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Endereço

Av. Prof. Lineu Prestes, 1.374
05508 – São Paulo – SP – Brasil
Fone: (011) 813-9647 – Telex: 011 35085 –

REVISTA DE MICROBIOLOGIA
Depto. de Microbiologia – ICB II – USP
Av. Prof. Lineu Prestes, 1374 – Cid. Universitária
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CHARACTERIZATION OF BACILLUS POLYMYXA ISOLATES FROM DIFFERENT BRAZILIAN SOILS

Elvira Cristina Neves Ferreira[1]
Lucy Seldin[1]*

SUMMARY

Seventy-eight *Bacillus polymyxa* isolates were obtained from different Brazilian soils and characterized for the presence of genetic markers which could be used in recombination experiments within this species. Their DNAs were obtained and it could be observed that 4 isolates showed the presence of plasmids with similar molecular weight. Thirty-two isolates (including those containing the plasmids) were chosen for further studies. All strains showed resistance to polymyxin-B and to bacitracin and sensitivity to the other antibiotics tested. Also, all isolates produced an inhibitory substance against one strain of *Staphylococcus aureus* and 7 against a *Pseudomonas* strain. From the 32 isolates, 25 were lysed by EPy-2 phage, specific to *B. Polymyxa* and 2 were able to fix nitrogen (acetylene reduction test). However, none of these characteristics showed by the different isolates could be correlated to the presence of the plasmids. The strains cured from the plasmid, obtained in this work, showed the same phenotipic characteristics presented by the strains harbouring the plasmids.

Key Words: *Bacillus polymyxa* , isolation, characterization.

INTRODUCTION

The genus *Bacillus*, which comprises the aerobic spore-forming soil saprophytes, is widely used in the microbiological industry as many of its strains excrete a number of products that find application in the pharmaceutical, food and agricultural industries.

B. polymyxa, one of the *Bacillus* species, is one example of applicability of these bacteria in the different industries. Its strains produce antibiotics, different enzymes capable of degrading various carbohydrates, polysaccharides (4) and, furthermore, some *B. polymyxa* strains are nitrogen-fixing ones (3, 11). However, until now selection of a *B. polymyxa* strain for industrial application has been based on the screening of naturally occurring strains for desired properties, followed by attempts at further improvement by mutagenesis and suitable selection (4). This fact can be explained by the lack of a gene transfer system known for this *Bacillus* species. There was also a great difficulty to initiate genetic studies with this group of microorganisms as no plasmids or genetic markers were found within the species. So, this work had the purpose of isolate *B. polymyxa* strains and characterize them for the presence of genetic markers which could be used in recombination experiments.

1. Deptº de Microbiologia Geral - Instituto de Microbiologia, Centro de Ciências da Saúde - Bloco I - Cidade Universitária, Ilha do Fundão, CEP 21944 - Rio de Janeiro, RJ, Brasil.
* Corresponding author.

MATERIALS AND METHODS

Bacterial isolates and culture media - All *Bacillus polymyxa* isolates used in this work, together with their sources, are listed in Table 1. The isolates were stored aerobically at room temperature on GB slants (11) supplemented with 1% CaCO3. Long-term preservation was in sterile soil by the method of Gordon *et al.* (7). The different media described below were used for the following purposes: TBN medium (13) changing the glucose for mannitol (0.5%), for the enrichment of *B. polymyxa*; neutral red-peptone-starch-agar medium (3), for the isolation of colonies belonging to *B. polymyxa*; GB broth (11), for growing *B. polymyxa* to test for the production of the inhibitory substance, to screen for resistance determinants, to test for plaque formation with EPy-2 phage, to isolate their DNA and to make the curing experiments. Agar (1.5 or 0.7%) was added to the GB to obtain solid or semi-solid medium, respectively. *Staphylococcus aureus* RN450 and *Pseudomonas* sp. were also grown in GB medium. TBNR medium (10) was used to detect acetylene-reducing activity in *B. polymyxa*. Unless otherwise stated, the preferred incubation temperature was 32°C.

TABLE 1 - *Bacillus polymyxa* isolates used in this work.

Isolates	Source and other information
CL2, CL3, CL4, CL7	From rhizosphere of lemon grass, Rio de Janeiro State.
SCE1, SCE2, SCE3, SCE4, SCE5	From Cerrado soil, Brasília, Federal District.
EC1, EC2, EC3, EC4, EC5, EC6, EC7, EC8, EC9	From "Ecologia" soil, Rural University of Rio de Janeiro, Rio de Janeiro State.
BN2	From rhizosphere of banana tree, Rio de Janeiro State.
CA1, CA2, CA3, CA4, CA5, CA6, CA7	From rhizosphere of sugar cane, Rio de Janeiro State.
JP1, JP2, JP3, JP4	From Garden soil, Rio de Janeiro State.
AG1, AG2	From "Agrícola" soil, Rio de Janeiro State.
LMD48..22	Strain received from Dr. J. van der Toorn, Laboratory of Microbiology, Delft, Holland.
L	Strain received from Dr. M. Loutit, University of Otago, New Zealand.

Soil sampling and sample treatment - Surface soil samples listed on Table 1 were collected aseptically, passed through a 0.71 mm mesh sieve to remove coarse particles and then, 1 g of each soil was mixed with 10 ml of sterilized water and pasteurized (10 min, 80°C) to select for *Bacillus* species. Samples of 0.1 ml of these suspensions were transferred to tubes containing 10 ml TBN plus mannitol and inverted Durham vials. After incubation for 2 or 3 days, tubes showing gas formation were selected.

Isolation and identification of B. polymyxa - From the tubes showing gas formation, appropriate dilutions were plated on selective medium containing 0.005% of neutral red. The slimy colonies showing red pigmentation were isolated, purified by three aerobic single-colony reisolation steps and stored in GB agar slants.

For their identification, most cultural and biochemical tests were performed using the methods and media of Gordon *et al.* (7). For all tests that required complex media appropriately adjusted GB solid or liquid media were employed. The basal medium of Gordon *et al.*(7) was used to test for acid and gas production from carbohydrates. Additional identification tests were performed using the API system, as described before (13).

Screening for resistance determinants - Antibiograms were made in agar plates as described by Giambiagi *et al.* (5) using an overlay of soft agar seeded with 0.2 ml of an overnight culture. Antibiotic containing disks (Difco) were applied with a dispenser and the following drugs were used: amikacin (30 μg), bacitracin (10 U), carbenicillin (100 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamycin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), penicillin G (10 U), polymyxin B (300 U), novobiocin (30 μg), rifampicin (5 μg), tetracycline (30 μg) and vancomycin (30 μg). Results were read after 24 h of incubation.

Demonstration of the inhibitory activity of B. polymyxa - The inhibitory activity of *B. polymyxa* isolates against *Staphylococcus aureus* RN450 and *Pseudomonas* sp. was assayed by the lawn-spotting technique as described before (14). *B. polymyxa* LMD 48.22, a polymyxin producer strain, was used as a positive control.

Nitrogen fixation assays - Acetylene reduction was tested by measuring the ethylene production of cultures in 18 ml vials as described previously (10, 11). The vials were incubated for 24 h at 32°C, after which acetylene was introduced. Readings were made after 1 h at 32°C. In all tests, *B. polymyxa L (8)*, a *nif*$^+$ strain, was used as a positive control.

Sensitivity to bacteriophage EPy-2 (12) specific for B. polymyxa - Different dilutions of EPy2 lysate were spotted onto lawns of young *B. polymyxa* cells, and plates were scored after 24 h incubation. All presumptive positive results were retested for observation of plaque formation as described by Adams (1). In these experiments, *B. polymyxa* L was also used as a positive control.

Plasmid DNA isolation - The technique used to isolate plasmid DNA from *B. polymyxa* strains was described previously by Giambiagi-Marval *et al.* (6). The protocol used was the same, only using lysozyme to promote the bacterial lysis.

Curing experiments - Growth of broth cultures with sodium dodecyl sulphate (SDS, 2 $\mu g.ml^{-1}$) was used to cure *B. polymyxa* SCE2, as described previously (2).

Agarose gel electrophoresis - Agarose gel electrophoresis of total DNA samples was performed in 0.8% agarose (IBI - International Biotechnologies, Inc.) in TEB buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.2) at 2V cm^{-1}, for 16 h at room temperature, as described before (9).

RESULTS AND DISCUSSION

Seventy-eight isolates identified as *Bacillus polymyxa* were obtained from different Brazilian soils (Table 1) in plates containing selective medium and neutral red. All slimy colonies showing red pigmentation were isolated, purified and identified based on Gordon *et al.* (7). All 78 isolates presented the same results in all biochemical tests confirming the homogeneity of the species suggested before (3). All isolates formed mucoid raised colonies on GB agar and cultures in GB broth showed high viscosity due to the formation of mucous matter. Also, they formed acid from glucose, xylose, arabinose and mannitol. The Voges-Proskauer test, starch and casein hydrolysis and nitrate reduction tests were positive. These isolates grew at pH 5.7, but not in 5% NaCl or at 45°C. In these tests, identical results were obtained for *B. polymyxa* L, which was used as a control.

For further studies, intending to select strains with genetic markers which could be used in recombination experiments, 32 isolates were chosen (Table 1) to test for sensitivity to a *B. polymyxa* specific phage, for their susceptibility to different antibiotics, and for their ability to fix nitrogen. All 78 isolates were previously tested for the presence of extrachromosomal elements. Table 2 shows part of these results. When phage EPy-2 (12) was tested for its infection capacity against the 32 strains, only 7 showed resistance to it. The great majority of the isolated strains were susceptible to phage EPy2 indicating that this phage could be useful as a vector in genetic transfer experiments. Also, the 32 isolates were tested for their acetylene reducing ability. Only 2 isolates showed positive results, but with a low efficiency of acetylene reduction (data not shown). Furthermore, when the percentage (6.25%) of strains which were able to reduce acetylene was compared with previous results (11), it could be concluded that without a selective pressure during the isolation procedure [anaerobic TB agar, (10)], the number of nitrogen-fixing *B. polymyxa* was quite low in the different Brazilian soils tested in this work. There is also the possibility that both nitrogen-fixing isolates represent only one strain, as they showed similar results in all tests and were isolated from the rhizosphere of sugar cane from Rio de Janeiro state. The same can be suspected in respect to strains SCE2, SCE3, SCE4, and SCE5. They have also showed the same characteristics including the presence of plasmids with identical molecular weight. They were considered to be the same plasmid and studies are being performed to character-

TABLE 2 - Some characteristics of *B. polymyxa* isolates obtained from different soils.

