp. *C.tropicalis,* and *Saccharomyces* spp.. Several workers associated this pattern with fecal and organic pollution (8).

Species diversity in the Bodocongó stream could be grouped into three levels: a high level from station BD4 to BD11, which had 16 taxa and received a greater variety of effluents (three different industries, abbattoir and domestic sewage); a middle level from station BD12 to BD17, with a maximum of 14 taxa and also characterized by a larger river bed and the lowest diversity associated with low water-flux and spore sedimentation; and a third level for the lower region from TM to FC, which showed a new increase in diversity, up to 15 taxa, due to further addition of new substrates and sewage effluents.

São Salvador stream showed a higher diversity during the rainy periods in 5 out of 7 stations, higher values (17 taxa) concentrating between point SS, with the stabilization pond discharge, and point SS3. In both stations, fecal coliform numbers were the highest. The results from both streams suggest a relationship between high fungal diversity and high levels of pollution and richness of substrates. In particular, *C.albicans* exhibited a strong association with fecal pollution in running waters. It was possible to conclude that the different levels of organic and fecal pollution determined the diversity of species and the frequency of isolation of fungi in both lotic environments.

Based on the results on fecal coliforms, the five water bodies were classified according to the Brazilian legislation (CONAMA Nº 20, 1986) in order to establish whether their uses were within recommended standards. The Brazilian classification of natural water according to bacteriological standards is based on a maximum number of fecal coliforms, which should not be exceeded in at least 80% of 5 samples collected during one month. Due to this regulation, and because this research was initiated with monthly or forthnightly monitoring, each dry and wet period was used as a whole, thus permitting a reasonable number of samples for analysis and comparisons between the rainy and dry seasons.

In general, the water body quality deteriorated during the rainy seasons. Açude de Boqueirão was classified as Class 1 during both summer periods and as Class 2 during both wet periods. It cannot be used for unrestricted irrigation or for drinking purposes without treatment. Açude de Bodocongó was found to belong to Class 3 during both dry seasons and as Class 4 during the wet seasons, meaning its use in the summer can be extended to domestic purposes after treatment, as a source of drinking water for animals and also for restricted irrigation. During the rainy seasons, its use should be interdicted. Açude Velho was classified as Class 4 during the four periods, and its use is limited to a landscape visual effect.

Riacho de Bodocongó was found to be of the worst quality, and was placed in Class 4. In some parts, this stream had bad odor with floating materials and oils indicating a diverse high pollution level. Its use must also be prohibited. Riacho São Salvador belonged to Class 3 in the dry and wet seasons and its use has the same limitation as that of Bodocongó lake summer waters. Only water from Açude de Boqueirão had the sort of quality compatible with bathing.

The results showed that in both lentic and lotic waters there was a low number of genera or species of fungi systematically associated with fecal and organic pollution, which could probably be used as indicators. In particular, *Candida* spp. *C.albicans* and NSF presented higher frequencies of isolation in environments with the highest fecal contamination, suggesting their use as complementary indicators of feces or sewage discharges in tropical semi-arid freshwaters. Regional studies on substrate diversity could lead to a better understanding of the distribution and richness of geofungi.


## RESUMO

### Distribuição espacial e temporal de coliformes fecais, colifagos, bolores e leveduras em água doce na região tropical semi-árida do nordeste brasileiro (Estado da Paraíba)

A distribuição de coliformes fecais, colifagos, bolores e leveduras foi avaliada durante as épocas de seca (verão) e de chuva (inverno) em três lagos e dois riachos localizados na região semi-árida do Nordeste brasileiro, no Estado de Paraíba.

O açude de Boqueirão foi o único apropriado para recreação de contato primário, mas não para irrigação irrestrita. A diversidade de fungos au-

mentou com a contaminação fecal e com a chegada da época de chuva. Os bolores estiveram presentes em todas as amostras, enquanto que as amostras positivas para leveduras aumentaram com a contaminação fecal ($\chi^2o = 69$; $\chi^2cr = 9.21$; $\alpha = 0.01$). *Candida* spp. e *Rhodotorula* spp. apresentaram maiores frequencias de isolamento nos ambientes mais poluidos, nos quais *Candida* spp. apresentou maior diversidade (7 espécies). A incidência de FNE, *Candida* spp. e *C.albicans* foi maior nas águas mais poluidas, evidenciando diferenças estatisticamente significativas (FNE: $\chi^2o = 26.2$; *Candida* spp. $\chi^2o = 12.96$; $\chi^2cr = 9.21$; $\alpha = 0.01$). Os riachos não apresentaram estas diferenças; entretanto, a incidência de FNE e de *C.albicans* associou-se com o nível de contaminação fecal. Os resultados sugerem que FNE, *Candida* spp. e *C.albicans* são indicadores potenciais da contaminação fecal em corpos lênticos e lóticos do trópico semi-árido. Estudos regionais sobre a diversidade de substratos podem contribuir para uma melhor compreensão da distribuição e riqueza dos geofungos.

**Palavras-chave:** colifagos, coliformes fecais, fungos, água doce, trópico, semi-árido

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# INCIDENCE OF *MYCOBACTERIUM TUBERCULOSIS* AND OTHER MYCOBACTERIA ON PULMONARY INFECTIONS IN ARARAQUARA - SP, 1993

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## ABSTRACT

The incidence of tuberculosis and other infections by mycobacteria was analyzed in 559 patients admitted to the Tisiology Section of the Special Health Care Unit of Araraquara (SESA). Mycobacteria were isolated from 78 individuals out of this total. Among these patients, 15 were also HIV positive. The occurrence of isolated species was: *M. tuberculosis*: 69 patients; *M. avium-intracellulare*: 5 patients; *M. fortuitum*: 2 patients; *M. chelonae*: 1 patient; and *M. simiae* 1 patient. The latter was for the first time isolated from humans in Brazil. In most cases, non tubercular mycobacteria (NTM) were found in the HIV positive patients.

**Key words:** tuberculosis, mycobacteria, *M. tuberculosis*

## INTRODUCTION

In spite of the accomplishment of extensive treatment programs with tuberculostatic drugs and BCG vaccination, tuberculosis is one of the major "causa mortis" associated with identified infectious agents (3). Recent epidemiological data show a net annual increase in the incidence of tuberculosis in Brazil (12). The figures are almost the same as in the rest of the world (9). During 1992, for example, 78581 new cases of tuberculosis were notified in Brazil, of which 16.610 were from the State of São Paulo; 378 cases of disease were recorded just in the São Paulo city itself (13). The tuberculosis control programs are faced today with a new challenge: drug resistance to antimicrobial agents (2, 12) and association with AIDS(8, 15). At present, in the big Brazilian cities, about 10% of the new cases of tuberculosis are shown to be related with AIDS, while 40% of the AIDS patients have tuberculosis (4). The clinical evolution of tuberculosis has changed with AIDS, from a usually chronic illness in immunologically competent individuals to dramatically progressive in HIV carriers with AIDS (8). AIDS also favours the development of opportunistic mycobacterial infection (19). In contrast to *M. tuberculosis*, these opportunistic mycobacteria are not transmitted from person to person (19), since they are ubiquitous in nature (7, 10). They resist to most tuberculostatic drugs (19) and, when isolated from clinical specimens, should not be necessarily seen as

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etiologic agents of disease (18). All these considerations show the importance of species identification after isolating mycobacteria from clinical specimens.

The Special Health Care Unit of Araraquara (Serviço Especial de Saúde de Araraquara; SESA) develops control programs of infectious diseases. For tuberculosis in particular, SESA centralizes its epidemiological control in the city of Araraquara and Araraquara region. The laboratorial diagnosis of tuberculosis is made there only by bacilloscopy and culture techniques using sputum and other clinical specimens. Since 1993, in collaboration with the Microbiology Laboratory of the Faculty of Pharmacy of UNESP, mycobacterial identification has been carried out up to the species level.

The aim of the present work was to investigate the occurrence of tuberculosis and other infections by mycobacteria, as well as their relationships with AIDS, in the Araraquara region during the year of 1993.

## MATERIALS AND METHODS

1677 sputum samples from 559 patients with presumptive pulmonary tuberculosis were analyzed in triplicates during 1993. Bacilloscopy was performed according to the Ziehl-Neelsen technique (6). Cultures were carried out in Lowestein-Jensen (LJ) medium after alkaline sputum decontamination (6). The culture tubes were incubated at 30°C, 35°C and 42°C for 40 days, and inspected weekly for bacterial growth. The mycobacteria were identified by analysis of mycolic acid profile using thin layer chromatography (5), some biochemical tests, like the niacin and nitrate assays (6), and growth tests on Nutrient Agar medium, MacConkey agar medium without crystal violet (6), and LJ medium supplemented with p-nitrobenzoate (PNB) (6) or thiophene-2-carboxylic acid hydrazide (TCH) (6).

## RESULTS AND DISCUSSION

Mycobacteria were isolated from 78 out of 559 patients suspected of tuberculosis; 15 patients were also infected with the human immunodeficiency virus. The results are presented in TABLE 1. The culture technique was more sensitive than the bacilloscopy, with detection of 14.0% positive responses by the former and 8,2% by the latter. This suggests that the bacilloscopy failed to diagnose mycobacterial carriers in 32 cases. Similar results have been reported by other researchers (9, 16). TABLE 2 summarizes the species of mycobacteria identified from 78 positive cultures, of which 88.4% were *M. tuberculosis*. This microorganism is strictly pathogenic and its presence in clinical material indicates a specific tuberculous infection. *M. tuberculosis* is the main etiologic agent of mycobacteriosis in the Araraquara region, even among HIV positive patients (53.3% of the infections caused by mycobacteria in this group). Silva *et al.* (17), of a total of 5.536 mycobacteria identified, found 99.9% to be *M. tuberculosis*, with *M. avium-intracellulare* and *M. szulgai* present only at low percentages (0,07 and 0,03%, respectively). These figures are close to the reported for other Brazilian regions[14].

Several other species of mycobacteria were identified in our laboratory from the pulmonary

**TABLE 1** - Bacilloscopy and cultures of samples from 559 patients suspected of pulmonary tuberculosis.

| Cases | Bacilloscopy | | Cultures | |
|---|---|---|---|---|
| | nº | % | nº | % |
| Positives | 46* | 8,2 | 78 | 14,0 |
| Negatives | 513* | 91,8 | 481 | 86,0 |
| Total | 559* | 100,0 | 559 | 100,0 |

* Results from triplicates.

**TABLE 2** - Species of mycobacteria isolated from HIV positive and negative patients.

| Mycobacteria | Patients | | | | | |
|---|---|---|---|---|---|---|
| | Isolation infection | | without HIV infection | | with HIV | |
| | nº | % | nº | % | nº | % |
| *M. tuberculosis* | 69 | 88,4 | 61 | 96,8 | 8 | 53,3 |
| *M. avium-intracelullare* | 5 | 6,4 | 1 | 1,6 | 4 | 26,6 |
| *M. fortuitum* | 2 | 2,6 | 1 | 1,6 | 1 | 6,7 |
| *M. chelonae* | 1 | 1,3 | 0 | 0 | 1 | 6,7 |
| *M. simiae* | 1 | 1,3 | 0 | 0 | 1 | 6,7 |
| Total | 78 | 100,0 | 63 | 100,0 | 15 | 100,0 |

infection specimens, among them *M. avium-intracellulare, M. fortuitum, M. simiae* and *M. chelonae*. These are considered potentially pathogenic bacilli (19) and cause a variety of clinical manifestations. *M. avium-intracellulare* came second in incidence, corresponding to 6.4% of all the mycobacterial isolates. It was more frequent among patients with AIDS (26,6%), a feature that is consistent with results reported by other researchers (4, 8, 14). *M. fortuitum* came in third place, with an incidence of 2.6%, being present in both HIV positive and negative patients. In the latter group of patients, the presence of *M. fortuitum* does not indicate that an infectious process is taking place, since this microorganism is capable of colonizing the human respiratory tract as a saprophyte. *M. chelonae* and *M. simiae* were found only in HIV positive patients. The isolation of *M. chelonae* from infections has already been described in Brazilian publications (14), but our finding of *M. simiae* is probably the first confirmed in Brazil. In other countries, *M. simiae* is associated with chronic pulmonary disease (1,18).

In order to evaluate a set of physiological and biochemical features, the mycobacteria were classified according to their growth time and mycolic acid profile. In the first group (TABLE 3) were included the fast growing and potentially pathogenic species, namely *M. fortuitum*, with mycolic acids I and V, and *M. chelonae*, with mycolic acids I and II. Both species are biochemically active and may be easily distinguished from other rapidly growing mycobacteria, normally non pathogenic, owing to their growth ability on Mac Conkey Agar and positive arylsulphatase test after 3 days of incubation (6). In the second group (TABLE 4) were included the slow-growing mycobacteria. Their mycolic acid profiles were as follows: I.III.IV for *M. tuberculosis*, I.IV.VI for *M. avium-intracelulare* and I.II.IV for *M. simiae*. In this group, the niacin assay is a very appropriate biochemical approach test for species identification, since *M. tuberculosis* is one of the few mycobacteria that produces nicotinic acid in detectable amounts (6). *M.avium-intracellulare* is resistant to PNB and TCH, and shows negative responses on niacin and nitrate tests (6). *M. simiae* may be mistaken for *M. tuberculosis* because its niacin reaction is variable (1). The *M. simiae* isolated in this study had mycolic acids I.II.IV, poorly positive reaction for niacin, showed negative test for nitrate and resistance to PNB and TCH. All these features are characteristic of *M. simiae* (18).

The National Tuberculosis Control Program of the Ministry of Health (11) regards bacilloscopy as the basic examination whereas *in vitro* culture is recommended only when the bacilloscopy provides no positive information yet the patient shows clinical symptoms and radiological signs of pulmonary tuberculosis. However, our investigation clearly shows the importance of species identification, since the opportunistic mycobacteria are often drug resistant and must be recognized at an early stage. A successful treatment depends on precise diagnostic and drug susceptibility tests, particularly for the debilitated AIDS patients. These considerations apply to *M. tuberculosis*, which is beginning to exhibit strain resistance to tuberculostatic drugs (12).

**TABLE 3** - Physiological and biochemical features of *M. fortuitum* (mycolic acids I.V) and *M. chelonae* (mycolic acids I.II).

| Characteristics | *M. fortuitum* | | | *M. chelonae* | |
|---|---|---|---|---|---|
| | ATCC 6841 | 2 isolated 1st | 2nd | ATCC 19.977 | 1 isolated |
| Growth for < 7 days | + | + | + | + | + |
| Growth at 30°C | + | + | + | + | + |
| 37°C | + | + | + | + | + |
| Growth in the presence of: | | | | | |
| PNB | + | + | + | + | + |
| TCH | + | + | + | + | + |
| Growth on: | | | | | |
| Nutrient Agar < 7 days | + | + | + | + | + |
| Mac Conkey Agar < 7 days | + | + | + | + | + |
| Biochemical tests: | | | | | |
| Niacin | - | - | - | - | - |
| Nitrate reductase | + | + | + | - | - |

Symbols: + = positive reaction; - = negative reaction; v = variable results;
% = percentage of strains positive.
Abridgment: ACT = American Type Collection Culture;
PNB = paranitrobenzoic acid 500 g/mL; TCH = tiophene-2-carboxylic acid 2 g/mL.

**TABLE 4** - Physiological and biochemical features of *M. tuberculosis* (mycolic acids I.III.IV), *M. avium-intracellulare* (mycolic acids I.IV.VI) and *M. simiae* (mycolic acids I.II.IV).

| Characteristics | *M. tuberculosis* | | *M. avium-intracellulare* | | *M. simiae* | |
|---|---|---|---|---|---|---|
| | ATCC 27294 | 69 isolated % | ATCC 25291 | 5 isolated % | ATCC 25275 | 1 isolated |
| Growth for > 10 days | + | 100 | + | 100 | + | + |
| Growth at 30°C | - | 69 | - | 0 | [+] | - |
| 37°C | + | 100 | - | 100 | + | + |
| Growth on PNB | - | 0 | + | 100 | + | + |
| TCH | + | 100 | + | 100 | + | + |
| Biochemical tests Niacin | + | 88,40 | - | 0 | v | [+] |
| Nitrate reductase | + | 91,30 | - | 0 | - | - |

Symbols: + = positive reaction; - = negative reaction; [+] = weak reaction; v = variable result;
% percentage of positive strains.
Abridgment: ATCC = American Type Collection Culture; PNB = paranitrobenzoic acid 500 g/mL; TCH = tiophene-2-carboxylic acid 2 g/mL.

In conclusion, our results bring to light the gravity of the new trends presently observed for tuberculosis itself and for other infections by mycobacteria in the Araraquara region.