Isolates	Nitrogen fixation (acetylene reduction)	Susceptibility to phage EPy 2	Plasmid detection
CL2, CL3, CL4, CL7, SCE1, BN2, EC1 to EC9, CA1 to CA5, JP1	-	+	-
SCE2 to SCE5	-	+	+
CA6, CA7	+	-	-
JP2, JP3, JP4, AG1, AG2	-	-	-

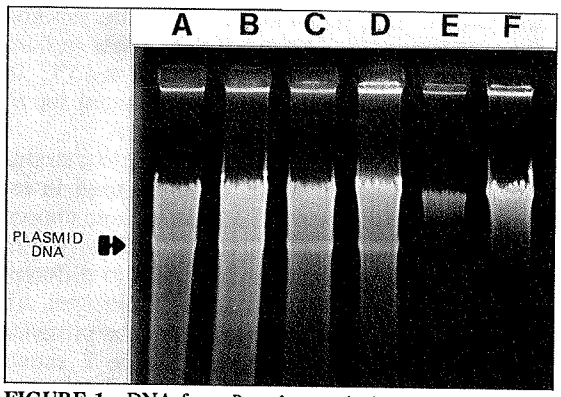

FIGURE 1 - DNA from *B. polymyxa* isolates (SCE2, lane A: SCE3, lane B; SCE4, lane C; SCE5, lane D) harboring plasmids and two cured derivatives from SCE2 (lanes E and F).

ize it genetically. Two strains cured from the plasmid, obtained by treating isolate SCE2 with SDS, were analysed with respect to its biochemical characteristics. No difference was observed between SCE2 and its cured derivatives.

The phenotypic characteristics analysed were the same in all strains. This fact indicates that no studied characteristic can be attributed to the presence of the observed plasmid. Figure 1 shows the plasmids isolated from strains SCE2, SCE3, SCE4, SCE5 and 2 SCE2 cured derivatives.

Fourteen different antibiotics were used to test the susceptibility of the 32 strains against these drugs. All strains showed to be sensitive to amikacin, chloramphenicol, carbenicillin, erythromycin, gentamycin, kanamycin, nalidixic acid, penicillin G., novobiocin, rifampicin, tetracycline, and vancomycin and resistant to bacitracin and polymyxin-B.

Studies on the susceptibility of *Bacillus* species to antibiotics are very few and nothing was known with respect to *B. polymyxa* strains (3). As its strains are not opportunistic pathogens, they have attracted little interest from medical microbiologists. However, drug markers have been used extensively in genetic studies concerning, for example, genetic exchange experiments.

B. polymyxa has been shown to produce a major group of antibiotics, the polymyxins. The ability of strains to produce antimicrobial substances can also be used as a genetic marker in gene transfer experiments. All isolates were tested for the production of an antibacterial substance using as indicator strains, *S. aureus* RN450 and *Pseudomonas* sp., usually sensitive to polymyxin. All isolates were able to inhibit growth of *S. aureus* RN450, the same observed with *B. polymyxa* LMD 48.22 used as a positive control. Only 7 isolates (CL2, CA3, CA5, JP1 to JP4) inhibited *Pseudomonas* sp.. This fact indicates that at least two different substances are being liberated by *B. polymyxa* isolates.

Studies are being developed concerning the introduction of foreign DNA in *B. polymyxa* strains (data not published). New methodologies have to be tested as, for example, electroporation for genetic transfer within this species. Certainly the isolates characterized here will be very useful for these studies.

RESUMO

Caracterização de isolados de *Bacillus polymyxa* oriundos de diferentes solos brasileiros

Setenta e oito isolados de *B. polymyxa* foram obtidos de diferentes solos brasileiros com intuito de se identificar estirpes com marcadores genéticos que pudessem ser utilizadas em experimentos de troca gênica nesta espécie de *Bacillus*. O DNA destes isolados foi extraído e observou-se que 4 deles apresentavam um plasmídio de tamanho semelhante. Trinta e dois isolados (incluindo os 4 contendo o plasmídio) foram escolhidos para estudos complementares. Todos eles mostraram-se resistentes à polimixina-B e à bacitracina e sensíveis aos outros 12 antibióticos testados. Todos os isolados produziram uma substância inibitória contra uma estirpe de *Staphylcoccus aureus* RN450 e 7 contra uma estirpe de *Pseudomonas* sp. Dos 32 isolados, 25 mostraram-se sensíveis ao fago EPy-2, específico para *B. polymyxa*, e 2 foram capazes de fixar o nitrogênio atmosférico (teste de redução de acetileno). Entretanto, nenhuma destas características acima mencionadas pode ser atribuída à presença do plasmídio, já que isolados curados (sem o plasmídio) apresentavam as mesmas características fenotípicas que aqueles contendo o plasmídio em questão.

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RECOMBINATION OF LAC$^-$ MAL$^+$ CELLS WITH LAC$^+$ MAL$^-$ VARIANTS ARISEN SPONTANEOUSLY FROM DIFFERENT CANDIDA ALBICANS STRAINS

Edson Marden Bonifacio e Souza[1]
Arlete Emily Cury[2]
Maria Helena P. Fungaro[3]
Zoilo P. Camargo[4]
João Lúcio de Azevedo[5]

SUMMARY

Candida albicans strains, maintained in usual culture media, formed colonies that, in auxonogram tests, showed an assimilation for lactose, but not for maltose. Spheroplasts prepared from these variants and from normal lac$^-$ mal$^+$ cells of different origins were fused by the polyethylene glycol method. This procedure yelded only lac$^+$ mal$^+$ cells. After reduction in ploidy, these cells originated 82% of lac$^-$ mal$^+$ colonies, 13.3% of lac$^-$ mal$^-$ and 4.4% of lac$^+$ mal$^+$.
Ploidy of the fusion product, occurrence of heterocaryose in this product, and the loss of chromosomes in the course of the segregation are discussed in this paper.

KeyWords: *Candida albicans*, natural variants, parasexuality.

INTRODUCTION

The characterization of *Candida albicans* has usually been made using assimilation and fermentation tests, as well as production of germ tubes and chlamydospores. Among the carbohydrate compounds employed, maltose is invariably assimilated and lactose is not (4).

In a re-evaluation of the morpho-physiological characteristics of *C. albicans* strains isolated from clinical specimens and stored in adequate conditions, it was found that some strains presented an intense growth when the carbon source was lactose and a poorer development when the source was maltose. Such a behavior occurred when the tests were performed by inoculating the strain on the surface of yeast nitrogen-base agar supplemented with the carbon source, but not with other basal medium or auxanographic method (4). Based on this observation, and in analogy with other reports (5,7,11,12), it seemed possible that these strains could originate spontaneously variants and normal cells with a requirement for lactose and maltose.

Isolation and characterization of spontaneous or induced variants are fundamental elements in the study of the genetics of *C. albicans*. Since

1. Universidade de São Paulo; Instituto de Ciências Biomédicas, Departamento de Microbiologia - ICB/USP. Av. Prof. Lineu Prestes, 1374, 05508 - São Paulo - SP. Brasil.
2. Universidade de São Paulo: Faculdade de Ciências Farmacêuticas, Departamento de Análises Clínicas e Toxicológicas. Av. Prof. Lineu Prestes, 580, B-17, 05580-900 São Paulo - SP. Brasil.
3. Universidade Estadual de Londrina, Centro de Ciências Básicas, Departamento de Biologia Geral. Campus Universitário, 86051 - Londrina - Paraná. Brasil.
4. Escola Paulista de Medicina, Disciplina de Biologia Celular. Rua Botucatu, 862, 8º andar, 04023 - São Paulo - SP. Brasil.
5. Universidade de São Paulo, Escola Superior de Agricultura Luiz de Queiroz, Departamento de Genética. Av. Pádua Dias, 11. 13400 - Piracicaba - SP. Brasil.

typical characteristics of sexuality have not been observed in this species, the parasexual cycle, based on the fusion of spheroplasts, has been used for genetic analysis and mapping purposes (8,9,12,13). With this protocol, the requirement for some aminoacids has been emphasized and auxotrophic and prototrophic variants obtained using mutagenic agents. Although these studies contribute for the knowledge of the genetics of *C. albicans.*, it was believed that the mutated cells may not represent a natural occurence in *C. albicans.*

Since the assimilation for lactose and maltose is considered a basic characteristics of *C. albicans*, the present study was carried out in order to confirm the presence of variants generated spontaneously, and to verify the genetic affinity between variant and normal cells derived from different strains.

MATERIAL AND METHODS

1. Strains

Twenty *C. albicans* strains were isolated from different patients, about one year ago, the maintenance of them being adequate (4). The morphophysiological characteristics of these strains were re-evaluated every three months using different procedures (4). In addition, the auxonograms were carried out by streaking the microorganisms on the surface of yeast nitrogen base (YNB, Difco) agar (2%) supplemented with carbohydrate (2%). Using this procedure it was possible to select two parental strains, Ca 1 and Ca 2.

2. Isolation of variants

Parental strains were maintained on Sabouraud dextrose agar (SDA, Difco) for 24-48h at 30°C. Harvested cells were centrifuged and suspended in distilled water at 10^3 cells/ml and 0.1 ml was plated on SDA and incubated at 30°C for 24-48h. Thirty isolated colonies were transferred to SDA slants. After incubation, each cell mass was washed three times by centrifugation in distilled water and a water suspension prepared at 10^3 cells/ml. A volume of 0.1 ml was plated on YNB agar supplemented with 2% glucose, lactose or maltose. The cultures were incubated at 30°C for 24-48h. Growth with glucose was used as control. Derivatives Ca 1.2 (lac^+ mal^-) and Ca 2.4 (lac^- mal^+) were them selected for subsequent studies.

The tests described below were carried out on the bases of protocol described by Poulter et al (8, 9).

3. Reversion test

Suspensions of each derivative, Ca 1.2 and Ca 2.4, containing 100 cells/0.1 ml were plated on YNB agar-glucose, YNB agar-lactose and YNB agar-maltose. After incubation at 30°C for 24-48h, the number of growing colonies were recorded.

4. Spheroplast formation

Blastospores of Ca 1.2 and Ca 2.4 were harvested from SDA slants and washed with distilled water by centrifugation. The cells were suspended in 0.1 M phosphate buffer, pH 7.2 (PB), at 10^7 cells/ml. A 5 ml suspension was centrifuged at 3000rpm for 10 min. The resulting pellet was suspended in 0.5 M MgSO4. 7H2O containing 0.1M Tris-HCI (pH 7.2) and 0.01 M B-mercaptoethanol. The cells were washed twice with stabilizer buffer (TSP) pH 7.2 [0.1 M phosphate buffer and 1M sorbitol (v/v)], suspended in 5 ml of TSP with 0.8 mg of lyticase (5000 UI, Sigma) and incubated at 30°C for 1h or until 95% to 100% of spheroplasts were formed.