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## RESUMO

**Incidência de *Mycobacterium tuberculosis* e de outras micobactérias em infecções pulmonares em Araraquara, SP, 1993**

Estudou-se a incidência da tuberculose e de outras micobacterioses entre os pacientes atendidos no Setor de Tisiologia do Serviço Especial de Saúde de Araraquara - SESA, no ano de 1993. Analisou-se 559 pacientes, verificando-se positividade em 78, dos quais 15 eram simultaneamente portadores do virus da AIDS. Dos 78 casos novos, 69 estavam infectados por *M. tuberculosis* e outros 9 por micobactérias oportunistas, sendo 5 por *M. avium-intracellulare*, 2 por *M. fortuitum*, 1 por *M. chelonae* e 1 por *M. simiae*. O isolamento deste último ainda não havia sido descrito em humanos no Brasil. Verificou-se maior incidência das micobacterioses por micobacterias oportunistas entre os pacientes HIV positivos.

**Palavras-chave**: tuberculose, micobactérias, *M. tuberculosis*

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# EVALUATION OF DIFFERENT TECHNIQUES FOR THE DIFFERENTIATION OF PATHOGENIC AND NON PATHOGENIC STRAINS OF *YERSINIA ENTEROCOLITICA*

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## ABSTRACT

Different methods and tests have been used to evaluate the pathogenic potential of distinct *Y. enterocolitica* serotypes and biotypes. We tested a total of 60 *Y. enterocolitica* strains, being 25 of human origin (serotype O3 biotype 4 and serotype O5 biotype 1); 6 of animal origin (serotype O3 biotype 4); 19 isolated from the environment (serotype O5.27 biotypes 1 and 2); and 8 isolated from food (serotype O5 biotype 1 and serotype O5.27 biotype 1). The methods used were based on plasmid gene expression (autoagglutination, calcium-dependence at 37°C and Congo Red absorption tests), chromosomal gene expression (assays for pyrazinamidase activity, salicin fermentation and esculin hydrolysis), and invasion of HEp-2 cells. All but one of the *Y. enterocolitica* O3 strains,were found to be potentially pathogenic when submitted to the pyrazinamidase-salicin-esculin tests. In contrast, the results obtained with the assays related to plasmidial gene expression were not so uniform, probably due to plasmid loss. The least homogeneous results were obtained with the HEp-2 cell invasion test. *Y. enterocolitica* O5 behaved in a uniform manner when tested with the first two groups of tests (based on chromosomal and plasmidial gene expression), but not when tested with the HEp-2 invasion assay. The strains of serotype O5.27 biotype 1 presented a uniform behavior when submitted to the chromosomic-related tests, showing no pathogenicity. However, they did not provide conclusive results with the tests related to plasmidial gene expression or HEp-2 cell invasion. We conclude that the tests related to chromosomal gene expression (esculin-salicin-pyrazinamidase) are simple and highly effective for the detection of potentially pathogenic *Y. enterocolitica* isolated from clinical cases.

**Key words**: *Yersinia enterocolitica*, pathogenicity, esculin hydrolysis, salicin fermentation, pyrazinamidase activity.

## INTRODUCTION

*Yersinia* has been considered to be an enteric pathogen even though some species or some serotypes within the same species are not correlate with human disease.

Until recently, the pathogenicity of these microorganisms was related to the presence of a 40-48 MDa plasmid (pYV)(5,8,13,17), and the expression of this genetic determinant was associated with several phenotypic properties such as autoagglutination at 37°C, adherence to HEp-2

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cells, Congo Red absorption, calcium dependence at 37°C, and resistance to killing by normal human serum, among others. Therefore, these tests were used to define the pathogenicity of *Yersinia* and were correlated with the presence of the virulence plasmid and hence with the ability of this microorganism to cause disease in man.

The involvement of chromosomal genes in the pathogenicity of *Yersinia* was later shown to be related to certain phenotypic characteristics such as activity of the enzyme pyrazinamidase and the abilities to hydrolyze esculin and to ferment salicin (1,4). These properties, not being plasmidial, are more stable.

Regarding mammalian cell invasion by *Y. enterocolitica*, it is now known that three independent pathways are involved: one plasmidial (pYV) and two chromosomal (*inv* and *ail* locus) (20).

Thus, several tests and methods have been recommended to separate pathogenic from nonpathogenic *Y. enterocolitica* (7,12,19). However, due to the diversity of the genetic factors involved, there is some controversy over defining which of them identify better the pathogenic potential of *Yersinia* strains and serotypes.

In an attempt to contribute to the development of a rapid method for the separation of pathogenic and nonpathogenic *Y. enterocolitica* strains, we compared the performance of the tests based on chromosomal gene expression, plasmidial gene expression and invasion of HEp2 cells commonly used to evaluate *Y. enterocolitica* virulence. Collection strains of *Y. enterocolitica* of different serotypes and biotypes and of different origins were used in the present study.

## MATERIALS AND METHODS

**Bacterial strains**: A total of 60 *Y. enterocolitica* collection strains previously activated in mice were studied: 26 *Y. enterocolitica* O3 biotype 4; 15 *Y. enterocolitica* O5 biotype 1; 4 *Y. enterocolitica* O5.27 biotype 1; 15 *Y. enterocolitica* O5.27 biotype 2. They were received at the Reference Laboratory on *Yersinia* in Brazil, Faculdade de Ciências Farmacêuticas, Araraquara, UNESP, for complete identification, after being isolated in the following different Institutions: Instituto de Microbiologia, UFRJ; CETESB, São Paulo; Faculdade de Medicina, USP, Ribeirão Preto; Faculdade de Ciências Farmacêuticas, USP, São Paulo; Faculdade de Ciências Farmacêuticas, UNESP, Araraquara; Secretaria da Agricultura do Estado do Paraná; Universidade de Londrina, Paraná; ITAL, Campinas; Laboratório Faillace, Porto Alegre and Laboratório LIAC, São Paulo.

The general characteristics of the strains are presented in TABLE 1.

TABLE 1 - General characteristics of the 60 *Y. enterocolitica* strains

| Origin | Bioserophage type | Total of strains | Number/Source |
|---|---|---|---|
| Human | 4/O3/VIII | 20 | 18 diarrheic feces<br>1 normal feces<br>1 ascitic fluid |
| | 1/O5/Xz | 5 | 5 normal feces |
| Animal | 4/O3/VIII | 4 | 1 normal feces<br>3 diarrheic feces |
| | 4/O3/IXa | 2 | 2 diarrheic feces |
| Food | 1/O5.27/Xz | 2 | 2 ground meat |
| | 1/O5/Xz | 6 | 2 milk<br>3 ground meat<br>1 beef hamburger |
| | 1/O5/Xo | 2 | 2 ground meat |
| Environment | 1/O5.27/Xz | 2 | 2 freshwater |
| | 2/O5.27/Xz | 15 | 15 freshwater |
| | 1/O5.27/Xz | 2 | 2 freshwater |

**Activation of the *Y. enterocolitica* strains**: Two female Swiss mice, five to six weeks old, were inoculated intravenously with 0.5 mL of a $10^8$ saline suspension of each strain. After 24 hours, the animals were killed and the spleens removed. A saline homogenate of each spleen was prepared with inoculated onto blood agar base plates, which were incubated at 25°C for 48h. The growth present on these plates was used for the different tests (6).

**Pyrazinamidase activity**: The test was performed according to Kandolo and Wauters (12) using pyrazinamide agar incubated at 25°C for 48 hours, and tested with 1 mL of a freshly prepared 1% solution of ferrous ammonium sulfate. A positive pyrazinamidase reaction was indicated by a pink to brown color that developed on the slant. A positive test indicates a nonpathogenic serotype

while a negative test indicates a pathogenic one.

**Salicin fermentation**: Salicin fermentation was determined according to Farmer III *et al* (7), using enteric fermentation base with 1% salicin and Andrade indicator, incubation at 25°C and final reading after 2 days. A positive reaction corresponds to a nonpathogenic serotype.

**Esculin hydrolysis**: Esculin hydrolysis was performed as recommended by Farmer III *et al* (7) using esculin broth incubation at 25°C and final reading after 2 days. A positive reaction indicates a nonpathogenic serotype.

**Detection of the 40-48 MDa plasmid**: Plasmidial DNA was obtained by the technique of Birnboim and Doly (2) and submitted to vertical electrophoresis on agarose gel (15). The gel was stained with 0.4 g/mL ethidium bromide and visualized under ultraviolet light.

**Autoagglutination at 37°C**: The test was performed in duplicate by the method of Laird and Cavanaugh (13), using Dulbecco modified minimal essential medium (MEM modified). One of the tubes was incubated at 25°C and the other at 37°C for 24 h. Readings were taken with special care to detect the formation of clusters, which indicated positive autoagglutination reactions.

**Calcium dependence at 37°C and Congo Red absorption (CR-MOX)**: The methodology used was that proposed by Ryley and Toma (19) using Congo red-magnesium oxalate agar (CR-MOX). The strains were first grown on blood agar plates at 22°C for 18 hours and then plated onto CR-MOX and incubated for 24 h at 37°C. Positive CR-MOX strains produced small red colonies and CR-MOX negative large colorless colonies.

**Invasion of HEp-2 cells**: For this test the technique recommended by Lawson *et al.* (14) was used to detect HEp-2 cell invasion by *Aeromonas* sp, with some modifications: a monolayer of $5.0 \times 10^4$ HEp-2 cells/mL in Dulbecco modified minimal essential medium (MEM modified) containing 5% fetal bovine serum was grown for 18 h in a 24-well tissue microplate at 37°C, with 5% $CO_2$. The monolayer was infected with $10^6$ cells grown overnight in BHI at 25°C and diluted in 2mL of MEM. After 3h of incubation, the cells were washed three times with PBS, pH 7.4. Fresh MEM was added to the monolayers, which were incubated for a further 2 h, washed again three times and reincubated for 2 h in fresh MEM. The cell monolayers were washed four times, fixed in a mixture of 3:1 methanol and acetic acid for 10 min., and stained with Giemsa for 15 min. The microplates were then examined under the light microscope and the number of infected cells was recorded. Enumeration of intracellular bacteria was not performed.

## RESULTS

The results of all the tests are summarized in TABLE 2.

All but one of the 26 strains of *Y. enterocolitica* O3 biotype 4, isolated from human and animal clinical cases, presented characteristics of virulence according to negative pyrazinamidase-esculin-salicin tests, but only 15 strains (57.7%) carried the 40-48MDa plasmid and were positive for the autoagglutination and CR-MOX tests. Also, only 50% of the 26 strains invaded HEp-2 cells.

Fifteen strains of *Y. enterocolitica* O5 biotype 1 isolated from food, from the environment or from non-diarrheic human feces were tested. All but one were found to be nonpathogenic on the basis of positive pyrazinamidase - esculin-salicin tests. However, a 40-48 MDa plasmid and its accompanying phenotypic characteristics (autoagglutination and CR-MOX) were observed in 46.7% of them. Also, 6 strains (40%) invaded HEp-2 cells.

The 19 strains of *Y. enterocolitica* O5.27 biotypes 1 and 2 were isolated from the environment and from food. All the 4 strains belonging to biotype 1 were found to be nonpathogenic according to positive pyrazinamidase-esculin-salicin tests, and 50% were noninvasive. Only one of the 15 strains of *Y. enterocolitica* O5.27 biotype 2 was found to be nonpathogenic as shown by the positive pyrazinamidase-salicin-esculin tests, while 60% of them were noninvasive. None of the *Y. enterocolitica* O5.27 presented the virulence plasmid or the characteristics associated to it.

## DISCUSSION

Cornelis *et al.*(4) showed that the expression of the 40-48 MDa plasmid (pYV) is related to the secretion of eleven proteins called YOPs and to the production of at least two outer membrane proteins

TABLE 2 - Reactions of the *Y. enterocolitica* strains to the various tests applied.

| Properties | Ye O3 biotype 4 | | Ye O5 biotype 1 | | Ye O5.27 biotype 1 | | Ye O5.27 biotype 2 | |
|---|---|---|---|---|---|---|---|---|
| | Positive | | Positive | | Positive | | Positive | |
| | nº | % | nº | % | nº | % | nº | % |
| Pyrazinamidase activity | 1/26* | 3.8 | 14/15* | 93.3 | 4/4* | 100.0 | 1/15* | 6.7 |
| Salicin fermentation | 1/26 | 3.8 | 14/15 | 93.3 | 4/4 | 100.0 | 1/15 | 6.7 |
| Esculin hydrolysis | 1/26 | 3.8 | 14/15 | 93.3 | 4/4 | 100.0 | 1/15 | 6.7 |
| 40-48MDa plasmid | 5/26 | 57.7 | 7/15 | 46.7 | 0/4 | 0.0 | 0/15 | 0.0 |
| Autoagglutination at 37°C | 15/26 | 57.7 | 7/15 | 46.7 | 0/4 | 0.0 | 0/15 | 0.0 |
| CR-MOX** | 15/26 | 57.7 | 7/15 | 46.7 | 0/4 | 0.0 | 0/15 | 0.0 |
| HEp-2 invasion | 13/26 | 50.0 | 6/15 | 40.0 | 2/4 | 50.0 | 6/15 | 40.0 |

* Total of strains studied.

** CR-MOX = calcium dependence at 37°C and Congo Red absorption.

called Yad A and YLp A. Yad A is a polymer consisting of 4 to 5 subunits with a molecular mass of 45 to 52.5 kDa that form a fine fibrillar structure on the surface of *Y. enterocolitica*. It has been demonstrated *in vitro* that secretion of this outer membrane protein leads to increased hydrophobicity of the cell surface and promotes, among other properties, autoagglutination (3). Thus, it may be inferred that all cells carrying the intact pYV plasmid should have the property of autoagglutination. Our data agree with this statement since the present study showed a close correlation between the presence of the pYV plasmid and the occurrence of autoagglutination, and are also in agreement with the data reported by other authors (18,21), who consider that the autoagglutination assay is the best test for detecting the presence of this plasmid.

Additionally, we also observed a correlation between results from the rapid and simple CR-MOX test and the occurrence of the 40-48 MDa plasmid; strains of pathogenic serotypes carried the *Yersinia* virulence plasmid and were CR-MOX positives.

Farmer *et al.* (7) have demonstrated a high correlation between positive CR-MOX tests and the presence of the 40-48 MDa plasmid. They also showed a high correlation between the behavior of *Y. enterocolitica* strains and the pyrazinamidase-salicin-esculin tests, which they indicated as tests of choice for the separation of pathogenic and nonpathogenic strains of *Y. enterocolitica*, as also demonstrated in our study. However, we observed that biotypes 1 and 2 of serotype O5.27 behaved differently with respect to these two group of tests: strains of biotype 1 behaved as nonpathogenic while those belonging to biotype 2 did not behave in a uniform manner.

The invasion test with HEp-2 cells presented the weakest correlation with the others. Nowadays it is known that three different gene products are responsible for the uptake of enteropathogenic *Yersinia* by mammalian cells: the chromosomal genes *inv* and *ail* and the plasmidial Yad A protein (9). Some authors (10,16,18,23) have shown that cellular penetration of enteropathogenic *Yersinia* species is mediated mainly by invasin, the product of a chromosomal *inv* gene that initiates entry by binding to mammalian cell receptors; *Y. enterocolitica* also has the chromosomal *ail* gene

product which facilitates bacterial adhesion and/or invasion. In addition to factors encoded by chromosomes, the *Yersinia* virulence plasmid encodes adhesins that also play a role in the adherence/invasion properties of the bacterium. One of such adhesins is the Yad A protein (22,23). Isberg (11) demonstrated that chromosomal insertion mutations which eliminate the production of the *inv* gene show residual internalization that is dependent on the presence of the *Yersinia* virulence plasmid.

We conclude that the HEp-2 cell invasion test and the assays related to plasmidial gene expression are not so good for differentiating pathogenic from non pathogenic *Y. enterocolitica* collections strains. On the other hand, the pyrazinamidase-salicin-esculin tests are of great value in the separation of pathogenic from nonpathogenic strains of *Y. enterocolitica*, with the exception of *Y. enterocolitica* O5.27 biotype 2.