5. Spheroplast fusion and regeneration

Spheroplasts were washed three times with TSP by centrifugation at 2000 rpm for 10 min. The resulting pellet was suspended in 4 ml of TSP and 1 ml of 1.2 M CaCl2. Identical spheroplast suspensions were prepared from different blastospores, combined and centrifuged at 1000 rpm for 10 min. The cells were suspended in 6 ml of PB and 1 ml of 40% polyethylene glycol, and incubated for 20 min at 30°C. Then, 3 ml of PB was added and the suspension centrifuged at 1000 rpm for 10 min. The sediment was washed with PB and suspended in PB to contain 10^7 cells/ml. Dilutions of this suspension were made to give 10^1,10^2,10^3 and 10^4 cells/ml. Then, 2 ml of each dilution was transferred to Petri dishes and mixed with 18 ml of SDA prepared in TSP. The plates were incubated at 30°C for 24-48h. Thirty colonies were transferred to SDA slants and maintained at 30°C for 24-48h. From each culture, cells were streaked on YBN agar-glucose, YNB agar-lactose and YBN agar-maltose, and incubated at 30°C. Three cultures maintained on SDA slants were randomly selected for ploidy reduction.

6. Reduction of fusion products

From each culture a suspension containing 10^5 cells/ml was prepared in PB. Then, 1 ml of

this suspension was added to 50 ml of yeast extract-peptone glucose (YEPG) containing 0.5 ml of Benlate solution (1), incubated at 30°C for 24-48h, and centrifuged. The resulting pellet was washed twice with distilled water and suspended at 10^4 cells/ml. A volume of 0,5 ml was plated on SDA. After incubation at 30°C for 24-48h, 15 colonies were respectively inoculated in SDA slants and incubated. The cell growth was then submitted to assimilation tests for glucose, lactose and maltose.

Controls were obtained from the SDA cultures by using the protocol described for reduction, with exclusion, of Benlate solution.

RESULTS

1. Phenotypic characteristics of cells derived from parental strains.

Six of the 20 *C. albicans* strains generated variants showed a requirement for lactose and/or maltose. The parental strain designated Ca 1 generated 56.6% of colonies lac^+ mal^+, 10% of lac^+ mal^-, and 33% of lac^- mal^+; the Ca 2 strain simply generated the lac^- mal^+ type. The derivative Ca 1.2 (lac^+ mal^-), one of the progenies of the Ca 1 strain, and Ca 2.4 (lac^- mal^+), progenie of Ca 2, were selected for reversion and fusion studies.

2. Revertion products.

Ca 1.2 submitted to reversion, gave rise to lac^+ mal^- (91%) and lac^- mal^+ (9%) colonies. Ca 2.4 originated only lac^- mal^+ colonies.

3. Requeriment for lactose and maltose of the spheroplast fusion products.

All colonies derived from spheroplast fusions were lac^+ mal^+.

4. Requeriment for lactose and maltose of the reduction products and control.

The first of the three colonies selected from spheroplast fusion products and submitted to reduction, gave rise to 20% of lac^- mal^- and 80% of lac^- mal^+ derivatives. The second colony gave rise to 6.6% of lac^+ mal^+, 13.3% of lac^- mal^- and 80% of lac^- mal+ derivatives. The third one originted 6.6% of lac^+ mal^+, 6.6% of lac^- mal^- and 86.6% of lac^- mal^+ (Tab.1). The average results obtained for the 45 derivatives are included in Fig. 1.

Control colonies gave rise only to lac+ mal+ cells.

TABLE 1 - Requeriment for glucose, lactose and maltose of deirvatives of reductions products.

reduction product (rCa) and derivatives	assimilation test		
	glucose	lactose	maltose
rCa 1			
1.1	+	-	+
1.2	+	-	+
1.3	+	-	+
1.4	+	-	-
1.5	+	-	+
1.6	+	-	-
1.7	+	-	+
1.8	+	-	+
1.9	+	-	+
1.10	+	-	+
1.11	+	-	+
1.12	+	-	+
1.13	+	-	-
1.14	+	-	+
1.15	+	-	+
rCa 12			
12.1	+	-	+
12.2	+	-	-
12.3	+	-	+
12.4	+	-	+
12.5	+	+	+
12.6	+	-	+
12.7	+	-	+
12.8	+	-	+
12.9	+	-	+
12.10	+	-	-
12.11	+	-	+
12.12	+	-	+
12.13	+	-	+
12.14	+	-	+
12.15	+	-	+
rCa 30			
30.1	+	-	+
30.2	+	-	+
30.3	+	-	+
30.4	+	-	+
30.5	+	-	+
30.6	+	-	+
30.7	+	-	+
30.8	+	-	+
30.9	+	-	+
30.10	+	-	+
30.11	+	+	+
30.12	+	-	-
30.13	+	-	+
30.14	+	-	+
30.15	+	-	+

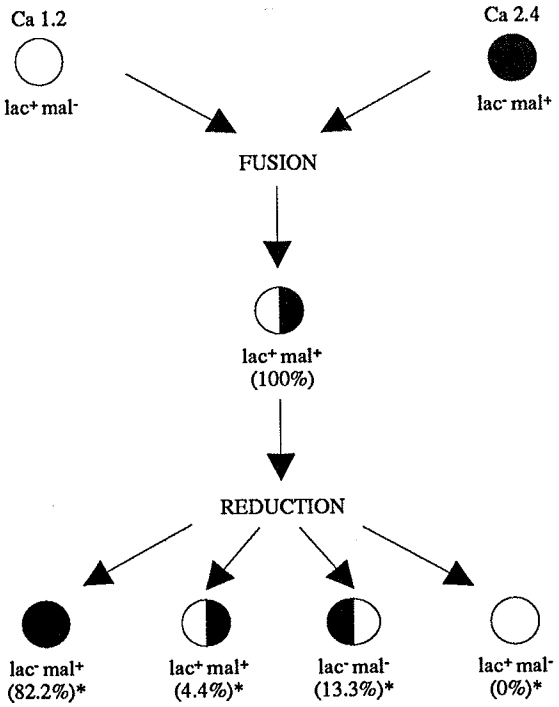

FIGURE 1 - Requirement for glucose, lactose and maltose of Ca 1.2 and Ca 2.4 derived from *C. albicans* strains, and their products of fusion and reduction.
* : average results obtained for 45 derivatives.

DISCUSSION

From strains maintained in usual culture conditions it was possible to isolate variants with requirements for lactose and maltose. Contrary to other studies, the frequency of variant derivatives was sufficiently high so that the use of mutagenic agents (2,8,9,14) was not necessary. Therefore this work could be developed maintaining the natural characteristics of the strains. In addition, the nutritional requirements for lactose and maltose, instead of aminoacids, were better phenotypic markers since, taxonomically, the lac- mal+ characteristic has been considered an invariable trait of *C. albicans* (4).

Based on the results obtained for the Ca 1 strain, it is possible to infer that strains classified as *C. albicans* can spontaneously form variants with different requeriments for lactose and maltose. In contrast, other strains behaving, as Ca 2, maintained in the same conditions are unable to originate similar variants.

The Ca 1.2, lac$^+$ mal$^-$ variant, showed a relatively low reversion (9%) for the typical characteristics of the species whereas the Ca 2.4, which resembled the parental strain, gave rise only to lac$^-$ mal$^+$ colonies. These findings permitted the use of Ca 1.2 and Ca 2.4 for spheroplast fusion analyses. Although all the fusion products had a lac$^+$ mal$^+$ requeriment most of the reduction products showed the characteristic behavior of *C. albicans* (lac$^-$ mal$^+$). The Ca 1.2 lac$^+$ mal$^-$ phenotype, was not detected among the reduction products, whereas lac$^-$ mal$^+$ and lac+ mal$^+$ types which were not observed in any of the original strains, appeared among these products.

It is probable that these segregants arose from mitotic crossing-over or from unstable polyploid nucleous of the fusion product (3,12,14). *C. albicans* was found to be diploid (6,8,10,12) and the fusion products were frequently tetraploids or hexaploids (6,8,12,14). These hybrids can undergo loss of chromosomes to yield segregants with ploidy similar to that of the parental cells (14). In this condition, the predominance of one parental phenotype among the segregants and the occurrence of cells showing phenotypes other than parental cells can be possible (3).

On the other hand, the characteristic lac$^+$ mal$^+$ can represent a heterocaryon fusion product. The probable transference of genetic information from one nucleous to another without nuclear fusion or the loss of some chromosomal material from one of two types of nuclei in heterokaryon (3,10), suggest another way that the segregants lac$^-$ mal$^+$, lac$^+$ mal$^+$ could arise.

Further studies are necessary to test the natural occurrence of these events in *C. albicans* and this inteference in taxonomic studies. Characteristics that arise from derived cells in the parasexual process and from naturally generated cells in *C. albicans*, should be submitted to more comprehensive biochemical and genetical analyses to establish their importance.

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We thank Prof. Dr. Sergio Olavo P. Costa for critical comments and suggestions.

RESUMO

Recombinação de células lac- mal+ com variantes lac$^+$ mal$^-$ naturalmente originadas de cepas de *Candida albicans*

Cepas de *Candida albicans*, mantidas em meios de cultura usuais, originaram colônias que

em testes de assimilação de fontes de carbono mostraram-se lac$^+$ mal$^-$.

Esferoplastos obtidos a partir desses variantes e esferoplastos preparados a partir de células normais, lac$^-$ mal$^+$, foram submetidos à fusão e produziram apenas células lac$^+$mal$^+$. Após redução na ploidia, essas clulas deram origem a colônias lac$^-$ mal$^+$ (82%), lac$^-$ mal (13,3%) e lac $^+$mal$^+$ (4,4%).

Discute-se neste trabalho, ploidia dos produtos de fusão, ocorrência de heterocariose nesses produtos e a perda de cromossomos durante a segregação.

Palavras-chave: *Candida albicans*, variante natural, parassexualidade.

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IDENTIFICATION, PROPAGATION AND SUBGROUP CHARACTERIZATION OF AN EQUINE ROTAVIRUS ISOLATED IN SÃO PAULO, BRAZIL

Maria Lucia Rácz[1]*
Veridiana Munford[1]
Maria Judite Bittencourt Fernandes[2]
Suzana S. Kroeff[2]
Ivanete Kotait[2]

SUMMARY

Two equine fecal samples (EQ/R28 and EQ/R29) were received for diagnostic purposes in Instituto Biológico. Samples were from Orlândia, SP, from two different animals with diarrhea. Identification of rotavirus was made by enzyme immunoassay (EIARA/FIOCRUZ) and by polyacrylamide gel electrophoresis (PAGE). The samples were positive for goup A rotavirus by both techniques, with electropherotype characteristic of equine rotavirus (gene segment 3 and 4 close together, segments 7, 8 and 9 spaced). Rotavirus was isolated in established cell line of fetal rhesus monkey kidney MA104, with added trypsin, in a roller apparatus, reaching the sixth passage, with characteristic cytopathic effect. Lysates of all the passages in MA104 cells were positive for rotavirus by EIARA and PAGE, with electropherotype similar to the original sample, in respect to segments 1-6 and with differences in migration in segments 7-11. Original samples and lysates of passages in cell culture were tested for subgroup determination, with group A, subgroup I and subgroup II specific monoclonal antibodies (MAb), by an ELISA test. Both samples and lysates reacted with group A specific MAb and could not be assigned to either subgroup I or II, similar to strain H-2 and other equine rotaviruses. This is the first report of equine rotavirus isolated in Brazil and it is important to elucidate the prevalence of this virus in normal and diarrheic foals.