## RESUMO

### Avaliação de técnicas diversas na diferenciação de cepas patogênicas de não patogênicas de *Yersinia enterocolitica*

Diferentes métodos e testes para avaliar o potencial patogênico de *Y. enterocolitica* de sorotipos e biotipos diversos têm sido utilizados. Testamos 60 cepas do microrganismo, sendo 25 de origem humana (sorotipo 03 biotipo 4 e sorotipo 05 biotipo 1): 6 de origem animal (sorotipo 03 biotipo 4); 19 isoladas do meio ambiente (sorotipo 05.27 biotipos 1 e 2) e 8 isoladas de alimentos (sorotipo 05 biotipo 1 e sorotipo 05.27 biotipo 1). Foram utilizados testes relacionados à expressão de genes plasmidiais (autoaglutinação, dependência ao cálcio a 37°C e absorção do corante Vermelho Congo), de genes cromossomais (presença de enzima pirazinamidase, fermentação da salicina e hidrólise da esculina) além do teste de invasão em células HEp-2. Todas as *Y. enterocolitica* O3 exceto uma, foram potencialmente patogênicas frente aos testes pirazinamidase-salicina-escalina, o mesmo não ocorrendo com os testes relacionados a genes plasmidiais, quando os resultados não foram tão uniformes, provavelmente pela perda do plasmídeo; o teste de invasibilidade em células HEp-2 apresentou os resultados menos uniformes. As cepas de *Y. enterocolitica* O5 também tiveram comportamento uniforme, sendo não patogênicas frente aos dois grupos de testes (plasmidiais e cromossomais); em relação ao teste de invasão em células HEp-2 não houve uniformidade. As cepas do sorotipo O5.27 biotipo 1, mostraram-se uniformes em relação aos testes cromossomais, sendo não patogênicas; não apresentaram no entanto, resultados conclusivos nos testes relacionados à genes plasmidiais e invasão em células HEp-2. Conclui-se que os testes relacionados a genes cromossomais (esculina-salicina-pirazinamidase) são simples e muito eficazes na detecção de *Y. enterocolitica* potencialmente patogênicas, originárias de casos clínicos.


**Palavras-chave**: *Yersinia enterocolitica*, patogenicidade, hidrólise da esculina, fermentação da salicina, atividade da pirazinamidase.

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# PATHOGENIC ACTION OF *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* ON INBRED STRAINS OF MICE

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## ABSTRACT

The ability of fifty-seven *Actinobacillus actinomycetemcomitans* strains to cause abscesses on inbred strains of mice raised with barriers in the "Breeding Unit for Isogenic Mice" of ICB/USP was tested. Mice were injected subcutaneously with the bacterial suspensions. Twenty-three of the tested *A. actinomycetemcomitans* strains were found to cause the development of ulcerated and/or pus-filled abscesses in mice. Bacteria were recovered from all the injection sites, irrespective of abscess formation.

**Key words**: *Actinobacillus actinomycetemcomitans*; inbred mice; pathogenicity.

## INTRODUCTION

Animal models such as mice, rats, hamsters, and non-human primates have been used in periodontal disease research (18). Several microbial species related or not to periodontitis may destroy periodontal tissues in rodents as monoinfectans (17). The experimental animal currently used are models where specific clinical features of periodontal diseases can be demonstrated or where the growth of specific etiology agents can be assessed (8).

Studies on experimental mixed infections suggest that a synergism exists between bacterial populations (3, 4). The relationship between putative periodontal pathogenic species has been of interest in elucidating the mechanisms of subgingival colonization. Among these species is found *Actinobacillus actinomycetemcomitans*, a gram-negative non-motile coccobacillus frequently recovered from individuals with localized juvenile periodontitis, which has also been isolated from patients with endocarditis, with septicemia, and from brain and lung abscesses (10, 20).

The pathogenic potential of *A. actinomycetemcomitans* has been extensively studied *in vitro*. This oral bacterium produces many virulence factors, including a heat labile leukotoxin that kills human polymorphonuclear leukocytes and monocytes, but not lymphocytes, fibroblasts nor epithelial cells (2, 11, 19). However, *A. actinomycetemcomitans* as monocontaminant or in associations has not been very well studied in animal models. Unmasking the complex interactions between bacterial growth conditions, bacterial products, host factors, and disease progression requires a suitable *in vivo* model (8).

In this work, abscess formation following the subcutaneous injection of pure cultures of *A. actinomycetemcomitans* in inbred strains of mice was studied.

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## MATERIALS AND METHODS

**Bacteria**. The 57 *A. actinomycetemcomitans* strains used were isolated from 30 patients with periodontal disease selected from the Periodontal Clinic of the College of Dentistry, University of São Paulo, SP, Brazil. The patients exhibited radiographic evidence of alveolar bone loss confined to molar and incisor teeth and periodontal pockets deeper than 5 mm. Samples for microbiological analysis were obtained with two sterile paper points (Dentsply Ind. Co. Ltda., RJ, Brazil) from selected periodontal pockets. The paper points were pooled and transported to the laboratory in prereduced anaerobically sterilized Ringer solution. From this pooled sample, 10-fold dilutions were prepared in the Ringer solution, plated onto a selective trypticase soy-serum-bacitracin-vancomycin (TSBV) agar (14) and incubated under anaerobic conditions (90% $N_2$ + 10% $CO_2$). *A. actinomycetemcomitans* was identified on the basis of its specific colonial morphology on TSBV plates (star-like inner structure), production of catalase and non-fermentation of lactose, starch, sucrose and trehalose (15). One, and in some cases up to two, colonies of *A. actinomycetemcomitans* were selected from each subgingival sample. All *A. actinomycetemcomitans* isolates were stored in 20% glycerol at -70°C. Reference strains *A. actinomycetemcomitans* ATCC 29522, ATCC 29523 and FDC Y4 were included.

**Animals.** The inbred mouse strains Balb/c, C57BL/6, A/Sn, C3H/HePAS, cAF1, A/J, 129, NZW, CBA/J, NZB, C3H/HeJ, AKR, MDX, and B10.A, bred with barriers in the "Breeding Unit for Isogenic Mice" of ICB, University of São Paulo, SP, Brazil, were used. All the animals were five weeks old males weighing 25-30g. Food and water were supplied *ad libitum*.

**Preparation of inoculum.** *A. actinomycetemcomitans* strains were grown on brain heart infusion agar (Difco) supplemented with 0.5% yeast extract (Difco) and 5% sheep blood, under microaerophilic conditions (candle method) at 37°C. After 48 hours of incubation, four colonies were subcultured in 5mL of brain heart infusion broth (Difco) and again incubated as described above. The cells were harvested by centrifugation in phosphate-buffered saline (PBS), pH 7.4, at 10.000g x 15 minutes, and each suspension was adjusted to a concentration of approximately $10^8$ to $10^9$ cells/mL. The size of the inoculum was verified by the colonies count method (1).

**Experimental design.** 0.1 mL of each *A. actinomycetemcomitans* strain suspension was injected subcutaneously into the abdominal region of mice, at approximately 1 cm from the midline. The skin was shaved, disinfected with ethanol 70% (v/v) and allowed to dry previous to the injection. Mice were inoculated in duplicates and monitored for up to 10 days post-injection for lesion size. After this period, all mice were killed. Skin fragments including the lesion site were excised and examined for pus formation. Abscesses containing or not pus were regarded as positive. The fragments were cut through the middle and homogenised in PBS (3.0 mL). Microorganisms were recovered in thioglycolate medium 135-C (Difco) or in TSBV agar and incubated for five days under microaerophilic conditions (candle method) at 37°C. Control animals were injected with 0.1 mL of PBS by the same route. Additional control animals were submitted to trauma by the same route.

## RESULTS AND DISCUSSION

Ten days after injection, twenty-three *A. actinomycetemcomitans* strains caused ulcerated and/or pus-filled abscesses, which were large, round and dome-shaped, with approximately 6.0 ± 2.5 mm in diameter. Reference strains produced abscesses in at least two of the mouse strains used in this work (TABLE 1). Mice injected as duplicates showed similar responses to infection.

Microbial sampling of all mice injected with *A. actinomycetemcomitans* strains demonstrated the presence of these bacteria at all the injection sites, irrespective of abscess formation.

Historically, animal models have been invaluable for the establishment of the etiology, pathogenicity and prevention of human infectious diseases (5). Slots and Genco (16) described four stages of the pathogenesis of human periodontal disease: colonization, invasion, destruction, and healing. *A. actinomycetemcomitans* produces a number of factors that may be responsible for its pathogenicity, including enzymes and cytotoxic

TABLE 1. Abscess formation in inbred strains of mice after subcutaneous injection with *A. actinomycetemcomitans* strains.

| Bacterial Strains[1] | Mouse strains | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Balb/c | C57Bl/6 | A/Sn | C3H/HePAS | cAF1 | A/J | CBA/J | C3H/HeJ | NZB | MDX | 129 | NZW | AKR | B10.A |
| 1 | - | - | - | - | - | - | + | - | - | - | - | - | - | - |
| 3 | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 6 | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| 8 | - | - | - | - | - | - | - | + | - | - | - | - | - | + |
| 11 | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 13 | - | - | - | - | - | - | - | - | - | + | - | - | - | - |
| 20 | - | - | + | - | - | - | - | - | - | - | - | - | - | + |
| 25 | - | - | - | - | - | - | - | - | - | - | + | - | - | - |
| 27 | - | + | - | - | - | - | - | - | - | - | - | - | - | - |
| 30 | - | - | - | - | - | - | + | - | - | - | - | - | - | - |
| 31 | - | - | - | - | - | - | + | - | - | - | - | - | - | - |
| 34 | + | + | - | - | - | - | - | - | - | - | - | - | - | - |
| 36 | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 40 | - | - | - | - | - | - | - | + | - | - | - | - | - | + |
| 48 | + | - | - | - | - | - | - | - | - | - | - | - | - | + |
| 50 | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 55 | + | - | + | - | - | + | - | - | - | - | - | - | - | - |
| 57 | - | - | + | - | - | + | - | - | - | - | - | - | - | - |
| 58 | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| 60 | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| 70 | - | - | - | + | - | - | - | - | - | - | + | - | - | - |
| 71 | - | - | - | + | - | - | + | - | - | - | - | - | - | - |
| 74 | - | - | - | + | - | + | - | - | - | - | - | + | - | - |
| 29522[2] | + | - | - | - | - | - | - | - | - | - | + | - | - | - |
| 29523[2] | - | + | + | - | + | - | - | - | - | - | - | - | - | + |
| Y4[2] | - | - | + | - | - | + | - | - | - | - | - | + | + | + |

[1] *A. actinomycetemcomitans* strains that promote abscess formation in at least one mouse strain
[2] Reference strains.

metabolites which may damage host tissues directly, or induce an inflammatory response thus causing host tissue damage indirectly, or still be responsible for evasion of certain host defenses by capsule formation and protease production.

The results obtained in this study showed that inbred mice injected with some *A. actinomycetemcomitans* strains developed local abscesses. The striking result from microbiological sampling was that *A. actinomycetemcomitans* could be recovered from all the injected sites, irrespective of abscess formation.

Injection of *A. actinomycetemcomitans* strains did not appear to affect the general health of the mice as assessed by weight changes. None of the bacterial strains were markedly pathogenic for the mice tested, nor was any of the inbred mouse strains particularly sensitive to infection with the bacteria. However, one isolate (55) was capable of producing abscess formation in three different mouse strains (Balb/c, A/Sn and A/J).

Reference strains ATCC 29523 and FDC Y4, on the other hand, were more virulent than the isolates recovered from patients, and also produced abscesses in four and five distinct mouse strains, respectively. However, in our study, the C3H/HePAS mouse strain, which is considered to be resistant to bacterial lipopolysaccharide (LPS), developed abscesses after inoculation with three isolates from patients. cAF1 and AKR mice were resistant to all *A. actinomycetemcomitans* capable of producing abscesses in other mouse strains but showed abscess formation when inoculated with the reference strains *A. actinomycetemcomitans* ATCC 29523 and FDC Y4 (TABLE 1).

The abscesses were large and dome-shaped, similarly to those reported by Chen *et al.* (6) on mice injected with either *A. actinomycetemcomitans* alone or with a mixture of *A.actinomycetemcomitans* and *Campylobacter rectus*.

All the strains of *A. actinomycetemcomitans* used in this study showed capsule formation when analyzed on Indian-ink preparations. It was surprising to find no correlation between the presence of capsule and virulence evaluated by abscess formation in our experiments, a situation that will need further study.

Lewis *et al.* (12) reported that lesions in mice resulting from a combination of two gram-negative oral microorganisms yielded both bacterial species in high concentrations. Farias *et al.* (7) demonstrated bacterial colonization and periodontal inflammation in germ-free rats which were inoculated with a strain of *A. actinomycetemcomitans* isolated from a patient with periodontal disease. The histopathology revealed an acute focal inflammatory process with abscess in the periodontal tissues. Holt *et al.* (9) demonstrated periodontal lesions in non-human primates with the implantation of *Porphyromonas gingivalis*.

Although the stimulus that promotes bone breakdown in periodontal disease is still unresolved, it is likely that bacterial components which can penetrate into the periodontal tissues are involved. Evidence has been given for an important role of bacterial LPS in this process, yet surface-associated material from *A. actinomycetemcomitans* not containing LPS was a better stimulator of bone resorption *in vitro* and significantly more active than LPS from the same organism (13).

Although much is known about host factors in periodontal disease, little is known on the *in situ* interaction of these factors with bacterial components and on how they relate to disease progression (9). There has been no previous report on abscess formation by this microbial group on inbred strains of mice. Using mice as experimental animals, further studies must certainly be performed to identify the factors from *A. actinomycetemcomitans* responsible for abscess formation when the microorganism is inoculated alone or in association with other microbial species.

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## RESUMO

**Ação patogênica de cepas de *Actinobacillus actinomycetemcomitans* em linhagens de camundongos isogênicos**

Foi testado o potencial de cinquenta e sete cepas de *A. actinomycetemcomitans* para produzir abscessos em linhagens de camundongos isogênicos criados com barreiras no "Biotério de Camundongos Isogênicos" do ICB/USP. Os camundongos foram injetados subcutaneamente com cepas de *A. actinomycetemcomitans*. Os animais desenvolveram abscessos ulcerados e/ou purulentos no sítio da injeção, dos quais as bactérias foram recuperadas. Vinte e três cepas de *A. actinomycetemcomitans* produziram abscessos.

**Palavras-chave:** *Actinobacillus actinomycetemcomitans*, camundongos isogênicos, patogenicidade

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# STAPHYLOCOCCI IN BREAST MILK FROM WOMEN WITH AND WITHOUT MASTITIS

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## ABSTRACT

Breast milk from nineteen women, ten with and nine without mastitis, was examined for the presence of staphylococci. Staphylococci was present in all breast milk samples, $10^2$ to $10^4$ cfu/mL. Plating on Baird-Parker agar plates revealed a variety of staphylococci, with typical Baird-Parker colonies present in all samples except one. *Staphylococcus aureus* (coagulase and thermonuclease-positive and anaerobic fermentation of mannitol) was present in 13 samples. Several typical and atypical colonies lacked one or two typical characteristics of *S. aureus*, such as coagulase or thermonuclease production and anaerobic mannitol fermentation. Some colonies lacked all three characteristics, but only one sample contained only colonies that lacked all three characteristics. Only eight of the nineteen samples contained staphylococci that produced enterotoxin detectable by gel diffusion. Some samples contained staphylococci that produced low amounts of enterotoxin or the toxic shock syndrome toxin, detectable by the reversed passive latex agglutination method.

**Key words**: breast milk, staphylococci, enterotoxin

## INTRODUCTION

Human breast milk is recognized as being superior to milk from other animals for the feeding of babies. Frequently it is not possible for a woman to breast-feed her baby because of inadequate production of milk or for some other reason. Sometimes a baby may be allergic to cow's milk, the most common substitute for human milk. In this case another source of milk is sought. It has become a practice in some hospitals to solicit milk from women who have an extra supply for use in feeding babies not able to consume cow's milk. There can be problems with such milk as it is not unusual for human milk to be contaminated with staphylococci. Previous investigations have shown that human milk is frequently contaminated with staphylococci, which can result in illness in the infants consuming the milk (1).

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The results of examining breast milk from a number of women contributing to a breast milk pool at a hospital in Belo Horizonte, Minas Gerais, Brazil, for the presence of enterotoxigenic staphylococci is reported.

## MATERIALS AND METHODS

**Isolation of staphylococci.** Each of the 19 milk samples (0.1 mL) was streaked on two Baird-Parker plates and incubated for 48 hours at 37°C. Both typical (jet black to dark grey, smooth, convex, entire margins, off-white edge, and may show an opaque zone and/or a clear halo beyond the opaque zone) and atypical colonies (gray and mucoid) were present on the plates. Several colonies of each type for each milk sample were individually transferred to two test tubes containing 1 mL of BHI broth and incubated for 24 hours at 37°C. Tests for coagulase and thermonuclease (TNase) production, anaerobic fermentation of glucose and mannitol, and production of hemolysin using sheep blood were made. Counts of each type were made from these plates.