Key Words: Rotavirus, equine.

INTRODUCTION

Rotaviruses are established as important etiological agents of acute gastroenteritis in mammalian and avian species. The genome of rotavirus consists of eleven double-stranded RNA segments, which form a characteristic pattern on polyacrylamide gel electrophoresis. Group A rotavirus have a common group antigen on the 42 kd major internal structural protein VP6, which contains also the subgroup specificity. Two distinct subgroup specificities have been described, designated subgroup I and II (9,12).

1. Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo. Av. Prof. Lineu Prestes, 1374. 05508-900 - São Paulo - SP.
2. Instituto Biológico. Av. Conselheiro Rodrigues Alves, 1252. 04014-002 - São Paulo - SP.
* Corresponding Author.

In Brazil, rotavirus diarrhea has been documented in various animal species, like human (6,16,17), bovine (14) and swine (3,8). In 1975, Flewett et al. (7) reported the identification of rotaviruses by electron microscopy, from feces of newborn foals. Since then, foal rotaviruses have been identified in various countries, like Australia (18,19), United States (9,11), Ireland (20) and others. Imagawa et al. (13) sucessfully propagated a foal rotavirus in MA104 cells.

This report describes the identification, propagation and subgroup characterization of an equine rotavirus from São Paulo, Brazil.

MATERIAL AND METHODS

Fecal samples:

Two equine fecal samples (EQ/R28 and EQ/R29) were received for diagnostic purposes in Instituto Biológico, São Paulo. Samples were from Orlândia, São Paulo, from animals with diarrhea. In the property, 5 animals died with diarrhea, and 3 were sick. The 2 samples were obtained from 2 different sick animals with diarrhea.

Identification of rotaviruses:

Samples were suspended in Tris/Calcium buffer (0.01M Tris, 1.5mM $CaCl_2$), pH 7.3. After clarification (5.900 g/30 minutes), samples were treated with equal volume of Freon TF (Du Pont), centrifuged at 2.600 g/5 minutes and supernatants were obtained and store in -20°C freezer. Samples were tested by enzyme immunoassay - EIARA/Fundação Oswaldo Cruz (15), specific for group A rotavirus and by polyacrilamyde gel electrophoresis, as described previously (16). The simian rotavirus SA11, cultivated in MA104 cells, was included in all electrophoretic runs as a standard.

Cell culture:

MA104, an established cell line of fetal rhesus monkey kidney was used for rotavirus isolation and propagation. Growth medium was Eagle minimum essential medium with 10% fetal calf serum and antibiotics. Maintenance medium was the same, without serum and with final concentration of 10 µg/ml trypsin (Difco).

Isolation and propagation of equine rotavirus in MA104 cells:

Samples were pretreated with antibiotics (penicillin, 2.000 U/ml and streptomycin 2.000 µg/ml, room temperature, 60 minutes), centrifuged (12.000 g/20 minutes) and treated with trypsin (final concentration 10 µg/ml) for 30 minutes at 37°C. Confluent monolayer culture tubes were washed three times with phosphate buffer saline (PBS) pH 7.2, and 5 tubes/sample were inoculated with 0.1 ml of treated fecal suspension.

After adsorption (60 minutes, 37°C), 0.9 ml of maintenance medium were added and tubes were incubated on a roller apparatus, at 37°C. At the time cells showed cytophatic effect (CPE), consisting of increased cell granularity, cell rounding and cell detachment from the glass, cells were frozen and thawed three times and cells lysates were inoculated into new cell culture tubes, as described above. Cell lysates were tested by EIARA and PAGE.

Subgroup antigen assay:

Group A monoclonal antibodies (MAb), and subgroup I and subgroup II specific MAb (1), were produced by H. Greenberg (Bethesda, MD, USA) and kindly supplied by G.M. Beards (Birmingham, UK). ELISA tests were performed as described previously (2), with some modifications. Briefly, ELISA plates (Hemobag) were coated with anti-rotavirus goat serum (15) in carbonate-bicarbonate buffer for 2 hours at 37°C. The original samples and lysates of cell cultured viruses were added and the plate was incubated overnight at 4°C. A 1/1.000 dilution of each monoclonal antibodies were added, followed by incubation at 37°C for 2 hours. Finally, an anti-mouse IgG-peroxidase conjugate (Sigma) diluted 1/400 was added and plates were incubated for 1 hour. The substrate used was hydrogen peroxyde/o-phenylenediamine (OPD) in citrate buffer. Phosphate-buffered saline plus 0.1% Tween 20 (PBS/T) was used as washing buffer between stages and the reagents diluent was PBS/T plus 10% bovine serum albumin. Optical densities (O.D.) at 492 nm were determined by spectrophotometry. Control samples included simian rotavirus SA11 (subgroup I) and an human rotavirus sample (F444) previously found to belong to subgroup II. Samples were considered to belong to a subgroup when O.D. obtained with one subgroup specific MAb was at least two times the O.D. obtained with the other subgroup especific MAb.

RESULTS

The two equine samples were positive for group A rotavirus by enzyme immunoassay (EIARA) and by PAGE and negative for adenovirus by EIARA. Electropherotypes of the two samples were the same (Figure 1). EQ/R28 sample, inoculated in MA104 cells showed 25% typical CPE beginning at day 6 and 100% CPE by day 7. EQ/R29 sample showed CPE at day 6 to 7, according to tube, and 100% CPE by day 7 to 10. In second passage, both EQ/R28 and EQ/R29 sample showed CPE beginning at day 7 and 100% CPE by day 11. In the third to fifth passage, MA104 cells showed 50% CPE by day 5 (Figure 2) and 100% by day 6 to 10. Cell lysates from all passages were positive by EIARA and PAGE, with segments 1-6 migrating at the same position and segments 7-11 in different position (Figure 1). Total CPE in the sixth passage was obtained by day 5, and supernatants, after freezing and thawing, were positive for rotavirus by EIARA and PAGE.

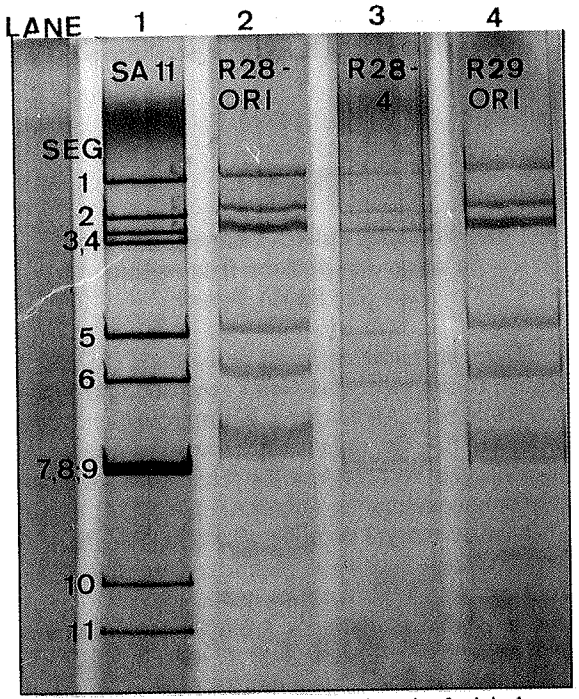

FIGURE 1 - Polyacrylamide gel ectrophoresis of original samples of equine rotavirus (R28/ORI, lane 2 and R29/ORI, lane 4) and 4th passage of cultivated virus (R28/4, lane 3) Lane 1: SA11 for comparison. Segments are numbered in left column. Migration was from top to bottom.

In the enzyme immunoassay for subgrouping, both original samples and lysates from second and fourth cell culture passages of the two samples reacted with group A specific MAb and did not react with subgroup I or subgroup II specific MAb.

DISCUSSION

Foal rotaviruses have been reported as an important cause of diarrhea in equine (9,11,18). In Brazil, to our knowledge, this is the first report of this virus.

The equine group A rotavirus isolated in this research is similar to other equine rotavirus reported in the literature. Electropherotype of original samples gave a pattern similar to most equine rotavirus, with gene segments 3 and 4 close together and segments 7, 8 and 9 more spaced (5). The subgroup characteristc of this virus, that could not be assigned to either subgroup I or II, is similar to strain H-2, described by Hoshino et. al. (12) and to other described equine rotaviruses (4). The majority of equine rotaviruses that have been subgrouped possess neither or both subgroup antigens on their viral protein VP6 (5). This is characteristic of equine rotaviruses and differentiate between equine and other animal rotaviruses, that belong most frequently to subgroup I (4,5).

Rotavirus have been reported to multiply in cell cultures with difficulty, and in Brazil, only porcine rotaviruses have been cultivated (10). Other rotaviruses have been identified mostly on a direct basis. In this paper, we describe the successful cultivation of the two samples of equine rotavirus, in MA104 cells, maintained in roller type cultures and with trypsin in culture media. The samples reached the sixth passage, with characteristic CPE, consisting of increased cell granularity, cell rounding and cell detachment from the glass (Figure 2). Lysates of the six passages were positive for rotavirus by EIARA and PAGE, with electropherotype similar to the original sample, in respect to segments 1-6 and with differences in migration in segments 7-11 (Figure 1).

This differences could reflect the possibility of mixture of different electropherotypes in original samples, as the electrophoretic pattern of original samples are diffuse (Figure 1, lanes 2 and 4). MA104 cell substrate could have selected, during multiplication, an homogeneous popu-