**Enterotoxin production.** For enterotoxin production, inocula were prepared by combining one to five colonies of identical characteristics for each sample and incubating them in brain heart infusion (BHI) broth over night at 37°C. Membrane-over-agar plates were prepared with 25 mL of BHI-agar and covered with a membrane disk made from Spectra/Por membrane dialysis tubing, 6000-8000, 100 mm flat width (Thomas Scientific, Philadelphia, PA, USA) (2). One-half milliliter of the inoculum was spread on the membrane and the plates were incubated at 37°C for 24 hours. The cultures were removed from the membranes by washing with 2.5 mL of 0.01 M $Na2HPO_4$ in three steps using 1 mL, 1 mL, and 0.5 mL of the phosphate buffer. The cultures were centrifuged and the culture supernatant fluids used for enterotoxin testing.

**Enterotoxin testing.** The optimum-sensitivity-plate (OSP) method was used (2). In this method, 3 ml of agar are placed in 50 mm plastic Petri plates with tight lids: wells are cut according to the original specifications. Specific antisera is placed in the center well, enterotoxin (4 mg/mL) is placed in the two smaller wells, and culture supernatant fluids are placed in the four larger outer wells. Different plates are required for each enterotoxin (SEA, SEB, SEC, SED). The plates are placed in a humidified container and incubated overnight at 37°C. Positive reactions are determined from precipitin lines formed by the culture supernatant fluids that joined with the control lines.

The more sensitive reversed passive agglutination (RPLA) method was used also for testing the culture supernatant fluid for enterotoxin (3). This method is based on the agglutination of enterotoxin antisera coated latex particles in the presence of enterotoxin. It is sensitive to less than 0.5 ng of enterotoxin. Only those tests positive at a one to ten dilution of the culture supernatants produced by the sac culture method were considered valid.

## RESULTS

**Staphylococcal counts in the milk sample.** The staphylococcal counts varied from $10^2$ to $10^4$ cfu/mL.

**Biochemical characteristics of the staphylococci.** Both the typical and atypical staphylococci varied in their characteristics (TABLE 1). Eight of the ten mastitic milk samples contained staphylococci that were positive for all of the characteristics tested for, whereas only two of the normal milk samples contained such staphylococci. Several of the typical colonies were negative for the hemolysis factor and others lacked other characteristics except glucose fermentation. Some atypical staphylococci were positive for all characteristics tested for.

**Enterotoxin production.** The enterotoxin production is given in TABLE 2. Four of the mastitic milk and four of the normal milk samples were positive for enterotoxin by the OSP method. Two additional normal milk samples tested positive for enterotoxin by the RPLA method. Many samples were weakly positive for the toxic shock syndrome toxin (TSST) by the RPLA method, but it is uncertain whether these results were reliable.

## DISCUSSION

All of the milk samples were contaminated with staphylococci, there being little if any difference between the normal and mastitic milk. The counts were too low to promote the production of enterotoxin, however, if the milk samples were

TABLE 1. Biochemical Characteristics of Breast Milk *Staphylococci*

| Milk sample | Colony type | Coagulase | TNase | Glucose[a] | Manitol[a] | Hemolysin |
|---|---|---|---|---|---|---|
| 2[b] | Typical | + | + | + | + | + |
| | Typical | + | + | + | + | - |
| | Typical | - | + | + | - | + |
| | Typical | - | - | + | - | - |
| | Atypical | - | - | + | - | - |
| | Atypical | - | - | + | - | + |
| 19 | Typical | + | + | + | + | + |
| | Atypical | + | + | + | + | - |
| | Atypical | - | - | + | + | - |
| 13 | Typical | + | - | + | + | + |
| | Typical | + | - | + | - | + |
| | Typical | - | + | + | + | + |
| | Atypical | - | + | + | - | + |
| 9[b] | Typical | - | + | + | - | - |
| | Typical | - | + | + | - | + |
| | Atypical | - | + | + | - | + |
| 10 | Typical | - | - | + | - | + |
| | Typical | - | + | + | - | - |
| | Typical | - | + | + | + | - |
| | Atypical | - | + | + | - | - |
| 15 | Typical | - | - | + | - | + |
| | Atypical | - | - | + | - | + |

[a] - Anaerobic fermentation
[b] - Mastitic milk

TABLE 2. Enterotoxin Production of Breast Milk Staphylococci

| Sample | Colony[a] | Coag | TNase | Glu[b] | Mani[b] | Hemo | SE by OSP | SE by RPLA |
|---|---|---|---|---|---|---|---|---|
| 1[c] | 4T | + | + | + | + | + | A | |
| 2[c] | 1T | + | + | + | + | + | A | |
| 16 | 5T+2AT | + | + | + | + | + | A | |
| 4[c] | 5T | + | + | + | + | + | A, B | |
| 5[c] | 2T | + | + | + | + | + | - | A |
| 19 | 4T | + | + | + | + | + | - | C |
| 2[c] | 1T | + | + | + | + | - | A | |
| 5[c] | 1T | + | + | + | + | - | A | |
| 13 | 1T | + | - | + | + | - | B | |
| 13 | 1T | + | - | + | - | + | C | |
| 14 | 2T | + | - | + | - | + | A | B, TSST |
| 13 | 1T | - | + | + | + | + | C | |
| 14 | 2AT | - | + | + | + | + | - | B |
| 5[c] | 2T+3AT | - | + | + | - | + | C | |
| 12 | 5T+3AT | - | + | + | - | + | B | |
| 1[c] | 1T+3AT | - | + | + | - | + | - | A |
| 13 | 3AT | - | + | + | - | + | - | B |
| 18 | 3AT | - | - | + | - | + | A | B |
| 17 | 3AT | - | - | + | - | + | - | B |
| 18 | 2T+1AT | - | - | + | - | - | A | B |

[a] - T, typical colony; AT, atypical colony
[b] - Anaerobic fermentation
[c] - Mastitic milk

mishandled before pasteurization, sufficient growth of the staphylococci could result in production of enterotoxin. Pasteurization would destroy the staphylococci but not the enterotoxin (4).

Although the staphylococci in the mastitic milk would be destroyed by the pasteurization it is not normal to use this milk. If milk containing enterotoxin were given to infants, it is likely that illness would result because children are more vulnerable to this type of illness. However, if the milk contained TSST, it is not likely this would affect the infant receiving it because in most cases they would have protective antibodies to this toxin, a carryover from the mother (5).

The RPLA method was developed primarily for the detection of enterotoxin in foods (6), however, it has been used for the testing of staphylococcal strains for enterotoxin production that were negative by the OSP method (7). Inadequate research has been done to determine how the strain testing should be done. The method is sensitive to less than 0.5 ng/mL, which is necessary in food testing, but not necessary for strain testing. Determination of how the enterotoxin should be produced and how much culture supernatant fluid should be diluted for the testing has not been done. Here, we arbitrarily chose a ten-fold dilution of the culture supernatant fluid as determination of positives. This may need further adjustment with further research, but the results are adequate for this work. Sufficient samples were positive for enterotoxin by the OSP method (TABLE 2) to warrant special care being taken in handling the breast milk after it was delivered and before pasteurization.

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## RESUMO

**Staphylococci em leite materno procedente de mulheres com e sem mastite**

Verificou-se a presença de staphylococci em leite materno procedente de 19 mulheres, nove consideradas sadias e dez apresentando sintomas clínicos de mastite. Staphylococci foram detectados em todas as amostras de leite, com contagens variando de $10^2$ a $10^4$ ufc/mL e revelando uma significativa variedade de colônias atípicas e típicas, estas últimas observadas em todas as placas de ágar Baird-Parker, exceto em uma amostra. *Staphylococcus aureus*, coagulase e termonuclease positivos e fermentadores de manitol em anaerobiose, estavam presentes em 13 amostras. Várias estirpes não apresentaram uma ou duas, outras evidenciaram todas e uma não apresentou nenhuma das características bioquímicas citadas. De um total de 19 cepas, 8 sintetizavam quantidades detectáveis de enterotoxinas pelo método de imunodifusão em gel e algumas mostraram-se produtoras de pequenas quantidades de enterotoxinas e de toxina da síndrome do choque tóxico, conforme detectado através do método de aglutinação reversa passiva em latex.

**Palavras-chave**: leite materno, staphylococci, enterotoxina

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# CHARACTERIZATION OF *ENTEROCOCCUS* STRAINS ISOLATED FROM READY-TO-EAT FOODS

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## ABSTRACT

Thirteen strains of *Enterococcus faecalis* and five strains of *E. faecium* isolated from foods were studied. Production of thermonuclease (TNase), hemolytic activity on human and rabbit blood and susceptibility to antimicrobial agents were analyzed. Only one strain was ß-hemolysis positive. No production of TNase and ß-lactamase or resistance to vancomycin and to high levels of aminoglycosides (HLAR) were detected.

**Key words:** *Enterococcus*; enterococci in foods; characterization.

## INTRODUCTION

Enterococci have been isolated from a variety of foods associated with foodborne diseases (5). Although attempts to demonstrate enterococci as food poisoning agents have not succeeded completely (5,10), there is a tendency to consider enterococci as causative agents during disease outbreaks whenever they are found in large numbers in suspected foodstuffs that do not contain other pathogens.

Some characteristics such as production of thermonuclease and hemolysis, lethality to animals (1), production of amines (21) and of enterotoxins (2) have been related to enterococcal virulence.

Enterococci are important agents of human infections (12,18) and have been especially implicated in cases of nosocomial infections. Most importantly, the resistance to antimicrobials frequently used for treatment (13,15,24) has been increasing among enterococcal strains.

Although characteristics of enterococcal strains isolated from human clinical specimens are largely documented in the literature, information on strains isolated from foods is still scarce.

The aim of this work was to identify enterococcal strains in eighteen samples of ready-to-eat foods and study some characteristics associated with enterococcal virulence, namely thermonuclease production, hemolytic activity and antimicrobial resistance.

## MATERIALS AND METHODS

Fifty five samples of ready-to-eat foodstuffs were purchased from commercial establishments, including 5 of each of the following items: ham and cheese pie, tuna sandwich, mozzarella and ham sandwich, cream pie, shrimp patty, chicken sandwich, potato and mayonnaise salad, ham, cream roly-poly, cream puff and sweet puff.

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The isolation of *Enterococcus* was carried out on KF *Streptococcus* agar (Difco Laboratories, Detroit, MI.) (4). The identification of *Enterococcus* strains was done by conventional tests as described by Facklam and Collins (8). Strains were also tested by the API 20S *Streptococcus* System (Analytab Products Ayerst Laboratories, Plainvew, N.Y.) according to the manufacturer's instructions.

Tests for detection of TNase were performed as described by Tatini *et al.* (22) and Lachica *et al. (15)*.

The hemolytic activity of the strains was determined on blood agar base (Merck) supplemented with 5% human or rabbit blood.

Antimicrobial susceptibility tests were performed as previously described (19). The following antimicrobial disks were used: ampicillin (10µg), chloramphenicol (30µg), erythromycin (15µg), gentamicin (10µg), kanamycin (30µg), norfloxacin (10µg), streptomycin (10µg), sulfazotrim (25µg), tetracycline (30µg) and vancomycin (30µg).

Tests for detection of high level aminoglycoside resistance (HLAR) were performed on Mueller-Hinton Agar supplemented with calcium and magnesium at final concentrations of 50 mg/liter and 25 mg/liter, respectively. After sterilization, different batches of media were supplemented with gentamicin, kanamycin or streptomycin (Sigma Chemical Co. St. Louis Mo.) at final concentrations of 500 and 2000 µg/mL.

Production of ß-lactamase was assayed as described by Facklam and Washington II (9).

## RESULTS AND DISCUSSION

Eighteen cultures classified as enterococci were catalase-negative Gram positive cocci. All the strains gave positive bile-esculin reactions and reacted with streptococcal group D antisera. Tolerance tests and growth in 6.5% NaCl broth at 10°C and 45°C showed positive results. Gas production in MRS broth was not observed. All the strains produced PYRase and gave positive results for trehalose, maltose, sucrose, salicin, mannitol and lactose fermentation, hydrolysis of arginine and acetoin production. None of the strains produced acid from inulin, sorbose or raffinose. Moreover, none of the strains produced pigments and all strains were nonmotile (TABLE 1).

**TABLE 1.** Percentage of positive reactions among *Enterococcus* strains from ready-to-eat foods. Tests for differentiation of enterococci into species.

| Tests | % of strains with positive reactions | |
|---|---|---|
| | *E. faecalis* (n=13)* | *E. faecium* (n=5)* |
| Acid from | | |
| Arabinose | 38.4 | 80 |
| Glycerol | 84.6 | 20 |
| Inulin | 100 | 100 |
| Lactose | 100 | 100 |
| Maltose | 100 | 100 |
| Mannitol | 100 | 100 |
| Mellibiose | 23 | 80 |
| Raffinose | 0 | 0 |
| Rhamnose | 92.3 | 80 |
| Salicin | 100 | 100 |
| Sorbitol | 100 | 0 |
| Sorbose | 0 | 0 |
| Sucrose | 100 | 100 |
| Trehalose | 100 | 100 |
| Deamination of Arginine | 100 | 100 |
| Resistance to Tellurite | 100 | 0 |
| Reduction of Tetrazolium | 76.9 | 0 |
| Motility | 0 | 0 |
| Production of pigment | 0 | 0 |
| Voges Proskauer test | 100 | 100 |
| Hemolysis | | |
| Alpha | 92.3 | 100 |
| Beta | 7.7 | 0 |

*n = number of strains tested

Thirteen strains identified as *E. faecalis* showed tolerance to tellurite and produced acid from sorbitol. Most of them produced acid from glycerol (84.6%) and reduced tetrazolium (76.9%). The strains classified as *E. faecium* did not tolerate tellurite and were not able to produce acid from sorbitol. Eighty percent of the strains produced acids from arabinose and melibiose.

The hemolytic activity of the strains was tested with human and rabbit blood. All strains were α-hemolysis positive except one, which was identified as *E. faecalis* and was found to produce ß-hemolysis in human and rabbit blood. None of the strains demonstrated TNase production.

The susceptibility to antibiotics of the strains as tested by the disk diffusion method showed resistance to erythromycin and to all the aminoglycosides used. However, all strains were susceptible to sulfazotrim, tetracycline, vancomycin, ampicillin and cloramphenicol. Three strains of *E. faecium* and two of *E. faecalis* were also susceptible to

not detected among the strains studied.

The intestinal tract of man and other animals is the natural reservoir of enterococci. These microorganisms are also commonly found in soil and water and on vegetables. Enterococci can survive in foods presenting low water activity and under a wide range of pH values (10). They have been isolated from cheese (14), baby food (2), milk, ice-cream and butter (24) and frankfurters (11).

Of the 55 samples of food examined in the present work, 32.7% contained enterococci. Strains were isolated at high counts from three samples of ham and cheese pie (average of final counts = 2.8 x $10^6$ CFU/g), three tuna sandwich samples (5.3 x $10^4$ CFU/g), four mozzarella and ham sandwich samples (1.4 x $10^4$ CFU/g) and three cream puff samples (3.5 x $10^3$ CFU/g). The strains were identified as *E. faecium* and *E. faecalis* by both the conventional method and the API 20S system. These two species are frequently found in poultry (6), milk and dairy products (24).

In this study, results on the E. *faecium* isolates were compatible with the species since no production of acid from raffinose and sorbitol was observed. Raffinose-positive strains since no production of acid from raffinose and sorbitol was observed. Raffinose-positive strains have been found in intestinal samples from poultry (6) and humans (8).

Facklam (7) has suggested that tolerance to tellurite, the ability to reduce tetrazolium and the formation of acid from sorbitol and glycerol are the main tests to indicate that an *Enterococcus* isolate belongs to the *faecalis* species. Of the thirteen *E. faecalis* strains analyzed in this work, 23.1% were not resistant to tellurite. It was also observed that 38.4% of the strains were positive for arabinose, a test which usually gives negative results for *E. faecalis* (6,8,20). In a study involving 171 *E. faecalis* strains isolated from human sources, Facklam (7) found only 4% strains positive for arabinose. Difference in sources could possibly explain differences in physiology. However, the actual sources of the strains isolated in our work are difficult to establish since the foods are made from different materials and the possibility of human contamination also exists.

The majority of the strains showed α-hemolysis activity and only one strain was ß-hemolytic. Batish *et al.* (1) found 5 ß-hemolytic strains (3.1%) among 161 enterococcal isolates obtained from frozen dairy products. They proposed that α-hemolysis and TNase production could be taken as the major criteria for establishing the *in vitro* pathogenicity of enterococcal strains. Although we found seventeen strains producing α-hemolysis, TNase production was not detected. Characteristics such as production of enterotoxin should also be investigated.