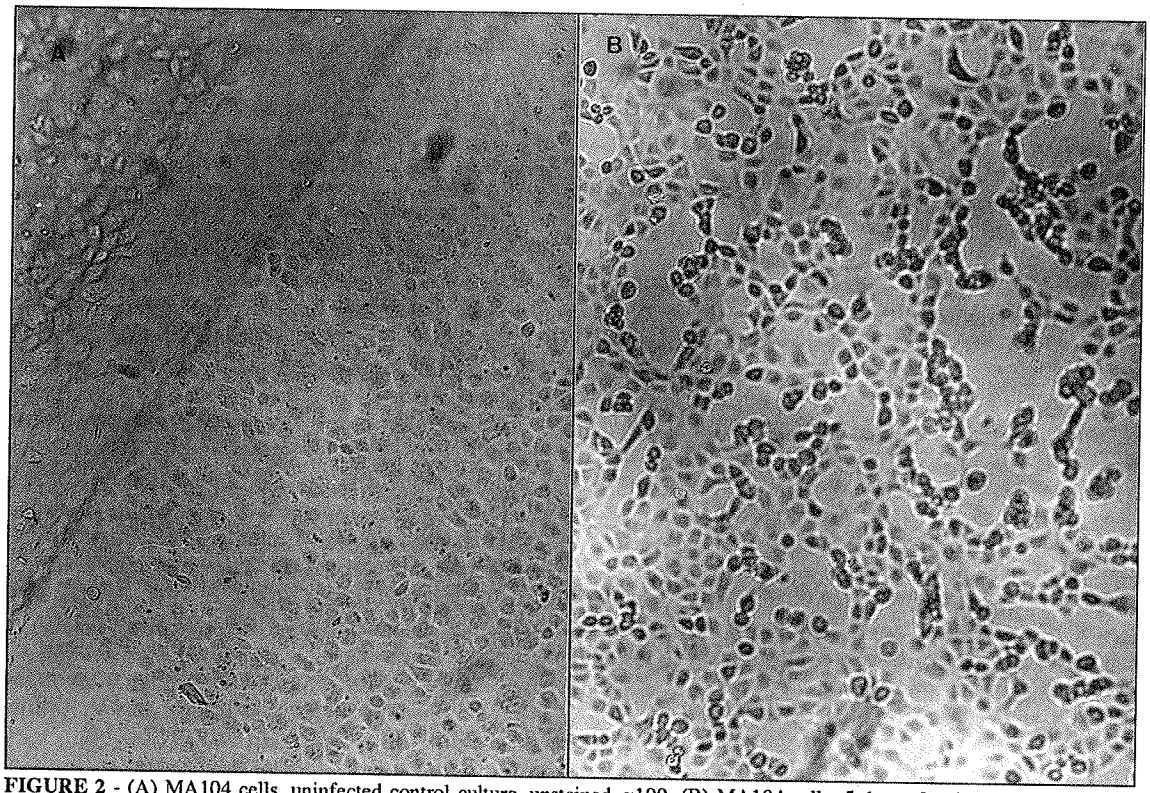

FIGURE 2 - (A) MA104 cells, uninfected control culture, unstained, x100; (B) MA104 cells, 5 days after inoculation with 4th passage of rotavirus EQ/R28, unstained, x100

lation of virus, giving the more sharp electropherotype obtained with isolated virus (Figure 2, lane 3).

The second and fourth passage of isolated virus showed the same sugroup specificity (non I, non II subgroup) as original samples, excluding the possibility of laboratory contamination with other samples during cultivation. Our laboratory was not cultivating, at the same time, any other samples with this subgroup characteristic. In each passage of samples, 100% CPE was obtained in shorter periods of time, suggesting adaptation of virus to the cell substrate. Isolation and serial propagation of rotavirus samples is very important, as with cultivated viruses it is possible to make more detailed immunological, physicochemical and molecular studies of the virus.

To make clear the relationship between rotaviruses and diarrhea of foals in Brazil, it is important to study the prevalence of this virus in normal and diarrhoeic animals.

RESUMO

Identificação, cultivo e caracterização do subgrupo de rotavírus eqüino em São Paulo, Brasil

Duas amostras de fezes de eqüinos (EQ/28 e EQ/29) foram recebidas para diagnóstico no Instituto Biológico. As amostras eram provenientes de Orlândia, SP, de dois animais com diarréia. A identificação de rotavírus foi feita através de ensaio imunoenzimático (EIARA/FIOCRUZ) e por eletroforese em gel de poliacrilamida (PAGE). As amostras foram positivas para rotavírus pelas duas técnicas, com eletroferótipo característico de rotavírus eqüino (segmentos 3 e 4 bem próximos e segmentos 7, 8 e 9 separados). O rotavírus foi isolado em linhagem celular de rim fetal de macaco Rhesus (MA104), com a adição de tripsina, em equipamento do tipo "roller", chegando a sexta passagem, com efeito citopítico caracteristico. Os lisados de cada passagem em culturas celulares

foram positivos para rotavírus por EIARA e PAGE, com eletroferótipo semelhante ao da amostra original, em relação aos segmentos 1 a 6, e com algumas diferenças na migração dos segmentos 7 a 11. As amostras originais e lisados das passagens em culturas de células foram testados para determinação de subgrupo, com anticorpos monoclonais (MAb) específicos para grupo A, subgrupo I e subgrupo II, através de um teste imunoenzimático. Tanto as amostras originais quanto os lisados reagiram com o MAb específico para o grupo A e não reagiram com os anticorpos anti-subgrupos I e II, da mesma forma que a amostra H-2 e outros rotavírus eqüinos. Este é o primeiro isolamento de um rotavírus eqüino no Brasil, tornando-se importante elucidar sua prevalência em animais normais e com diarréia.

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SEROLOGICAL CHARACTERIZATION OF 23 ISOLATES OF BACILLUS SPHAERICUS, PATHOGENIC TO MOSQUITO LARVAE, SCREENED FROM BRAZILIAN SOIL

Rose Gomes Monnerat Schenkel [1]
Simoni Campos Dias [2]
Josélia Batista Lopes [2]

SHORT COMMUNICATION

SUMMARY

This paper deals with serological classification of 23 entomopathogenic *Bacillus sphaericus* isolates obtained in soil from several regions of Brazil. All the isolates are pathogenic to *Culex quinquefasciatus* and belong to the serotype H5. This serological characterization showed that among Brazilian *Bacillus sphaericus* isolates there are no regional differences. The serology protocol was adaptated and seemed to be convenient because it decreased the reaction time and quantity of material.

Key Words: *Bacillus sphaericus*, serology, characterization.

The entomopathogenic bacteria *Bacillus sphaericus* and *B. thuringiensis* are currently being used for the control of several mosquito species. These bacteria offer an environmentally friendly alternative to chemical pesticides for controlling these important vectores of diseases. Both bacteria have different degrees of specificity, *B. sphaericus* is highly pathogenic to larvae of *Culex quinquefasciatus, Anopheles gambiae, Anopheles culicifacies and Anopheles albimanus* (6) while *B. thuringiensis* subsp. *israelensis is* effective against *Aedes aegypti* (5).

In Brazil there is considerable interest in the use of *B. sphaericus* in biocontrol programmes. Most studies to date have focused on find out new isolates that could be more adapted than the exotic ones (4).

Serological characterization of B.Sphaericus and B.Thuringiensis is based on the H Flagellar agglutination test developed by de Barjac et alli (2) and de Barjac and Bonnefoi (1). this test has shown that the strains of B.sphaericus most pathogenic to Culex quinquefasciatus belong to serotypes H5, H6 and H25 (2,3). This methodology,

TABLE 1 - Distribution of Bacillus sphaericus isolates according to regions.

Regiona	city	Isolates
South	S. Marinho (RS)	S64
South-Eastern	Vitória (ES)	S3
	Cubatão (SP)	S56
Central	Corumba (MS)	S4,S6,S7,S9,S12, S14,S15,S17,S20, S21,S22,S27,S28
	Cáceres (MT)	S23,S24,S25,S26
	Rondonópolis (MT)	S102
	Brasília DFY	S44,S46

1. EMBRAPA/CENARGEN , C. P. 02372, Brasília DF, Brazil.
2. CNPq - Felowship grant.

with slight modifications, was used to characterize 23 isolates of B.sphaericus screened from soils from central, south and south-eastern regions of Brazil (Table 1). All the isolates used in this study are kept in the *Bacillus* bank at "Centro Nacional de Recursos Genéticos e Biotecnologia, Brasília".

The modified protocol is as follows:

(i) An ELISA microtitre plate (MAKE) was used instead of tubes. To each well 10 µl of antiserum diluted in 0.85% (w/v) NaCl, pH 7.0 was added. The dilutions used were 1:10, 1:20, 1:40. Antiserum to 48 different serotypes was kindly provided by Institute Pasteur, Paris, France.

(ii) A suspension (90µl) of formaldehyde-treated *B. sphaericus* was added to each of the wells and incubated for 30 min. at 37°C.

(iii) Controls consisted of three strains of *B. sphaericus* which are positive for H5, H6 and H25. Another control consisting just of buffer was also included.

(iv) The wells were examined using a stereomicroscope to detect the presence of absence of agglutination.

This protocol was found to be convenient and easy to perform compared with the conventional methodology. Furthermore it facilitated examination of many isolates in a short period of time. The results of this study showed that all 23 Brazilian isolates of *B.sphaericus* were of the serotype H5 and this fact suggests that among Brazilian *B.sphaericus* isolates there is no serological difference they are highly pathogenic to *Culex quinquefasciatus*.

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RESUMO

Caracterização sorológica de 23 isolados de *Bacillus sphaericus* patogênicos para larvas de mosquitos, obtidos de solos brasileiros

Através da sorologia flagelar foram caracterizados 23 isolados de *Bacillus sphaericus* obtidos em amostras de solo provenientes de diferentes regiões do Brasil. Todos são patogénicos para *Culex quinquefasciatus* e todos pertencem ao sorotipo H5. Esta caracterização sorológica mostrou que entre os isolados de *Bacillus sphaericus* brasileiros não existem diferenças regionais. Adaptação da metologia se mostrou eficiente, diminuindo o tempo de reação e a quantidade de material utilizado.

Palavras-chave: *Bacillus sphaericus*, sorologia, caracterização.

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VIRULENCE FACTORS PRODUCED BY AEROMONAS HYDROPHILA STRAINS ISOLATED FROM DIFFERENT SOURCES

Angela Correa Freitas[1]
Arlete Moreira Milhomem[1]
Marly Paiva Nunes[1]
Ilvan Delgado Ricciardi[1]*

SUMMARY

Virulence factors produced by *Aeromonas hydrophila* derived from water, pasteurized milk, urine and a standard strain were compared. All four strains were hemolytic, and had protease and staphylolytic activity. Production of enterotoxin like activity in suckling mice and cytotoxin (Vero cell) were observed in *A. hydrophila* isolated from food, water and standard strains. Autoagglutination in broth culture was found only for two strains (standard and food strain).

The biological properties of lipopolisaccharide from *A. hydrophila* were analysed by the mouse LD 50 determination and dermonecrotic factor. Tests of purified LPS demonstrated toxicity assayed by LD50 in mice. The characteristic lesions of local Schwartzman reaction in albino rabbits with all strains tested demonstrated the endotoxic potential of the LPS assayed.

Mice challenged by the intraperitoneal route showed mortality rates of 50, 55,75 and 92%, respectively, for *A. hydrophila* isolated from food, water, standard and urine strains.

Production of virulence factors by *A. hydrophila* strains isolated from food and water had a significative correlation with virulence markers found in the strains isolated from urine and standard.

This finding indicates that strains of *A. hydrophila* isolated from food or environment sources may play a significant role as potential pathogens associated with human infections.

Key Words: *Aeromonas hydrophila*, virulence factors, LPS (biological properties).

INTRODUCTION

Aeromonas hydrophila is an important etiological agent of several diseases in human and animals. This organism can be associated with gastrointestinal symptoms (1,14) and various extra intestinal disorders such as wound infections, meningitis, endocarditis, conjunctivitis and urinary tract infections (2).

Evidences indicate that the pathogenicity of *A. hydrophila* may involve the action of several extracellular toxins and enzymes, including cytotoxin, enterotoxin, hemolysins, and proteases (6). Further evidences support the presence of outer

1. Departamento de Microbiologia Médica - Instituto de Microbiologia - Universidade Federal do Rio de Janeiro - CCS - Bloco I - Ilha do Fundão, CEP: 21.944 - RJ - Brasil.
* Corresponding author - Fax nº: (021) 270-8793.

membrane protein layers. O antigens fimbriae and adherence mechanism (18) which could evoke the capacity to colonize animal tissues. However, the role of these factors in the exact mechanism of virulence has not yet been established.

A.hydrophila occurs widely in natural aquatic habitats and may be also found in food of animal and vegetable origin (7, 19, 23). Nevertheless, the association between food contaminated with *Aeromonas* species and human illness is not established definitively.

The purpose of this study was to investigate the presence of vilurence factors in *A.hydrophila* strains isolated from water and food, and to compare these properties with those presented by a clinical isolate (urinary infection) and a standard strain (Culture Institute Pasteur, *A.hydrophila* CIP 7614).

MATERIALS AND METHODS

Bacterial Strains

Four strains of *A.hydrophila* were used in the present study; one strain isolated from urinary infection, one isolated from estuary water and another one from pasteurized milk. A standard strain obtained from Institute Pasteur, Lille, France (*A.hydrophila* CIP 7614) was also used. The stock cultures were lyophilized and stored at room temperature.

Biochemical characterization

The three test strains of *A.hydrophila* were isolated directly from a selective medium for *Pseudomonas - Aeromonas* GSP agar (Merck) contraining ampicillin (10mg/l) or deoxycholate citrate agar (BBL). Plates were grown at 28°C for 48h. Mucoid yellow colonies from GSP agar or mucoid pink (lactose positive) and colorless colonies (lactose negative) from deoxycholate citrate agar were submitted to oxidase, motility, sensivity to the vibriostatic agent (2,4-diamino-6,7-diisopropylpteridine) in 150 µg disks and fermentation of glucose tests. Gram negative rods, motile, oxidase positive, glucose fermentative, and resistant to the vibriostatic agent were considered as *Aeromonas* spp. Isolated strains were identified to the species level by biochemical tests proposed by Popoff and Véron (26) and confirmed by using the API20E system (Analytab Products, Palinview, N.Y.).

Hemolysin assay

A hemolysin assay was performed by the method described by Hugh (15). The strains were grown in 0.5 ml of a medium containing 1% of casitone (DIFCO) in 0.85% sodium chloride at 28°C for 24 h. An equal volume of sheep erythrocytes, suspended in buffered saline to a final concentration of 5% (v/v) was then added to each culture. The test tubes were read for the presence of beta-hemolysis after incubation at 37°C for 24h, and at 4°C for 18-24h. All strains were also tested for beta-hemolysis production on sheep blood agar plats incubated at 37°C for 48h.

Cytotoxin assay

Production of cytotoxin on Vero cell monolayers was measured as described by Donta and Shaffer (11). Briefly samples (50 ul) of serial two fold dilutions of culture supernatants in buffered saline (pH 7.4) were added to Vero cells grown in well tissue culture plates and incubated at 37°C in a 5% CO2 atmosphere for 24-48h. Cytotoxin titer was expressed as the highest dilution causing complete rouding of the cells, in 50 ul of the sample.

Autoagglutination test

The method described by Janda et al. (17) was performed for the evaluation of selfpelleting (SP) and precipitation after boiling (PAB) ability of the strains. Individual strains were grown in 6ml of brain heart infusion broth for 18h at 37°C. After incubation cultures were observed for evidence of self pelleting which was manifested by the appearance of a large aggregate of cells at the botton of the tube. The cultures were vortexed for 30s and a 3 ml fraction was heated for 1h at 100°C in a water bath and then cooled for 10min and compared with the unheated control. Strains exhibing a reduction in turbidity were considered positive for PAB.

Protease and staphylolytic activity

For protease production, cultures were grown at 37°C for 24-48h on brain heart infusion supplemented with 3% skim milk and 2% agar (16). The activity was evidenced by a clear halo around the colonies. The staphylolysin activity was determined according to the method described by Satta et al. (28). An overnight culture of *Staphylococcus aureus* (ATCC 25923) was used in this assay.

Enterotoxin assay

The suckling mouse assay was used to detect *Aeromonas* enterotoxin activity by the method established by Dean et al. (10).

Pathogenicity for mice

Four groups of six albino male mice each (4 to 6 week-old) were injected intraperitoneally with approximately 10^8 CFU/ml of the test samples, according to the following conditions. (A) Strains were grown in 3 ml of brain heart infusion for 37°C overnight, cells were pelleted (4000 xg) at 4°C for 10min, and suspended in 3ml of sterile saline (0.85%). (B) Supernatants obtained as described for the condition i. (C) Cultures uncentrifuged, and (D) diluted 1:100 in 3 ml of sterile saline. Control mice (E) were inoculated with brain heart infusion and sterile saline.

Extraction of LPS

Freeze-dried cells were extracted by the aqueous phenol method of Westphal and Jann (29) modified by Dooley et al. (12).

Biological properties of LPS

(i) Mouse LD50 determination

Appropriate dilutions of the LPS samples (1000 µg to 62.5 µg/0,1ml) were prepared in sterile saline for injection into groups of six 4 to 6 week-old albino male mice, and 100 µl of each dilution was injected intraperitoneally. Control mice were inoculated with sterile saline. Fifty percent lethal doses were calculated by the method of Reed and Muench (27).

(ii) Dermonecrotic factor

White male rabbits weighing 2 kg were used. LPS serial 2-fold dilutions of 500 µg to 62.5 µg/0,1ml) and negative control (sterile saline) were injected intradermal in several places of the back of the aninals, previously depilated. LPS doses of *Escherichia coli* OIIIB4 were also tested in the same animals, as well as in the same conditions. After 18h the rabbits were injected intravenously with a provocative dose of *E.coli* OIIIB4 LPS (10 µg/0.1ml). The presence of an hemorrhagic and necrotic inflammatory lesion at each site of the initial intradermal infection, was observed. Each preparation was tested on two separate rabbits.

RESULTS AND DISCUSSION

This study compares some virulence factors yielded by *Aeromonas hydrophila* strains isolated from the environment (estuary water), food (pasteurized milk), clinical origin (urine) and a standard strain (*A.hydrophila* CIP 7614).

The biochemical characteristics of the three strains of different sources were similar to those of the standard strain. Our findings are in agreement with Palumbo et al. (24) and Paniagua et al. (25) which reported similarity in the biochemical traits of the strains isolated from food and clinical origin and environmental strain. Table 1 shows that all strains were hemolytic (complete hemolysis), protease and staphylolytic activity positive. Enterotoxin and cytotoxin were produced by 3 *A.hydrophila* strains (from food, water and standard strain) but not by the isolated from urine. Positive relationships between *Aeromonas* strains isolated from vegetables (7), drinking water and clinical origin (22) and foods of animzal origin (23) were found particularly for cytotoxin and hemolysin production.

Burke et al. (5) and Majeed et al. (21) reported that the majority of clinical, environmental and food isolates of *A.hydrophila* were enterotoxin and hemolysin producers. Despite of these studies, Figura et al. (14) found a dissociation between production of hemolysin, cytotoxin and enterotoxin among the *Aeromonas* strains investigated. This report may support our results since only one strain (*A.hydrophila* isolate from urine) was cytotoxin and enteroxin negative but hemolysin positive. On the other hand Chakraborty et al. (8) demonstrated by genetic identification that enterotoxic, cytotoxic and hemolytic activities of *A.hydrophila* result from the expression of distinct genes which would explain such different virulence factors profiles among the strains. Our finding also agree with Kindschuh et al. (20) that found discrepancies among the virulence factors produced by *Aeromonas* strains from various geographic locations, suggesting the existence in different parts of the world of aeromonads with different virulence properties.

Staphylococus aureus cell lysin (staphylolysin), protease, pyrazinamidase and some others enzimes may contribute to the pathogenicity producing tissue damage or enhancing invasiveness

(18). Positive correlation between staphylolysin and protease production were observed in our study and by Palumbo et al. (24). These data support the concept that additional properties could define the pathogenicity patterns in *A.hydrophila* strains (18).

Other characteristics such as, autoagglutination in brain heart infusion broth (17) presence of a surface-layer protein (13), and resistence to bacteriophage Ae h1 (9), may also be markers of *A.hydrophila* virulence. It was difficult to correlate the autoagglutination test with other virulence factors assayed, since only the standard and the food strains were positive (Table 1). In addition, Paniagua et al. (25) described that negative SP and positive PAB (SP-/PAB+) phenotypes were more frequent among the avirulent *A.hydrophila* and *A.caviae* isolates than in the most virulent group.

TABLE 1 - Production of virulences factors by *A. hydrophila* strains isolated from differents sources.

Virulence factors	*A. hydrophila* strains (origin)			
	CIP 7614	Urine	Food	Water
Hemolysin	+	+	+	+
Protease	+	+	+	+
Staphylolysin	+	+	+	+
Enterotoxin[a]	+	-	+	+
Cytotoxin[b]	+	-	+	+
Autoagglutination (SP - / PAB+)[c]	-/+	-/-	-/+	-/-

[a] A ratio ≥ 0,08 was considered a positive result.
[b] Assay in Vero cells.
[c] Results negative for selfpelleting (SP-) and positive for precipitation after boiling (PAB+).

The relative virulence of *A.hydrophila* strains studied was also evaluated by biological properties of lipopolysaccharide (LPS) that are important components of the outer membrane of gram-negative species. Purified LPS from *A.hydrophila* strains were biologically analysed by the mouse LD50 determination and dermonecrotic factor. The LD50 were characterized by intraperitoneal inoculation of serial 2-fold dilution (1000 µg to 62.5 µg/0.1ml) of each bacterium in this assay. The standard *A.hydrophila* LD50 was 1000 µg, in contrast to the strain isolated from urine (250 µg). For the other two strains isolated from food and water the results indicated similarity to the LD50 (645 µg and 550 µg, respectivelly). This biologycal properties of purified LPS demonstrated that, *A.hydrophila* isolated from urinary infection had a high toxicity assayed by LD50 in mice. The 250 µg dose found is similar to that found for other pathogens, as for example, 187 µg for *E.coli* OIII B4 and 250 µg for *Moraxella bovis* (3), 274 µg for *Campylobacter jejuni* and 225 µg for *Yersinia enterocolitica* 0:3 (4). The high LD50 of the standard strain may be a result of long term storage in the laboratory, with several "in vitro" passages. In this situation the structural arrangement of LPS could be altered by loss of some fractions, thus interfering in the ability to express virulence properties.