Although there is a great deal of information available on antimicrobial susceptibility of enterococci isolated from human clinical sources (13,16,17), such characteristic has not been systematically investigated in enterococci isolated from foods (3). Recent studies have focused on human clinical isolates because of the large number of strains presenting resistance to high levels of aminoglycoside as well as production of ß-lactamase (16,17) and resistance to vancomycin (23). No strains with these characteristics were detected in our investigation. Although only a small number of strains was analyzed, our results indicate that there are differences between the characteristics of enterococci isolated from ready-to-eat foods and those of strains isolated from human clinical specimens.


## RESUMO

### Caracterização de estirpes de *Enterococcus* isoladas de alimentos

Treze estirpes de *Enterococcus faecalis* e 5 de *E. faecium* isoladas a partir de alimentos foram estudadas em relação a produção de termonuclease (TNase), atividade hemolítica em sangue humano e de coelho e sensibilidade a antimicrobianos.

Não foi observada a produção de TNase e ß-lactamase, resistência a vancomicina e a altos níveis de aminoglicosídeos.


**Palavras-chave**: *Enterococcus*; enterococos em alimentos; caracterização

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# SCREENING OF ß-FRUCTOFURANOSIDASE PRODUCING MICROORGANISMS FOR PRODUCTION OF FRUCTOOLIGOSACCHARIDES AND STUDIES OF SOME ENZYME PROPERTIES

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## ABSTRACT

One strain of yeast which produced the highest ß-fructofuranosidase activity was isolated from the leaf of tropical fruit and the strain was identified as *Aureobasidium pullulans*. The strain of *A. pullulans* produced both extra and intracellular ß-fructofuranosidase which catalyzed production of fructooligosaccharides from sucrose. The optimum pH and the optimum temperature of enzyme activity were 5.2 and 55°C, respectively. Thermostability of the enzyme was also about 55°C. The enzyme converted sucrose to 61.2% fructooligosaccharides with a 50% concentration of sucrose as substrate.

**Key words**: *Aureobasidium pullulans*, ß-fructofuranosidase, fructooligosaccharides, fructosyl transfer, sucrose

## INTRODUCTION

Neosugar is an example of a fructooligosaccharide (FOS), these being naturally occurring sugars that can have beneficial effects as food ingredients (12). Fructooligosaccharides are $1^F$ -(1-ß-fructofuranosyl) $_{n-1}$ sucrose oligomers and n may vary from 2 to 4, with n=2 being 1-kestose ($GF_2$); n=3, nystose ($GF_3$); and n=4, $1^F$-ß-fructofuranosyl nystose ($GF_4$) (9). These common sugars are found in a variety of edible plants that have been used as human and animal food sources for many years, including banana, barley, garlic, honey, onion, rye, brown sugar, tomato, asparagus root, Jerusalem artichoke and wheat (2, 12).

These fructooligosaccharides are also produced from sucrose by the action of fructosyl transfer using ß-fructofuranosidase from *Aspergillus oryzae* (11), *Fusarium oxysporum* (3), *Aureobasidium pullulans* (5, 7, 8), *Aspergillus niger* (6, 10) and *Aspergillus japonicus* (4).

The aim of this work was to isolate a new strain of microorganism which produced high ß-fructofuranosidase activity and could be used for the production of fructooligosaccharides and to study the characteristics of the enzyme

## MATERIALS AND METHODS

**Screening of ß-fructofuranosidase producing-microorganisms**. The respective carrier materials such as pollen, honeycomb and plant secretions were added to 50mL conical flasks containing 10mL of enrichment culture medium, which consisted of 40% sucrose and 1% yeast

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extract. The flasks were incubated at 30°C on a rotating shaker. When good growth had occurred, the cultures were streaked onto agar plates containing 20% sucrose, 1% yeast extract and 2% agar. Then, individual colonies were picked and transferred to slants containing the same culture medium. The isolated microorganisms were inoculated into 50mL conical flasks containing 10mL of the same culture medium (except for the sucrose concentration, which was 10% instead of 40%) and incubated at 30°C on a rotating shaker for 3 days. After incubation, the culture medium was centrifuged and the supernatant obtained was examined by paper chromatography.

**Production of ß-fructofuranosidase from isolated strains of microorganisms**. An enzyme-producing strain of microorganism was inoculated into 250mL conical flasks containing 50mL of the same culture medium (10% sucrose) as mentioned above and incubated at 30°C. Periodically, the cultures were centrifuged to obtain both supernatant and cell mass. The precipitated cells were resuspended in deionized water and centrifuged to obtain the washed cell mass. This procedure was repeated three times, after which the cells were resuspended in deionized water to the original volume to examine cell growth and intracellular enzyme activity. Alternatively, part of the washed cells was mixed with a small volume of 0.1 M citrate-phosphate buffer pH 5.5 and the cells were then ruptured by sonic oscillation using Lab-Line Ultra Tip Labsonic System. After removal of cellular debris by centrifugation, the cell free solution was used as a source of intracellular enzyme.

**Assay of ß-fructofuranosidase activity**. The culture supernatant (as extracellular enzyme) and both the washed cell suspension and sonic oscillation treated supernatant (as intracellular enzyme) were used as a source of enzymes. The enzyme activity was determined by incubating a mixture of 0.9mL of 10% sucrose solution in 0.1 M citrate-phosphate buffer, pH 5.5 and 0.1mL of the respective enzyme solution at 55C for 2 hr, followed by inactivation of the enzyme by boiling in water for 10 min. The concentrations of glucose were determined using a glucose-oxidase/peroxidase method (kit CELM). Alternatively, glucose, fructose and 1-kestose were determined by HPLC. One unit of fructofuranosidase activity (transfructosylation activity) was defined as the amount of enzyme activity which catalyzed the formation of 1 mole glucose per min, and the hydrolytic activity of ß-fructofuranosidase was determined by measuring concentrations of fructose.

**Analysis of sugars**. The qualitative analysis of sugars was performed by paper chromatography using borate-treated Whatman n. 1 paper developed with ethyl acetate/isopropanol/water (6:3:1). Sugars were detected with aniline-diphenylamine-phosporic acid (13). Quantitative analysis of sugars was performed by HPLC using a chromatograph equipped with a differential refractometer detector and a Shodex Ionpak KS-802 column, the eluent being water with a flow rate of 1mL/min. Authentic standards were fructose, glucose, sucrose, 1-kestose ($GF_2$), nystose ($GF_3$) and fructofuranosyl nystose ($GF_4$).

**Preparation of extracellular ß-fructofuranosidase**. Approximately, 1 liter of fermented supernatant was dialyzed against deionized water for 48 hr, and the dialyzed supernatant concentrated using a Model TCF2A Micro-Thin-Channel Ultrafiltration System (Amicon Co.) to contain 12.8 units/mL of the enzyme activities. This preparation was used to determine the enzymatic characteristics and also for the production of fructooligosaccharides from sucrose.

**Production of fructooligosaccharides from sucrose**. A total of 15mL of sucrose solution (30% and 50% respectively) in 0.1 M citrate-phosphate buffer, pH 5.5 containing 20 units of extracellular ß-fructofuranosidase, were incubated at 55°C for 12 hr.

## RESULTS AND DISCUSSION

A total of 150 strains of microorganisms were isolated from samples obtained in the State of Amazonas. One strain of yeast was found that produced the highest ß-fructofuranosidase activity and, more importantly, with more activity of fructosyl transfer to other sucrose molecules as acceptors than to water as acceptor. This is a very important point because if water is the preferred acceptor for the fructosyl units, the result is hydrolysis. It was also found that this strain produced both extra and intracellular enzymes.

The characteristics of the cultures, morphological studies and some physiological studies are described in TABLE 1. The isolated strain of yeast was classified as *Aureobasidium pullulans* (De Bary) in accordance with the description by Cooke (1).

TABLE 1 - Taxonomy of Isolated Microorganisms

The microorganism which was isolated from leaf of a tropical fruit tree (*Annona densicoma*) in Manaus, Amazonas, Brazil, was cultivated on potato dextrose agar and malt extract agar and observed as follows:
Colony color: White to brown or pink creamy flesh, then black.
Colony appearance: Usually mucoid to dry velvety and colonies composed of mycelium and yeast-like cell.
Colony growth: Fairly slow.
Micelium: Hyphae radiating from the center of the colony, becoming irregularly entangled and septet, branched, often thickened, producing arthrospore-like hyphal segments or chlamydospores or radulaspores.

Conclusion:

The microorganism was identified as *Aureobasidium pullulans* (De Bary) in accordance with description by Cooke, 1962 (*Mycopathol. et Mycol. Appl.* XVII, 1, A taxonomic study in the "Black Yeasts").

Production of the enzyme by isolated *Aureobasidium pullulans* 79 was performed as described above. Measurements of the enzyme activity and cell growth at time intervals were made to determine the relationship between amounts of both extra and intracellular ß-fructofuranosidase and cell growth. Production of both extra and intracellular enzymes accompanied the cell growth phase as shown in FIGURE 1. The maximum level of production of the enzyme was observed after 40 hr. (3.4 units/mL of extracellular and 1 unit/mL of intracellular enzymes).

Some enzymatic characteristics are shown in FIGURES 2, 3 and 4. The optimum pH and the optimum reaction temperature of the enzyme was 5.2 and 55°C. The enzyme loses thermostability at temperatures above 55°C.

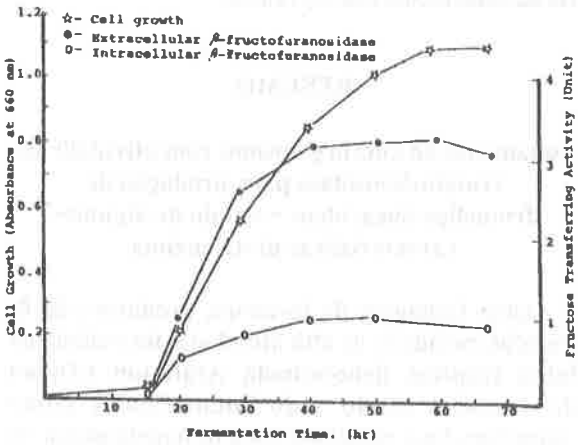



FIGURE 1 - Time course of enzyme production by *Aerobasidium* sp.

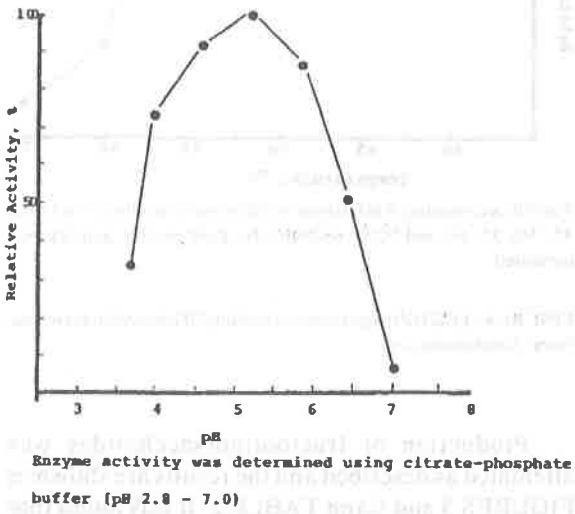



FIGURE 2 - Effect of pH on activity of extracellular enzyme from *Aerobasidium* sp.

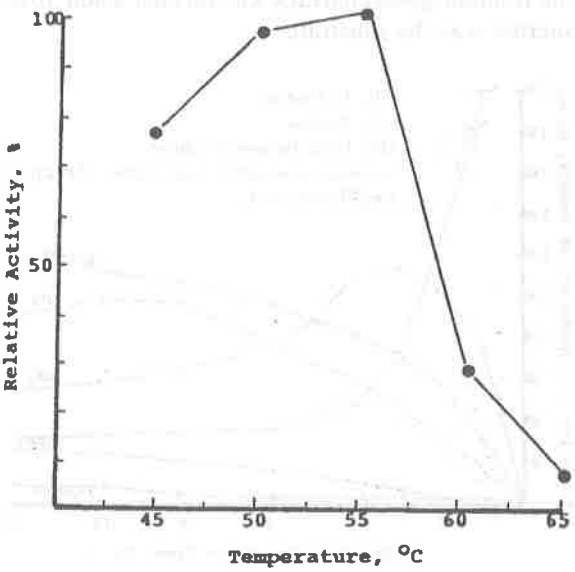
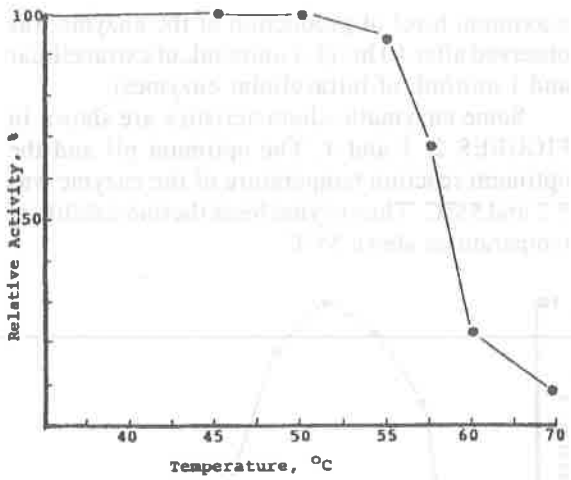



FIGURE 3 - Effect of temperature on activity of extracellular enzyme from *Aerobasidium* sp.




Test tubes containing 5 ml enzyme solution were incubated for 1 hr at 45, 50, 55, 60 and 70°C respectively, then enzyme activity was measured.

FIGURE 4 - Effect of temperature on stability of extracellular enzyme from *Aerobasidium* sp.

Production of fructooligossaccharides was attempted as described and the results are shown in FIGURES 5 and 6 and TABLE 2. It was found that 61.2% of the fructooligosaccharides, consisting of 21.9% 1-kestose, 29.3% nystose and 10% fructofuranosyl nystose was formed when the sucrose concentration was 50%, whereas 52.4% of the fructooligosaccharides was formed when 30% sucrose was the substrate.

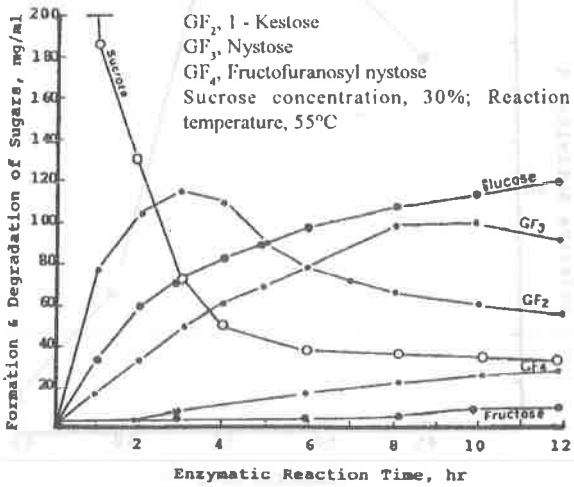



FIGURE 5 - Production of fructooligosaccharides from sucrose by the *Aerobasidium* enzyme.

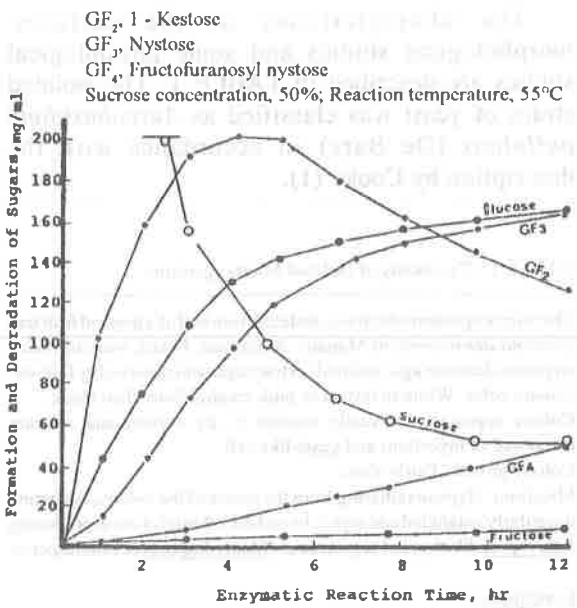



FIGURE 6 - Production of fructooligosaccharides from sucrose by the *Aerobasidium* enzyme.

TABLE 2 - Formation of Fructooligosaccharides from Sucrose by *Aureobasidium enzyme*

| | Concentration of Sucrose | |
|---|---|---|
| | 30% | 50% |
| Sucrose | 9.9% | 9.0% |
| 1-kestose | 17.0% | 21.9% |
| Nystose | 27.2% | 29.3% |
| Fructofuranosyl nystose | 8.2% | 10.0% |
| Glucose | 35.0% | 28.4% |
| Fructose | 2.7% | 1.4% |

The enzymatic reaction time was 12 hours

## RESUMO

**Isolamento de microrganismos com atividade de frutofuranosidase para produção de frutooligossacarídeos e estudo de algumas características desta enzima**

Uma linhagem de levedura, produtora de ß-frutofuranosidade de alta atividade, foi isolada da folha tropical denominada Araticum (*Anona densicoma*) tendo sido identificada como *Aureobasidium pellulans*. Esta linhagem produziu ß-frutofuranosidade extra e intracelularmente que

catalizaram a produção de frutooligossacarídeos a partir da sacarose. O pH e temperatura ótimas de atividade da enzima foram 5.2 e 55°C, respectivamente. Sua termoestabilidade foi de 55°C. Esta enzima converteu sacarose a 61,2% de frutooligossacarídeos quando se utilizou sacarose como substrato na concentração de 50%.