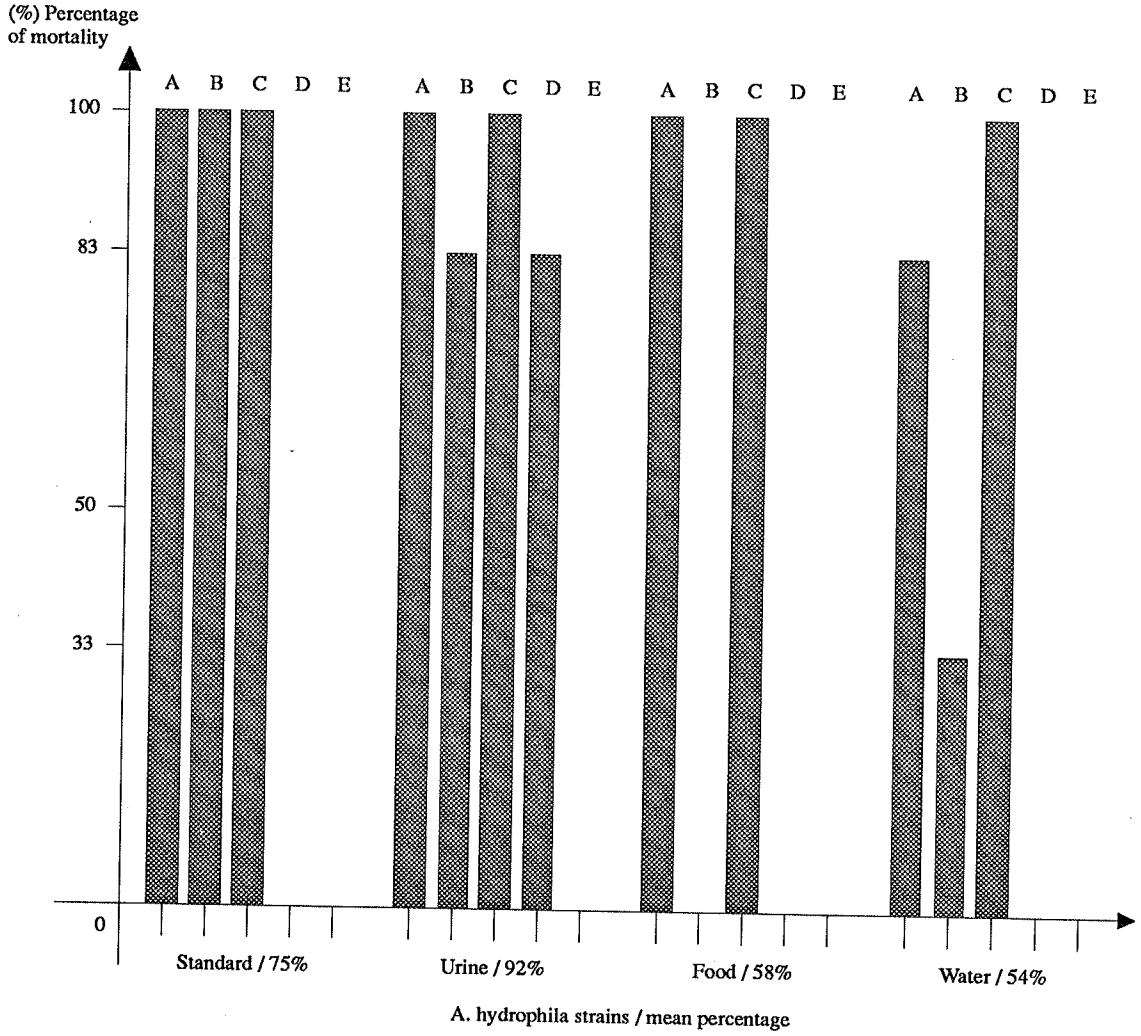
All the animals assayed for dermonecrotic factor (Shwartzman phenomenon) induced by the purified endotoxins of the four strains tested showed, in each infection site, an hemorrhagic-necrotic zone, 6-18h after the intravenous inoculation of the provocative dose. The minimal LPS dose necessary to the induction of the *A.hydrophila* (CIP 7614) reactions, was 2 µg and 8 µg for *E.coli* OIII B4.

These characteristic results of the Shwartzman reaction and the minimal dose of 2 µg demonstrated the endotoxic potential of the LPS assayed, in comparison with the doses of 8 µg, 5µg and 50 µg from LPS of *E.coli* OIII B4, *Y.enterocolitica* O3 and *C.jejuni*, respectively (3, 4).

Pathogenicity was also assayed by intraperitoneal inoculation, of 10^8 CFU/ml, in adult mice (Fig.1). All results were expressed by mean percentage. *A.hydrophila* standard strain produced 75% of mortality, remaining alive only the mice tested with uncentrifuged culture diluted 1:100. *A hydrophila* from urinary source killed 92% of the mice and the strains isolated from food and water produced respectively 50 and 54% of mortality. Control mice, were not affected.

Supernatant of the standard *A.hydrophila* produced 100% of mortality and cultures of *A.hydrophila* from urine source killed 83% of the mice. Supernatant of *A.hydrophila* culture isolated from food caused an intestinal disorder with solidity alteration in the feces. These results suggest the presence of exotoxins in this supernatant, confirmed by the positive result of enterotoxin test, in the Dean et al. (10) tecnique.

For the *A.hydrophila* isolated from water we observed that it also killed 92% of the mice when pelleted cell or uncentrifuged culture were injected. The supernatant promoted 33% of death and surviving animals had an intestinal disorder, similar to the observed with *A.hydrophila* isolated from food.



A - Pelleted cells suspended in sterile saline (0,85% NaCl)
B - Supernatant obtained for the condition A
C - Culture uncentrifuged in brain heart infusion
D - Culture uncentrifuged diluted 1:100 in sterile saline
E - Control mice in sterile saline and brain heart infusion

FIGURA 1 - Experimental pathogenicity in mice challenged intraperitoneally with Aeromonas hydrophila strains from different sources.

Results obtained show that the production of virulence factors by *A.hydrophila* strains isolated from food and water environment had a significative correlation with virulence markers found in the standard strain and the *A.hydrophila* isolated from clinical material. This finding indicates that strains of *A.hydrophila* isolated from food or environment sources may play a significant role as potential pathogens, associated with human infections.

RESUMO

Fatores de virulência produzidos por amostras de *Aeromonas hydrophila* isoladas de diferentes origens

Foram comparados alguns fatores de virulência produzidos por amostras de *Aeromonas hydrophila* isoladas do meio ambiente aquático, de leite pasteurizado, de material clínico (urina) e

de uma amostra padrão. Todas as amostras foram positivas para a produção de hemolisinas, protease e enzima estafilolítica. A detecção de uma enterotoxina e de uma citotoxina, somente não foi observada na amostra isolada de urina. A positividade no teste de autoaglutinação foi verificada entre as amostras padrão e de alimento. A avaliação de algumas propriedades biológicas do lipopolissacarídeo extraído das amostras estudadas foi realizada através de determinação da DL50 em camundongos e pelo fator dermonecrótico. O estudo "in vivo" dos LPS purificados demonstraram toxicidade evidenciada pela DL50 em camundongo. Lesões dermonecróticas localizadas foram detectadas pela reação de Schwartzman, demonstrando o potencial endotóxico do LPS analisado. Camundongos inoculados intraperitonealmente com as amostras estudadas produziram percentagens médias de mortalidade de 50, 55, 75 e 92%, respectivamente para amostras isoladas de alimento, água, amostra-padrão e de urina. Uma significativa correlação foi encontrada entre os fatores de virulência produzidos pelas amostras estudadas. Esses resultados sugerem que amostras de *A.hydrophila* isoladas de alimentos e de meio ambiente podem desempenhar um importante papel como patógenos, associados com infecções humanas.

Palavras-chave: *Aeromonas hydrophila,* fatores de virulência, LPS (propriedades biológicas).

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PECTIN LYASE ACTIVITY OF PENICILLIUM GRISEOROSEUM RELATED TO DEGUMMING OF RAMIE

Maria Helena Nasser Brumano
Jorge Luiz Cavalcante Coelho
Elza Fernandes de Araújo
Daison Olzany Silva

SUMMARY

Penicillium griseoroseum Dierckx produced pectin lyase, when grown in liquid culture containing citrus pectin as the sole carbon source.

Temperature, pH, buffers and calcium ion for the action of pectin lyase (PL) from *P. griseoroseum* were studied. PL cleaved citrus pectin most efficiently at pH 6.0 and 50°C. Sorensen's phosphate buffer allowed the greatest enzymatic activity, and calcium ions did not stimulate enzyme activity. PL exhibited activity on ramie fibres and citrus pectin but did not cleave polygalacturonic acid.

Key Words: Beta - elimination; *Penicillium griseoroseum*; Pectin lyase; Ramie.

INTRODUCTION

Pectic enzymes have been used for retting processes based on the ability of certain pectolytic microorganisms to liberate cellulose fibres from the stems of fibrous plants (10). Pectic enzymes are classified in pectinesterase and depolymerases (polygalacturonase, pectate and pectin lyase) (10). Pectinesterase (PE) catalyses the hydrolysis of methoxyl groups on pectin, forming free carboxyl groups and methanol. Polygalacturonase (PG) attacks preferentially pectic acid by hydrolysis. Pectin lyase (PL) catalyzes the cleavage of α-D-(1,4)-glycosidic bonds of pectin by the mechanism of beta-elimination and pectate lyase (PAL) can break the same bond by the same mechanism on pectic acid (1,8). PL is able to decompose highly esterified pectins without the action of pectinesterase, which is an advantage in the food industry (1,15).

The use of crude fungal pectinases for degumming of ramie fibres has been investigated in our laboratory (4,5) and the present study was undertaken to investigate some conditions for enhancing PL activity produced by *Penicillium griseoroseum* in liquid medium.

MATERIAL AND METHODS

The *Penicillium griseoroseum* strain (Dierckx) used in this work was isolated from seeds of forest plants by Dr. James J. Muchovej, Departamento de Fitopatologia, Universidade Federal de Viçosa. The *P. griseoroseum* was grown on solid medium (oat meal agar) for nine days at 28°C and them used to produce a spore suspension.

The fungus was grown for enzyme production, for 120 h at 28°C in a rotary shaker (150 rpm) in 125 ml Erlenmeyer flasks containing 50

1. Departamento de Microbiologia, Universidade Federal de Viçosa. Viçosa-MG - 36570-000 - Brasil. *corresponding author*: Daison Olzany Silva.

ml of modified mineral medium (6), pH 6.0, of the following composition ($g.l^{-1}$): KH_2PO_4, 2: K_2HPO_4, 0.62; $MgSO_4,7H_2O$,1.1; $(NH_4)_2SO_4$, 1; 0.1% citrus pectin (ESKISA) as carbon source and 0.06% yeast extract. Each flask was inoculated with $5X10^4$ spores per ml of medium. All experiments were carried out three times with duplicate samples.

The culture liquid was separated from the mycelium by filtration through a 400 mesh (37 μm) sieve, and the filtrate assayed for PL activity by measuring the increase in absorbance at 235 nm of the substrate solution [SIGMA citrus pectin (2.5 $g.l^{-1}$) in 0.05 M Sorensen's phosphate buffer, pH 6.0, at 40°C. The enzyme activity was expressed as mU (nmoles of unsaturated uronide produced per ml per minute), based on the molar extinction coefficient (5500) of the unsaturated products (2).