**Palavras-chave:** *Aureobasidium pellulans*, ß-frutofuranosidade, frutooligossacarídeos, transferência de frutosila, sacarose

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# SEROTYPING, PROTEINASE AND PHOSPHOLIPASE PRODUCTION ASSOCIATED WITH THE PATHOGENICITY OF *CANDIDA ALBICANS* STRAINS ISOLATED FROM FROG MEAT

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## ABSTRACT

Strains of *C. albicans* isolated from frozen frog legs marketed in Brazil were serotyped and assayed for proteinase and phospholipase production and also evaluated for *in vivo* pathogenic effects by intraperitoneal inoculation into mice. Strain serotyping revealed the predominance of serotype B. All the *C. albicans* analyzed were phospholipase producers. Extracellular proteinase was detected in 66,6% of the isolates. The *in vivo* pathogenicity study demonstrated the occurrence of an acute renal disease with cortical abscesses and a chronic renal disease with unilateral pyelonephritis.

**Key words:** *C. albicans*; meat yeast; frog legs

## INTRODUCTION

Previous studies (15) demonstrated that 6,25% of the yeast flora of frog meat (*Rana catesbeiana* - SHAW, 1802) destined to human consumption in Brazil during the period of 1988 to 1990 corresponded to *C. albicans*. This species is of particular interest for it can cause different diseases in man and animals and also because it is not commonly found in meat and derivatives.

Reis *et al.* (18) isolated *Candida* species from the intestinal contents of frogs at different growth stages but failed to detect the presence of these microorganisms in internal organs of any of the frogs examined.

Carmo Souza (5) regards the species *C. albicans* as an obligatory animal parasite. Although the occurrence of this microorganism has been reported in fresh marine, chlorine and recreational waters (4,6,19), in such instances it was probably associated with fecal contamination. Unsanitary conditions often lead to contamination of the environment or of foodstuffs with *C. albicans*.

In 1961, Hasenclever and Mitchell (7) identified two serotypes of *C. albicans*, namely A and B. Epidemiological studies have revealed the prevalence of serotype A over serotype B in man and in recreational waters. Serotype B, however, can be isolated from many different sources (1,8,19).

The ability of *C. albicans* to produce cytolytic

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enzymes such as proteinases (13) and phospholipases (2) may be associated with the pathogenicity of this fungal species.

The aim of this study was to determine the most common serotypes of *C. albicans* strains isolated from marketed frog meat and to investigate some factors related to their pathogenicity.

## MATERIAL AND METHODS

**Specimens:**

Fifteen *C. albicans* strains isolated from frog legs as previously described (15) were studied. The strains were characterized and identified according to conventional methods (10). Stock cultures were maintained in Glucose-yeast-peptone medium, stored at 4°C and subcultured at 4-month intervals. All strains were subcultured on Sabouraud Dextrose Agar for three consecutive times before use.

**Serotyping:**

Specific antiserum: Total antiserum was obtained by inoculation of rabbits with serotype A *C. albicans* EM-USP 464. Specific anti-A antiserum was prepared adsorbing the total antiserum with *C. albicans* serotype B EM-USP 461 cells according to Hasenclever and Mitchel (7).

Slide agglutination technique: The pattern of serological reactions was established using the slide agglutination test. A drop of the specific antiserum was placed on a microscope slide, mixed with the same amount of the suspension of the studied strain ( $10^6$ cell / mL) in formalized saline solution and turbidimetrically adjusted with the Wickerham card technique (10). The formation of clusters in a 30 seconds interval classified the strain as serotype A.

**Proteinase assay:**

Proteinase production was determined as described by Odds and Abbot (16). The plates were incubated at 37°C for 6 to 8 days; the production of a clear ring around the colony was taken as positive proof of proteinase production. Total proteolysis was visualized by staining with amido black (1 g $l^{-1}$ in 3,5M acetic acid) and subsequent differentiation in 1,2M acetic acid.

**Phospholipase activity:**

Phospholipase activity was determined by a plate assay according to the method of Price *et al.* (17). After incubation at 37°C for 24 h, the plates were analyzed and the phospholipase activity (Pz) was determined by the ratio between the diameter of the colony and the total diameter of the colony plus the precipitation zone. Thus, Pz = 1.00 means that the isolate in question was phospholipase negative and Pz < 1.00 means that the test strain was phospholipase positive.

**Mouse infections:**

Preparation of inocula: Cells of each *C. albicans* strain were suspended in sterile saline solution and the concentration was estimated by the Wickerham card technique (16). Inocula consisted of $10^8$ cell per mL, the exact number being determined by standard pour plate enumeration techniques.

From these initial suspensions, 4 distinct mixtures of strains were prepared (M1, M2, M3 and M4 strain groups) on the basis of variation in proteinase production, serotype and biochemical activities (TABLE 1).

TABLE 1 - Phenotypic characteristics used for grouping *C. albicans* strains.

| Mixture | Strains | % | Serotype | Mz** | Proteinase |
|---|---|---|---|---|---|
| M1 | RaDF 54, RaDF 57 RaDF 60, RaDF 65 RaDF 66 | 20 | B | + | + |
| M2 | RaDF 63, RaDF 67 RaDF 68, RaDF 72 RaDF 73 | 20 | B | - | + |
| M3 | RaSP 199, RaSP 209 RaSP 215 | 33 | B | + | - |
| M4 | RaSP 196, RaSP 201 | 50 | A | - | - |

* Proportion of *C. albicans* strains in the suspension used as the inoculating mixture.

** Melizitose assimilation.

Animals and inoculation: Female mice of the conventional *Mus musculus* strain were infected intraperitoneally with 0,5 mL of a suspension containing a given mixture of strains. Four mice were challenged with each mixture and one control group was injected with 0,5 mL of sterile saline solution. Mortality rates were recorded over a 30 days period.

Evaluation of *C. albicans* pathogenicity: The number of survivors and any unusual external

feature or behavior were recorded daily. Mice which died due to *Candida* infection and those near death within a 15 days period were subjected to necropsy. Surviving mice were sacrificed with ethyl ether on days 15, 21 and 30 after inoculation

During necropsy, the peritoneal wall, abdominal viscera, heart and lungs were examined for the presence of whitish lesions characteristic of *C. albicans* infection.

Visceral organs were fixed in 10% buffered formalin for histopathology. Blocks of various organs were dehydrated, embedded in paraffin, sectioned, and the tissue sections were stained by the hematoxylin-eosin, Grocott's methenamine silver and periodic acid-Schiff procedures (12).

## RESULTS

**Phenotipic characterization and serotyping:**

Results are shown in TABLE 1. Of the 15 strains studied, 13 (86,7%) were serotype B and 2 (13,3%) were serotype A.

**Production of extracellular enzymes:**

Proteinase: Ten (66,6%) strains were positive for proteinase production, as shown by the appearance of a clear ring around the colonies. Five (33,3%) strains gave negative results.

Phospholipase activity: One hundred percent of the *C. albicans* isolates were phospholipase producers, with varying degrees of activity. Pz values ranged from 0,58 to 0,87.

**Pathogenicity of *C.albicans* in mice after intraperitoneal inoculation of the fungus:**

A variation in pathogenic effects was observed among the different *C. albicans* isolates.

One mouse inoculated with M1 strains and another with M2 strains died 19 and 14 days after inoculation, respectively. One mouse infected with the 4th mixture group (M4) died within two days of inoculation. No deaths were recorded among mice challenged with the M3 strains.

Surviving mice were sacrificed on days 15, 21 and 30 post-inoculation. At necropsy, the peritoneum and abdominal viscera showed evidence of mycotic infection, such as the presence of whitish nodular lesions and visceral adhesions on days 15 and 21 post inoculation. The kidneys were enlarged, with numerous abscesses, and also presented discoloration.

The pathological findings for mice infected with M4 strains and for those which were dying on the 10th day of infection were similar.

There was no evidence of mycotic lesions in the lungs, brain and heart.

Two types of renal lesions were observed. Mice inoculated with the M4 strains developed cortical renal abscesses, while those infected with strains M1 and M3 showed massive destruction of the right kidney or asymmetry of the kidneys with the smaller sometimes being reduced to half the normal size. Animals injected with the M2 strains showed no evidence of fungal growth on day 30 post-infection.

The earliest histological changes seen in the kidneys for both types of diseases were similar. There was an increase in interstitial tissue and leukocyte infiltration ( mostly polymorphonuclear leukocytes). Both yeast and pseudomycelia were seen in the lesions. Extrarenal tissues showed a similar picture but usually with a reduced infection. No fungi were observed in the severely damaged renal parenchyma at the final stage of the mycotic disease caused by M1 and M2 strains.

## DISCUSSION

Epidemiologically, the anurans constitute a significant reservoir of fungi because of their natural susceptibility to these microorganisms. Researchers have found an association between asymptomatic anurans and pathogenic fungi ( 14, 15, 18 ). The frog is of special interest because it is used as food for human consumption. Therefore, the presence of *C. albicans* in frog meat is a matter of Public Health concern and the importance of studies that address this matter must be duly emphasized.

In our study, the observed predominance of serotype B in frog meat agrees with the data from most of the available epidemiological surveys, which report that serotype B can be isolated from many sources (8). Another explanation for this predominance is the fact that serotype B appears to be more resistant to antimicrobial treatment (Chlorine) than serotype A (9). The chlorine concentration used during the handling and processing of frog legs was possibly not sufficient to kill *C. albicans* of serotype B.

Barret Bee *et al.* (2) found a correlation between phospholipase activity, adherence and virulence of *C. albicans*. MacDonald and Odds (13) found essentially the same relationship concerning the production of proteinases. These studies showed that the enzymes play an important role in the invasion of host tissue by fungal cells and in the *C. albicans* resistance to killing by host phagocytes.

The *in vivo* findings presented in this work show that the kidney was the organ most susceptible to infection with *C. albicans* in mice, a result which is in agreement with the data reported in the literature (11).

The results of this study demonstrate that isolates of *C. albicans* from frog meat (marketed frog legs) exhibited appreciable virulence. The severity of disease varied with different serotypes and proteolytic capacities. The proteinase deficient fungal strains (M3) were less virulent for mice than the proteinase producing strains (M1 and M2), demonstrating a correlation between proteinase activity and tissue colonization.

Besides the ability to produce proteinase, the other phenotypic differences between M1 and M2 strains were not considered importance for the virulence.

The *C. albicans* M4 strains were considerably more virulent than the other ones studied. Abscesses in both kidneys of M4 infected mice were visible 30 days post inoculation. The pathogenicity of these strains can be related to their being serotype A positive, despite their lack of proteinase production, as serotype A has been found to be more virulent for mice than serotype B (1).

One important factor widely reported as significant for *C. albicans* virulence is the ability of this species to produce hyphae. Borg and Rüchel (3) found that distinct strains belonging to serotype B expressed the proteinase antigen only on blastoconidia, while filamentous cells of these strains appeared non proteolytic. As this difference in the expression of proteinase is typical of serotype B, it may account for its reduced virulence.

The results of our study indicate that *C. albicans* strains isolated from frog meat were able to produce the same spectrum of diseases in mice as that found in man. The presence of this fungus in processed frog legs is caused by cross-contamination during handling and processing of the animals.

The source of C. albicans in the frog legs is apparently contaminated water. Nonetheless, handlers cannot be discarged as carriers, because *Candida* paronychia and onyquia occur as occupational diseases among workers whose hands are damaged by prolonged immersion in water.

The data presented in this study might prompt future legislation to be more stringent regarding conditions for processing of frog meat.

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## RESUMO

**Sorotipagem, produção de proteinase e fosfolipase relacionadas à patogenicidade de amostras de *Candida albicans* isoladas de coxas de rãs**

Quinze amostras de *Candida albicans* isoladas de coxas de rãs (*Rana catesbeiana*, Shaw-1802) comercializadas no Brasil, foram estudadas quanto ao sorotipo e fatores de virulência "in vitro" e "in vivo". A sorotipagem revelou predominancia do sorotipo B. Todas amostras estudadas foram produtoras de fosfolipases e 66,6% produziram proteinases. No estudo da patogenicidade foram observados dois tipos de alterações renais: uma do tipo agudo com abscessos corticais e outra do tipo crônico com pielonefrite e envolvimento unilateral.

**Palavras-chave:** *C. albicans*; leveduras em carnes; coxas de rãs.

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# INFLUENCE OF CULTURE MEDIA AND AIR SUPPLY ON THE PRODUCTION OF ADHESINS BY *MORAXELLA BOVIS*

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**Carlos Gil-Turnes***

## ABSTRACT

The production of hemagglutinating adhesins by *Moraxella bovis* GF 9 in four culture media at three levels of air supply was studied. The highest yields of adhesins and bacterial mass were produced in media containing meat peptone, such as Brain Heart Infusion (BHI) and peptone and meat extract medium (DCF). The influence of air supply on the production of adhesins differed with the media used. In BHI the yields obtained with 1 volume of air per volume of medium (vvm) were 2.5 times higher than those with 0.5 vvm. In DCF the titers were lower than in BHI, while in Mueller Hinton medium (MH) and Tryptic Soy Broth (TSB) adhesins were not produced. The maximal production of adhesins was obtained in BHI aerated at 1 vvm after 12 hours of incubation. Dissolved oxygen levels dropped to 7 and 30 %, at the beginning of the exponential phase of growth, under aeration of 0.5 and 1.0 vvm, respectively.

**Key words:** *Moraxella bovis*, adhesins, fermentation, vaccine.

## INTRODUCTION

Adhesins of the gram negative bacterium *Moraxella bovis* are used as antigens to prevent the most important ocular disease of cattle, Infectious Bovine Keratoconjunctivitis (IBK) (3, 4, 11). Even though a reasonable amount of information on several aspects of these antigens has been published, most of it is related to experimental vaccines prepared with antigens grown on blood agar (6, 12).

For the industrial production of vaccines, *M. bovis* is grown in submerged cultures inside fermenters, under growth conditions which differ considerably from those found in nature, where these bacteria form part of the microflora of the eyes. Information regarding the influence of medium composition and aeration on the synthesis of adhesins is, however, very scarce.

The purpose of this work was to compare the yields of adhesins produced by a strain of *M. bovis* grown, in fermenters, in four culture media extensively used for the industrial production of vaccines against IBK and under three different levels of air supply.

## MATERIALS AND METHODS

### Isolate

*M. bovis* GF 9, a strain recovered from an animal with IBK, characterized biochemically (1) and tested for adhesin production (2), was used throughout the experiments.

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**Culture media**

The media tested were: Tryptic Soy Broth (TSB)[a], Brain Heart Infusion (BHI)[b], Mueller Hinton (MH)[a] and a medium containing peptone 1.0 %, meat extract 0.25 %, NaCl 0.25 %, $Na_2HPO_4$ 0.25 %, in distilled water, designated as DCF. All of them had the pH adjusted to 7.2 at the beginning of the experiments. The media were autoclaved in the fermenter at 121°C for 30 min.

**Air supply and measurement**

Oxygen was supplied to the media at 0.5 and 1 volume of air per volume of media per minute (vvm). Dissolved oxygen levels (DO) were measured with a galvanic probe (NBS DO 40, probe 1016-0770, New Brunswick Scientific, NJ, USA) sterilized with the vessel. DO values, expressed as percent of saturation with air at atmospheric pressure, were only determined for cultures in BHI.

**Determination of growth parameters**

Bacterial growth was determined with a Coleman Junior 6A spectrophotometer at 600 nm and expressed as Transmittance (T). Adhesin concentration was estimated as previously reported (2). Briefly, twofold dilutions of the culture in 0.15 M NaCl were mixed with the same volume of a 0.5 % chicken red blood cells suspension, agitated and left at 4°C. Readings for hemagglutination were done 1 and 24 hours later.

The reciprocal of the highest dilution at which hemagglutination (HA) was produced was the HA titer. One hemagglutination unit (HAu) was defined as the least quantity of bacteria contained in 0.5 mL that agglutinated an equal volume of 0.5 % chicken red blood cells. pH was determined using a combined glass electrode.

**Fermentation**

The experiments were performed in a Multigen F-2000 fermenter (New Brunswick Scientific, NJ, USA) containing one liter of medium. The cultures were agitated at 350 rpm and the air sterilized by absolute filters. Silicone at a final concentration of 1:5000 was added with the inoculum and during fermentation whenever excessive foam formation made it necessary. Samples were collected at two hour intervals between 0 and 24 hours of incubation. The experiments were repeated two to four times.

## RESULTS

**Bacterial growth**

The highest yields of bacterial mass were produced in DCF medium aerated at 0.5 vvm, the ones obtained with 1 vvm being slightly lower, whereas those produced in the experiments without air supply were markedly reduced. Transmittances at the end of the logarithmic phase (around 12 hours) were 72.5, 15.5 and 17.5 for the experiments performed without aeration, at 0.5 vvm and at 1 vvm, respectively.

Yields in BHI were slightly lower than in DCF. At the end of the logarithmic phase the transmittances for the three levels of aeration (0, 0.5 and 1 vvm) were 87, 33 and 28, while in TSB they were 80.5, 75 and 99.

No growth was observed in MH.

**Production of adhesins**

Adhesins were first detected in DCF at time 12 hours of incubation in the experiments without aeration and at time 10 hours in those aerated at 0.5 vvm. With 1 vvm of air, adhesins were not detected in this medium. HA titers were 2 and 4 HAu/ mL, respectively.

Adhesins were not produced in BHI when air was not supplied. They were first detected at time 8 hours of incubation in the experiments with 0.5 vvm and at time 6 hours in those with 1 vvm. HA titers remained constant in the cultures aerated at 0.5 vvm (mean 5 HAu/ mL), while in those aerated at 1 vvm they grew steadily for up to 12 hours of incubation, decreasing afterwards. The titers were 5.2, 13 and 6.6 HAu/ mL at times 6, 12 and 24 hours of incubation, respectively (FIG. 1).

Adhesins were not produced in TSB.

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[a] Difco Laboratories, Detroit, Michigan, USA.

[b] Biobras, Montes Claros, MG, Brazil

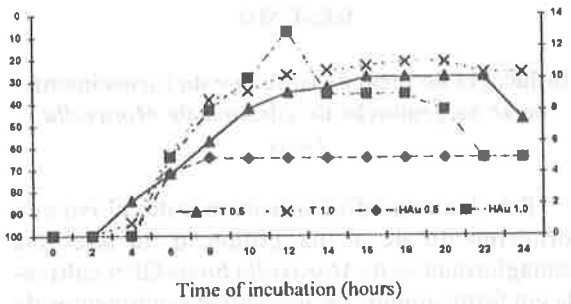


FIGURE 1. Growth (%T, left) and adhesin production (HAu/ ml, right) of *Moraxella bovis* in BHI aerated at 0,5 and 1 vvm

**Dissolved Oxygen (DO)**

Dissolved Oxygen levels dropped from 100 % to 5 % after six hours of culture in the experiments with 0.5 vvm, returning to the initial values at time 10 hours (FIG. 2). When 1 vvm was supplied, the lowest DO level was 30 % at time 4 hours, returning to saturation at time 14 hours (FIG. 2).

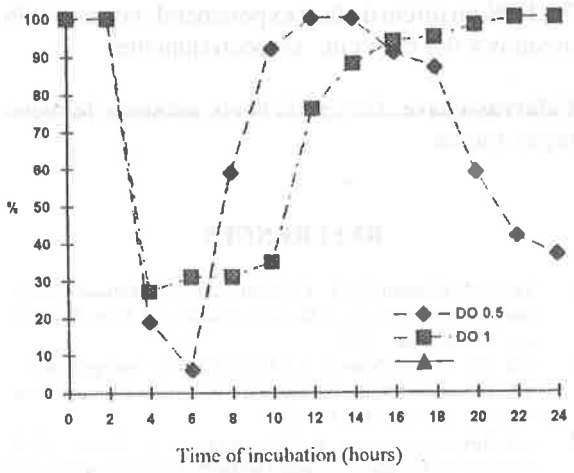


FIGURE 2. Dissolved oxygen levels of BHI during cultivation of *Moraxella bovis* GF 9 aerated at 0.5 vvm and 1 vvm.

**pH**

*Moraxella bovis* GF 9 grew at pH 7.2 but not at pH 6.2, 6.4 and 6.9. During the growth cycle, the pH remained almost unchanged in unaerated cultures, while increasing steadily in the experiments where air was being supplied. Under aeration at 0.5 vvm the pH was 8.0 in BHI and DCF and 7.6 in TSB after 24 hours of incubation. When air was supplied at 1 vvm the pH values for these media were 8.3, 8.6 and 7.0, respectively.

## DISCUSSION

Our observations show that both the constitution of the media in which the bacteria were grown and the supply of air during bacterial multiplication affected the synthesis of adhesins by *Moraxella bovis* GF 9. The four media tested are complex media that differ mainly in the nature of the peptones used for their formulation. The higher yields of bacterial mass were obtained in DCF and those of adhesins in BHI, which are media that contain peptones obtained from meat. Proteose Peptone, used in BHI, has twice the concentration of tryptophane, tyrosine, cysteine and organic sulfur than peptone, used in DCF. It has been postulated that the source of organic sulfur influences the growth of *M. bovis* (5).

*M. bovis* GF 9 did not grow in Mueller Hinton medium and grew very poorly in TSB, which are media that contain peptones obtained from casein. Using an enzymatic hydrolysate of casein to study the growth requirements of *M. bovis* strain CCM 3942 in submerged culture, Mandl *et al.* (7) found that growth and respiration were depressed after six hours of culture due to the limited supply of carbon and energy by the aminoacids, making necessary the addition of fresh medium in order to continue the culture. This procedure did not seem to be necessary with BHI, that supported growth until the end of the exponential phase. Riley (13) showed that complex media such as BHI were more efficient for the cultivation of *M. bovis* than basal media to which aminoacids and vitamins had been added. Nekvalisová *et al.* (9) showed that the nature of the proteins used in media for toxin production by *Clostridium perfringens* also influenced the yields obtained.

Air supply affected both bacterial mass and adhesin yields in DCF and BHI. Bacterial mass was higher in aerated cultures compared to the unaerated ones. For both these media, the exponential phases in aerated cultures ended more rapidly at 1 vvm than at 0.5 vvm.

The highest titers of adhesins were obtained four hours after the end of the logarithmic phase in BHI aerated at 1 vvm, being 2.5 times higher than

those obtained at 0.5 vvm and decreasing rapidly during the stationary phase. Ostle and Rosembusch (10), studying the kynetics of hemolysin expression in *M. bovis*, found that they were also produced at the end of the logarithmic phase, the production falling rapidly afterwards. It is possible that exotoxins with proteolytic activity, which are also synthesized by these bacteria during the growth cycle, destroy the adhesins thus leading to such reduction.

A very high oxygen demand was detected in BHI during the logarithmic phase. DO levels fell to 5 % and 30 % when air was supplied at 0.5 and 1 vvm, respectively. Mooyman (8) reported that several bacteria do not grow when DO levels are lower than 15 %. Even considering that *M. bovis* has great affinity for Dissolved Oxygen (7), it could be said that the lower titers of adhesins detected in the experiments with low air input were caused by insufficient availability of oxygen during the exponential phase. This could irreversibly hamper bacterial growth, due, probably, to the limited utilization of other nutrients.

The continuous increase in pH observed in our experiments was also reported by Mandl *et al.* (7), who showed that it was due to the accumulation of ammonia produced by the utilization of aminoacids and that it did not inhibit bacterial growth.

Our observations suggest that media that support good growth, such as DCF, are not necessarily the best choice when *M. bovis* must be cultivated to produce specific antigens such as adhesins. For the production of these antigens by *Moraxella bovis* GF 9, BHI with aeration at 1 vvm should be recommended.

The influence of nutrient supply on bacterial synthesis and expression of fimbriae used for vaccine production has rarely been reported. The results presented herein indicate that such influence should be further investigated, in order to optimize the use of media and equipment for the obtention of good quality antigens at low costs.

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## RESUMO

**Influência do meio de cultura e do fornecimento de ar na produção de adesinas de *Moraxella bovis***

Estudou-se a influência do meio de cultivo e do fornecimento de ar na produção de adesinas hemaglutinantes de *Moraxella bovis* GF 9 cultivada em fermentador. Os mais altos rendimentos de adesinas e massa bacteriana obtiveram-se em meios preparados com peptona de carne, Infuso de Cérebro e Coração (BHI) e medio com peptona e extrato de carne (DCF), respectivamente. A influência do fornecimento de ar foi diferente nos meios estudados. Em BHI a produção de adesinas foi 2.5 vezes maior nos cultivos aerados a 1 volume de ar por volume de meio (vvm) que com 0.5 vvm. Em DCF os títulos não alcançaram a metade dos obtidos em BHI, enquanto que em meio de Mueller Hinton (MH) e Caldo Soja Tripticaseína (TSB) não se produziram adesinas. O título mais alto se obteve em BHI aerado a 1 vvm às 12 horas de cultivo. Os níveis de oxigênio dissolvido cairam a 7 e 30 % ao inicio da fase exponencial, nos cultivos aerados a 0.5 e 1 vvm., respectivamente.

**Palavras-chave**: *Moraxella bovis*, adesinas, fermentação, vacina

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# EFFECTS OF SODIUM DODECYL SULFATE ON THE GROWTH OF *CANDIDA LIPOLYTICA*

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## ABSTRACT

The effects of an ionic surfactant, sodium dodecyl sulfate, on the growth of *Candida lipolytica* were studied. Surfactant concentrations of 0.07%, 0.09% and 0.11% were added to the culture medium at different time points of the growth curve (0, 8, 16 and 24 hours of culture). The growth of *Candida lipolytica* at 27°C was evaluated over 96 h by viable cell counts (CFU/mL), maximum growth rate and generation time. The inhibition of growth observed was dependent on the addition time and concentration of sodium dodecyl sulfate used; no inhibitory effect was detected when the surfactant was introduced at time 24 h of culture.

**Key words:** *Candida lipolytica*, sodium dodecyl sulfate, growth.

## INTRODUCTION

Sodium dodecyl sulfate is an anionic surfactant with a number of industrial and biochemical applications, owing to its surface-active properties and solubilizing potency. Indeed, it has been used in technical procedures involving solubilization, isolation and characterization of proteins (5,6,9,18).

Considerable interest has been focused on the potential value of surfactants for the stimulation of growth and production of exoenzymes by different fungi, through their capacity to change the permeability properties of cytoplasmic membranes. Recent experiments have shown a positive effect of non-ionic surfactants on the growth and enzyme production of microorganisms. On the other hand, ionic surfactants have been associated with inhibition of growth and secretion of exoenzymes (11,17,7,2,10,14,8). However, the effect of surfactants on different growth phases of microorganisms has never been established.

The present study was undertaken to investigate the effects of sodium dodecyl sulfate on different growth phases of *Candida lipolytica*, a yeast with a potential industrial use related to its properties.

## MATERIALS AND METHODS

### Organism and media

The microorganism used in the experiments was *Candida lipolytica* IA 1055. The yeast was grown in the CYM medium described by Cirigliano and Carman (3), for 96 h at 27°C in a reciproc shaker (120 Hz). Sodium dodecyl sulfate was added to the medium at 0, 8, 16 and 24 h of culture, at concentrations of 0.07%, 0.09% and 0.11%.

### Evaluation of *C. lipolytica* growth

Cell viability was monitored by serial dilution of culture samples collected after 0, 8, 16, 24, 48, 72 and 96 hours of growth, which were plated in YMA medium as described by Cirigliano and

Carman (3). Triplicate plates were incubated at 27°C for 24h. Viable cells counts were expressed as the mean of three counts.

The parameters used to evaluate growth kinetics were maximum growth rate (mMax) and generation time (Tg), which were calculated according to the method described by Pirt (16).

## RESULTS

The growth of *Candida lipolytica* at 27°C as determined by viable cell counts was affected by sodium dodecyl sulfate (SDS), at all the concentrations tested, when the surfactant was added at the beginning of the culture (0 h) (FIGURE 1A). A drastic reduction in cell viability could be observed.

Cultures treated with the surfactant at the start of the exponential phase (8h - FIGURE 1B) and during middle exponential phase (16 h - FIGURE 1C) showed a higher cell viability than those where SDS was added at time 0 h. However, cell viability was related to the SDS concentrations used: cultures treated with 0.11% were less viable and cultures treated at time 16 h with 0.07% and 0.09% presented a similar viability. Both treatments (SDS addition at times 8h and 16h) induced a reduction in cell viability with respect to control cultures.

On the other hand, cultures treated with SDS at 24 h of culture (end of the exponential phase / beginning of the stationary phase) did not show any cell viability inhibition compared to controls (FIGURE 1D).

The results on maximum growth rate (mMax) and generation time (Tg) are presented in TABLE 1.

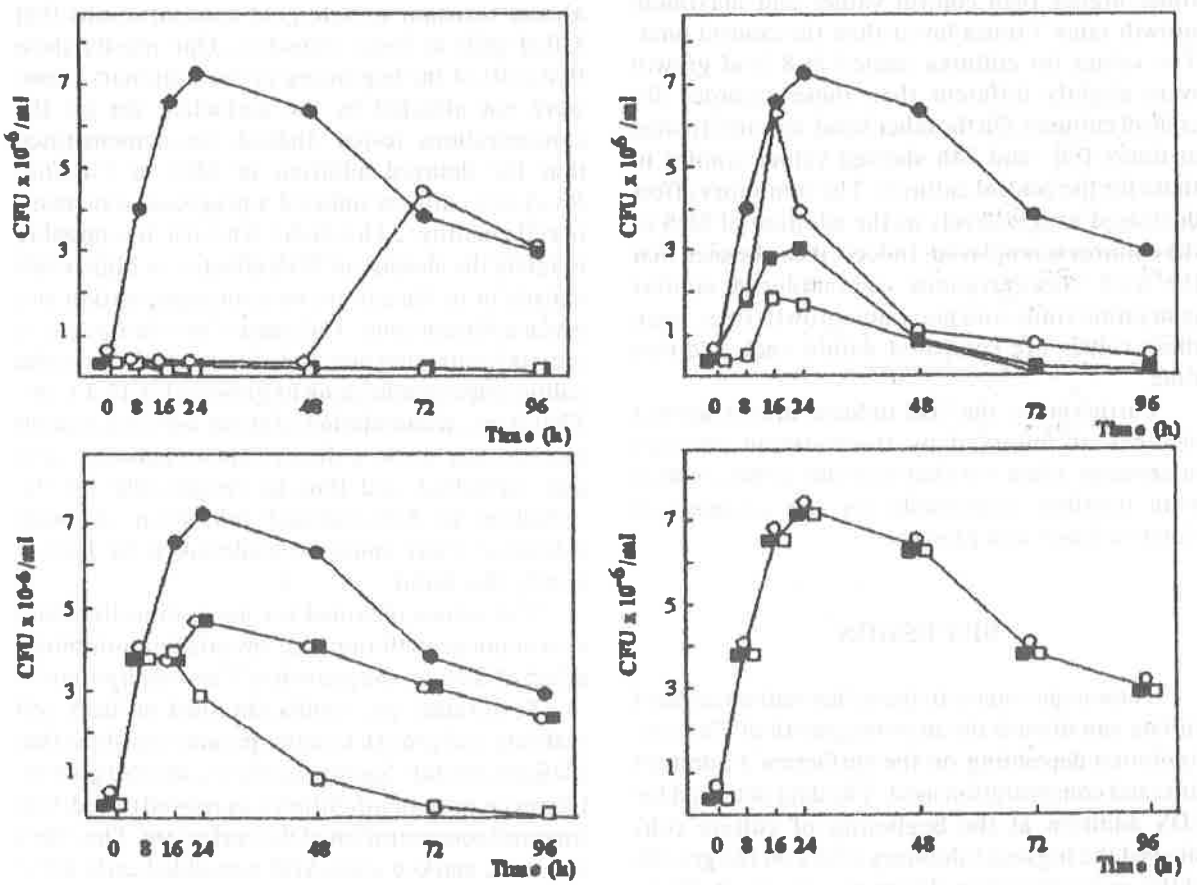


FIGURE 1 - Viability of *Candida lipolytica* treated with SDS at the beginning of growth (T 0 h - 1A) and after 8 (T 8 h - 1B), 16 (T 16 h - 1C) and 24 (T 24 h - 1D) hours of culture. SDS concentrations: (○) 0.07%, (■) 0.09% and (□) 0.11%. (●) Control culture.

TABLE 1 - Kinetics of *Candida lipolytica* growth in cultures treated with SDS at different growth phases.

| SDS addition Time (h) | Generation Time Tg (h) | | | Maximum Growth Rate mMax (h ) | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| 0 (T0) | 8.92 | 9.18 | 9.45 | 0.07 | 0.07 | 0.07 |
| 8 (T8) | 4.15 | 4.52 | 5.03 | 0.17 | 0.15 | 0.14 |
| 16 (T16) | 3.48 | 3.49 | 3.57 | 0.20 | 0.20 | 0.20 |
| 24 (T24) | 3.45 | 3.63 | 3.72 | 0.20 | 0.20 | 0.20 |

SDS Concentrations: 1 - 0.07%, 2 - 0.09% and 3 - 0.11%.
Control Culture: Tg = 3.38h and Max = 0.20h.

The values obtained reveal the inhibitory effect of sodium dodecyl sulfate on the growth of *Candida lipolytica*. It was possible to observe that SDS had the highest effect on these parameters when added at the beginning of culture (0 h). In this case, treated cultures presented generation times about 3 times higher than control values and maximum growth rates 3 times lower than the control ones. The values for cultures treated at 8 h of growth were slightly different than those recorded for control cultures. On the other hand, cultures treated at times 16h and 24h showed values similar to those for the control cultures. The inhibitory effect decreased progressively as the addition of SDS to the cultures was delayed. Indeed, it can be seen that the SDS concentrations used induced similar generation times and maximun growth rates when these values are compared within each addition time.

Furthermore, the SDS induced ultrastructural changes as observed by transmission electron microscopy (data not shown in this paper), which were possibly responsible for the decrease in *Candida lipolytica* growth.

## DISCUSSION

These experiments indicate that sodium dodecyl sulfate can disturb the *in vitro* growth of *Candida lipolytica* depending on the surfactant's addition time and concentration used. The data obtained for SDS addition at the beginning of culture (0h) showed the highest inhibitory effect on the growth of this microorganism. These results are similar to those reported by Reese and Maguire (17), Jagger *et al.* (7), Asther and Corrieu (2) and Long and Knapp (10). Additionally, Jagger *et al.* (7) showed that SDS concentrations of 0.01% inhibited growth and production of ligninases in *Phanerochaete chrysosporium*. This microorganism could grow in the presence of SDS 0.001%, though ligninase activity was still absent at this lower concentration of the surfactant.

Newton (15) has demonstrated that phospholipids, proteins, nucleic acids and soaps form complexes with surface active anions and cations and prevent their bactericidal action if they are present in a culture before introduction of the surface active agent, but not after the combination of the agent with the cells. Miller *et al.* (14) reported the effect of a lysosomotropic detergent on BHK cells. The authors concluded that the sensitivity of the cells was strongly density dependent; cells at the highest cell density were almost resistant to detergent concentrations that killed cells at lower densities. Our results show that cells at the beginning of the stationary phase were not affected by the surfactant for all the concentrations tested. Indeed, we demonstrated that the delayed addition of SDS to *Candida lipolytica* cultures induced a progressive increase in cell viability: 24 h > 16 h > 8 h > 0 h. It is possible to relate the absence of SDS effects to a higher cell density or to the culture medium composition at a given addition time. The yeast *Candida lipolytica* secrets lipids, enzymes and a bioemulsifier into the culture supernatant along its growth (1,4,12,13,19). Therefore, accumulation of these molecules could increasingly avoid a direct contact between cells and surfactant and thus be responsible for the reduction in SDS-induced inhibition. A more extensive study must be undertaken to further clarify this point.

The values obtained for generation time and maximum growth rate also revealed an inhibitory effect of SDS on the growth of *Candida lipolytica*.

To resume, the results obtained on both cell viability and growth kinetics parameters show that SDS can inhibit *Candida lipolytica in vitro* growth. However, growth inhibition was related to addition time and concentration of the surfactant. The effect was more marked when SDS was added early to the cultures as compared to later additions.

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## RESUMO

### Efeitos do dodecil sulfato de sódio sobre o crescimento de *Candida lipolytica*

Neste trabalho o efeito do dodecil sulfato de sódio sobre o crescimento de *Candida lipolytica* foi analisado. O surfactante foi adicionado ao meio de cultura, nas concentrações de 0,07%, 0,09% e 0,11% nas diferentes fases de crescimento celular 0, 8, 16 e 24 h. As culturas foram crescidas por 96 horas a 27°C e o crescimento foi acompanhado pela contagem de células viáveis, velocidade máxima de crescimento e tempo de geração. A inibição do crescimento pode ser relacionada ao tempo de adição e concentração do surfactante. Por outro lado, a adição do composto após 24 h de cultivo não demonstrou qualquer efeito sobre o microrganismo.

**Palavras-chave:** *Candida lipolytica*, dodecil sulfato de sódio, crescimento

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# SEROLOGICAL CHARACTERIZATION OF *MORAXELLA BOVIS* BY IMMUNODIFFUSION AND IMMUNOFLUORESCENCE: A COMPARISON

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## SHORT COMMUNICATION

## ABSTRACT

Antisera to surface antigens of six *Moraxella bovis* strains (five adherent and one non adherent) were used to analyze antigenic differences. Immunodiffusion with absorbed antisera detected differences, but the Fluorescence Antibody Technique did not.

**Key words:** *Moraxella bovis*, serological characterization, fluorescence.

The antigenic diversity of isolates of *Moraxella bovis* recovered from cattle with Infectious Bovine Keratoconjunctivitis (IBK) has been demonstrated using double immunodiffusion (DI) (3), ELISA (5), seroagglutination (8) and hemagglutination inhibition (HI) tests (4). The fluorescence antibody technique (FAT) was used to detect *M. bovis* in eye secretions of diseased and normal cattle (5, 9) and to identify colonies of *M. bovis* grown in culture media (7). The use of FAT has been proposed by several workers interested in reducing the time taken to characterize bacterial isolates.

The aim of the present study was to determine whether FAT could replace time-consuming techniques used to detect antigenic differences among *M. bovis* isolates.

Six isolates of *M. bovis* (five adherent and one non-adherent) recovered from cattle with IBK were used. They were characterized biochemically and their adherent properties determined using chicken red blood cells (2). Hyperimmune antisera to each isolate were raised in rabbits. The immunization schedule and the absorption of antisera were done as previously reported (3). The *M. bovis* strains were identified as follows: 1 (ML 1), 2 (ML 2), 3 (JUR), 4 (LO 2), 5 (A 1429) and 6 (B 1429). Only isolate 5 was non adherent.

To carry out the FAT, a loopful of culture of each isolate grown for 24 hs at 37°C on Tryptose Blood agar was suspended in a drop of sterile distilled water on the surface of a glass slide, dried in air and fixed gently by heat. One drop of undiluted serum was placed over each dried *M. bovis* suspension and the glass slides were then incubated in a moist chamber for 30 min at 37°C. The serum was discarded, the slides rinsed three times with PBS and dried in air. Next, a drop of appropriately diluted Fluorescein Isothyocyanate conjugated goat anti-rabbit antiserum (Difco, Michigan, USA) was placed over the reacting surface of the slides. Incubation was carried out in a moist chamber for 30 min at 37°C. After incubation, the serum was

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discarded and the slides were rinsed three times with PBS. The preparations were analyzed with an Olympus CBB-FL microscope by epifluorescence and interpreted as -, +, ++ and +++. Each isolate was tested with its homologous antiserum, with unabsorbed antiserum 6 and with antiserum 6 absorbed with antigen 4. Isolate 2 was also tested with four antisera used in a previous work (3).

The immunodiffusion tests were done as previously described (3). Each isolate was tested with the unabsorbed antisera to the isolates mentioned above, and with these same antisera absorbed with heterologous strains (TABLES 1 and 2).

TABLE 1 - Pattern of precipitation reactions by immunodiffusion with absorbed antisera to six isolates of *Moraxella bovis*

| Antiserum | absorbed with antigen | tested with antigen 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| 1 | 2 | + | - | + | + | + | + |
| 2 | 1 | - | + | + | + | + | + |
| 3 | 6 | - | - | - | - | - | - |
| 4 | 6 | + | + | + | + | + | - |
| 6 | 4 | + | + | + | - | + | + |

+ = one band of precipitation

TABLE 2 - Pattern of precipitation reactions by immunodiffusion with unabsorbed antisera to six isolates of *Moraxella bovis*

| Isolate | Sera 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| 1 | ++ | ++ | ++ | ++ | + | ++ |
| 2 | ++ | ++ | ++ | ++ | + | ++ |
| 3 | ++ | ++ | ++ | ++ | + | ++ |
| 4 | ++ | ++ | ++ | ++ | + | ++ |
| 5 | + | + | + | + | ++ | + |
| 6 | ++ | ++ | ++ | ++ | + | ++ |

+ = one band; ++= two bands

The adherent isolates produced two bands of precipitation with unabsorbed antisera, while the non adherent produced only one. Antisera No 1, 2, 4 and 6, after absorption with antigens 2, 1, 6 and 4, respectively, produced only one band with their homologous antigens and none with the absorbing strain (TABLE 1).

The immunodiffusion test using absorbed antisera showed that the isolates were antigenically different, with the exception of isolates 3 and 6. The precipitin band shared by the adherent isolates suggests that their adhesins are antigenically identical, a fact apparently confirmed when absorbed antisera were used. This observation, however, is not in agreement with the results obtained using quantitative methods. The hemagglutination inhibition titers for antiserum 6 were 1:128 and 1:32 when determined with its homologous antigen and with antigen 3, respectively, but the former titer fell to 1:32 (Gil Turnes, C., 1983, M.Sc. Thesis) when antiserum 6 was absorbed with isolate 4 and afterwards tested with its homologous antigen. This shows that quantitative methods are more sensitive than the qualitative ones. Lepper and Hermans (5) using quantitative ELISA, and Gil Turnes and Aleixo (4) using the hemagglutination inhibition test with monoclonal antibodies, also demonstrated differences among fimbrial antigens of *M. bovis*, which were not revealed by immunodiffusion.

The antigenic differences reported were not detected by FAT. The fluorescence observed when testing the isolates with unabsorbed serum 6 might have been related to the antigenic identity between adhesins shown by the immunodiffusion technique mentioned above. Yet fluorescence intensity remained unchanged when serum 6 was absorbed with isolate 4, while the band produced by adhesins disappeared. The failure of FAT in detecting such differences was also evident when antisera 7 (strain FLA 64, isolated in the USA) and 8 (strain IBH 64, also from USA), which did not react with adhesins, produced fluorescence of the same intensity as that observed with antiserum 6, which reacted with adhesins (TABLE 3). McDonald and Pugh (7)

TABLE 3 - Pattern of fluorescence reactions by FAT using homologous and heterologous antisera to six isolates of *Moraxella bovis*

| Isolate | Antisera Homologous | 6* | 6** | 7 | 8 |
|---|---|---|---|---|---|
| 1 | +++ | +++ | +++ | - | - |
| 2 | +++ | +++ | +++ | +++ | +++ |
| 3 | +++ | +++ | +++ | - | - |
| 4 | +++ | +++ | +++ | - | - |
| 5 | + | + | + | - | - |
| 6 | +++ | + | +++ | - | - |

* = non absorbed; ** = absorbed with antigen 4
7 = antisera prepared with strain FLA
8 = antisera prepared with strain IBH

observed that the fluorescence obtained with strain FLA-64 antifimbrial antiserum was more intense than that obtained with strain IBH-64 homologous antiserum, suggesting that fimbriae of this latter strain could be less antigenic than the former.

The non adherent isolate 5 gave a weaker fluorescence than the adherent isolates. Lepper and Barton (6) observed that "spreading and corroding" strains produced stronger fluorescence than non-corroding ones when tested with a heterologous antiserum. The capacity of *M. bovis* to corrode some culture media has been associated with the presence of fimbriae (1).

From the observations stated it may be concluded that FAT is not adequate for the detection of antigenic differences between adhesins or somatic antigens of *M. bovis*, and that other methods, such as ID using absorbed antisera or quantitative methods, should be used for this purpose.


## RESUMO

### Caracterização sorológica de *Moraxella bovis* mediante imunodifusão e imunofluorescência

Soros contra antigenos de superfície de cinco amostras aderentes e uma não aderente de *Moraxella bovis* recuperadas de bovinos com Ceratite Infecciosa, foram utilizados para detectar diferenças antigênicas entre elas, mediante as técnicas de imunodifusão e imunofluorescência indireta. Soros adsorvidos foram eficientes na detecção de diferenças antigênicas mediante a técnica de imunodifusão. Com imunofluorescência indireta, porem, não foi possível detectá-las.


**Palavras-chave:** *Moraxella bovis*, caracterização sorológica, imunofluorescência

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MATERIALS AND METHODS: This section should provide enough information for other investigators to repeat the work. Repetition of details of procedures which have already been published elsewhere should be avoided. If a published method is modified, such modification(s) must be described in the paper. Sources of reagents, culture media and equipment (company, city, state, country) should be mentioned in the text. Names that are registered trade marks should be so indicated. Subheading often make this section easier to read and understand.

RESULTS: This section should, by means of text, tables and/or figures, give the results of the experiments. If a *DISCUSSION* section is to be included, avoid extensive interpretation of results but do so in the *DISCUSSION* section. If *Results* and *Discussion* are combined, then results should be discussed where, in the text, it is most appropriate. Tables should be numbered independently of the figures using Arabic numerals. All tables and figures must be mentioned in the text. The approximate location of tables and figures in the text should be indicated.

DISCUSSION: The discussion should provide an interpretation of the results in relation to known information.

ACKNOWLEDGMENTS: This section is optional and follows the *DISCUSSION*. It aknowledges financial and personal assistance.

REFERENCES: Arrange the references in alphabetical order, by last name of the author. All authors must be cited. Number the references consecutively. Cite each reference in the text by its number. Journal names should be abbreviated according to the style of *Biological Abstracts* or *Chemical Abstracts*. All references given in the list should be cited in the text and all references mentioned in the text must be included in the list. List references according to the style shown in the following examples.

a. Paper in a journal
Campos, L.C.; Whittam, T.S.; Gomes, T. A.T.; Andrade, J.R.C.; Trabulsi, L.R. *Escherichia coli* serogroup O111 includes several clones of diarrheagenic strains with different virulence properties. *Infect. Immun.*, 62: 3282-3288, 1994.

b. Paper or chapter in a book
Nelson, E.B. Current limits to biological control of fungal phytopathogens. *In*: Arora, D.K.; Rai, B.; Mukerji, K.G.; Knudsen, G. (eds). *Handbook of applied mycology; soils and plants*. Marcel Dekker, New York, 1991, p.327-355.

c. Book by author(s)
Salyers, A.A.; Whitt, D.D. *Bacterial pathogenesis. A molecular approach*. ASM, Washington, 1994, 418p.

d . Patent
Hussong, R.V.; Marth, E.H.; Vakaleris, D.G. Manufacture of cottage cheese. *U.S. Pat. 3,117,870*. Jan. 14, 1964.

e. Thesis
Calzada, C.T. Campylobacter jejuni e Campylobacter coli - *caracterização em sorogrupos e biotipos das cepas isoladas no município de São Paulo, no período de 1983-1989*. São Paulo, 1991, 131p. (Ph.D. Thesis. Instituto de Ciências Biomédicas. USP).

f. Publication with no identifiable author or editor
Anonymous. The economy of by-products. *Álcool Alcoolquim.*, 2;33-40, 1985.

g. Communications in events (Symposia, conferences, etc)
Simão, G.S.; Silva, J.; Toledo, A.S.; Gontijo Filho, P.P. *Micobactérias não tuberculosas isoladas de pacientes com a síndrome de imunodeficiência adquirida.* XVII Congresso Brasileiro de Microbiologia, Santos, 1993, p.41.

REFERENCES citing "personal communication" or "unpublished data" are discouraged, although it is recognized that sometimes they need to be used. In these cases, they should be cited in the text and not in the list of references. References consisting of papers that are "accepted for publication" or "in press" are acceptable. However, references of papers that are "submitted" or "in preparation" are not acceptable.

TABLES

Tables should not be included in the text. Each TABLE must be typed in a separate page and numbered sequentially with an Arabic number. The title of a TABLE should be placed in the top of it and should be brief but fully descriptive of the information in the TABLE. Headings and subheadings should be concise with columns and rows of data carefully centered below them.

FIGURES

Arabic numbers should be used for numbering the Figures. Data in Tables should not be repeated in FIGURES. The legend of the FIGURES should be placed in the bottom of them.

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Only those photographs which are strictly necessary for the understanding of the paper should be submitted. Photoprints must be of sufficient quality to ensure good reproduction. They should be numbered on the back and identified with the nominated author's name. Legends of line drawings and photographs should not exceed the printing area. All elements in the drawing should be prepared to withstand reductions. Drawings and line figures should be drawn in black ink on tracing paper and should be prepared as indicated for the photographs. Colored illustrations are not accepted.

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