Cellulase activity was estimated as described and modified by Halliwell (7).

Ramie fibres (1g) were used as substrate for *P. griseoroseum* PL (15 ml of culture filtrates), and for Flaxzyme (Novo Industri, Denmark) used as a control (0.1 ml of enzyme plus 14.9 ml of Sorensen's phosphate buffer, pH 6.0, with three drops of formaldehyde to avoid contamination). The experiments were run at 40°C on 50 ml beackers without shaking.

RESULTS AND DISCUSSION

Substrate specificity: Citrus pectin (SIGMA) was the best substrate for enzymatic beta-elimination of the various pectic materials tested (Table 1). No PL activity from *P. griseoroseum* filtrate was detected when polygalacturonic acid was used as a substrate, showing that this enzyme is specific for pectin. Pectate lyase (PAL) and PL can be distinguished by their preference for highly esterified or less esterified pectin (11).

Effect of temperature and pH: The effect of temperature on the enzymatic reaction was studied within the 25-65°C range. Measurements were made after 60 min of incubation. The substrate concentration was 0.25% (w/v) and the pH 6.0. The optimal temperature was 50°C (Fig. 1). The optimal pH was investigated in the 3.0-8.0 range. The substrate was prepared in sodium acetate-Tris-K_2HPO_4 buffer (3). Optimun pH for PL activity was pH 6.0 (Fig. 2).

TABLE 1 - *P. griseoroseum* PL activity on different substrates.

Substrates [%(w/v)]	Activity (mU)
Citrus pectin (SIGMA)	1.56
Apple pectin (SIGMA)	0.69
Citrus pectrin (ESKISA)	0.67
Polygalacturonic acid (SIGMA)	0.05

All substrates were prepared in 0.05 M Sorensen's phosphate buffer, pH 6.0.

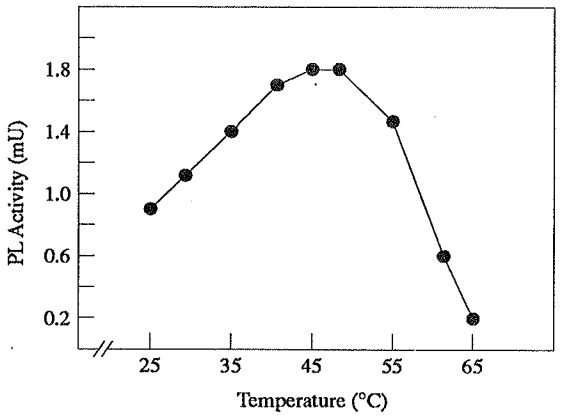

FIGURE 1 - Effect of temperature on pectin lyase activity of *P. griseorosum* culture filtrates. Enzymatic assay conditions as described in the analytical methods.

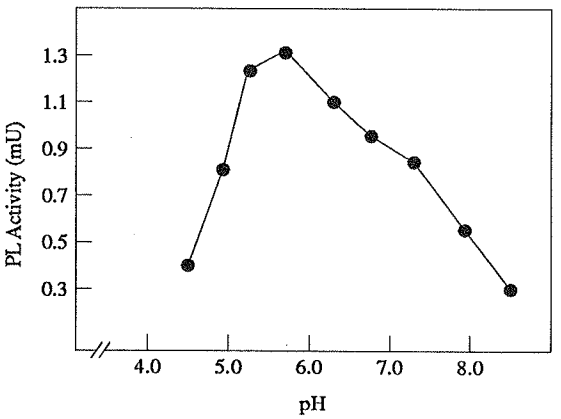

FIGURE 2 - Effect of pH on pectin lyase activity from *P. griseoroseum*.

Enzyme activity depends to a large extent on temperature and pH. These factors affect the rate of the enzymatic reaction, substrate affinity, and enzyme stability. Optimun temperature for PL activity from *P. griseoroseum* was in accordance with PL from *Aspergillus oryzae* A3 which exhibited greatest activity at 50-55°C (8). Similarly, the

optimum pH for pectin clevage by PL from *P. expansum* (13), *A. niger* (14) and *Aureobasidium pullulans* LV 10 (9) was around 6.0.

Optimal activity for pectinolytic enzymes was near neutral pH and elevated temperatures. These conditions are ideal for use in the processing of plant material because these high temperatures are sufficient to limit the growth of mesophilic contaminants and can act as a pasteurization process. Furthermore, the enzymes can be applied directly to the plant tissue without the need for a pH modification (12).

Effect of different buffers on PL activity: Citrate/phosphate (McIlvaine), Na_2HPO_4-KH_2PO_4 (Sorensen's phosphate), succinate, citrate, and sodium acetate-Tris-K_2HPO_4 buffers (50 mM respectively) were investigated. All buffers were adjusted to pH 6.0. The substrate was 0.25% citrus pectin (SIGMA). Only Sorensen's phosphate buffer, showed significantly higher enzymatic activity as compared with citrate buffer (Table 2). This is possibly due to the a chelating effect of citrate on some essential ion for enzyme action.

Influence of calcium ions on PL activity: Calcium ions (0 to 10 mM) were tested for the ability to stimulate enzyme activity. In contrast to other various fungal enzymes, PL from *P. griseoroseum* had no requirement for calcium ions (Table 3). This is an advantage, since pectin coagulates in the presence of high concentrations of calcium ions.

TABLE 2 - Effect of various buffers on PL activity from *P. griseoroseum* filtrate.

Buffer System	Activity (mU)*
Sorensen's phosphate	1.67 A
Succinate	1.54 AB
McIlvaine	1.47 AB
Na acetate-Tris-K_2HPO_4	1.17 AB
Citrate	1.09 B

* Values followed by the same letter were not significantly different at the 5% level by the Tukey test.

TABLE 3 - Effect of Calcium ions on PL activity from P. griseoroseum.

$CaCl_2$ Concentration (mM)	Activity (mU)*
0.0	2.25
1.0	2.24
7.0	2.23
10.0	2.24

* Activity measured in a reaction mixture containing 0.25% citrus pectin in 50 mM Sorensen's phosphate buffer (pH 6.0).

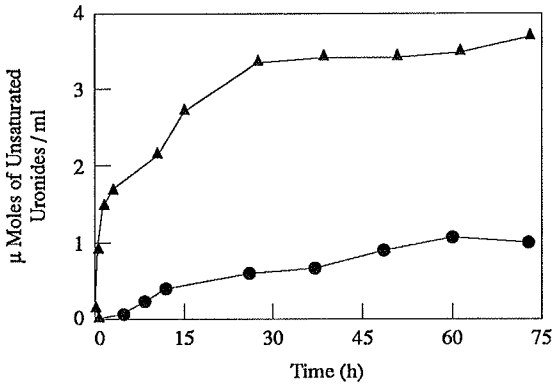

FIGURE 3 - Action of *P. griseoroseum* (— ● —) and Flaxzyme (— ▲ —) pectin lyases on ramie fibers.

Degumming of ramie fibers: Ramie fibers were significantly softer after 72 h of treatment with Flaxzyme than after 72 h of treatment with *P. griseoroseum* PL. However, the Flaxzyme-treated fibers were weak in tension, probably due to the presence of cellulases. The action of *P. griseoroseum* and Flaxzyme PLs on ramie fibers is illustrated in Figure 3. The Flaxzyme and *P. griseoroseum* PL activities used on this experiment were respectively 18 and 2 mU. The cellulase activity was none on *P. griseoroseum* culture filtrate and 2.6 nmoles of reducing sugar ml^{-1}. min^{-1} on Flaxzyme suspension used. It must be emphasized that the *P. griseoroseum* strain used in this report is wild type, therefore, studies of strain improvement by mutagenesis are in progress in order to increase PL activity.

RESUMO

Atividade de pectina liase de *Penicillium griseoroseum* relacionada à degomagem de rami

Foram estudados os efeitos de temperatura, pH, diferentes tampões e íon cálcio, sobre a atividade da enzima pectina liase extracelular (PL), produzida pelo fungo *Penicillium griseoroseum*. PL degrada pectina cítrica mais eficientemente à pH 6,0 e 50°C. O tampão de Sorensen proporcionou maior atividade enzimática, enquanto o íon cálcio não afetou a mesma. Foi observada atividade de PL sobre fibras de rami (Boehmeria nivea, Gand) e pectina cítrica, enquanto ácido poligalacturônico não é substrato para a enzima.

Palavras-chave: Beta - eliminação; *Penicillium griseoroseum*; Pectina liase; Rami.

ACKNOWLEDGEMENTS

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THE EFFECT OF ALGINIC MATRIX ON CADMIUM UPTAKE BY AN IMMOBILIZED GREEN MICROALGAE

Selma G. F. Leite[1],
Gustavo A. S. Pinto[1],
Antonio Carlos Augusto da Costa[2]

ABSTRACT

This work describes some studies on the absorption of cadmium by free and alginate immobilized *Chlorella homosphaera* cells, as well as the absorption detected by cell-free polymeric matrix, in cadmium-containing solutions in the range of concentrations from 8.7 to 45.0 mg/l. The uptake showed to be most effective with the use of free cells up to 26.8 mg/l cadmium solution. The results obtained for alginate and alginate immobilized cells are probably associated to the porosity of the matrix and cell density inside the alginate particles.

Key Words: cadmium, uptake, alginate, *Chlorella.*

INTRODUCTION

Some heavy metals, like mercury, lead cadmium, and zinc are widely launched in aquatic environments, poisoning a broad range of living organisms. These metals are found in many regions of Rio de Janeiro City (8), and studies to recover these metals, based on adsorption processes and conventional technologies are being tried. Metal ions can be strongly absorbed in the surface of algal cells (4), filamentous fungi (9), and bacteria (10), through interactions between functional chemical groups present in the cell wall.

There is a growing interest in the use of immobilized algae for the uptake of heavy metal ions like mercury (14), or the recovery of precious metals (3). Some investigations with bacterial cells immobilized in gelatine (13) or polyacrilamide (10) are also being considered. Cadmium, as a poisoning metal, exhibits a strong deleterious effect on some enzymatic systems (12), damaging the environment nearby industries, often launching these metals (6,12).

This work describes some studies on cadmium absorption by free and alginate entrapped *Chlorella homosphaera* cells, as well as by the absorption promoted by the absorption promoted by the alginic matrix.

MATERIALS AND METHODS

1) Microorganism and cultivation - The microbial species tested for cadmium uptake was the green algae *Chlorella homosphaera*, isolated from a lake at Quinta da Boa Vista (Rio de Janeiro City). The microorganism was maintained in tubes containing 5.0 ml of a salts solution, with the following composition (g/l): $NaNO_3$, 0.10; KH_2PO_4, 0.25; $MgSO_4.7H_2O$, 0.51; $CaCl_2.6H_2O$, 0.05; $FeCl_3$, 0.08; NH_4Cl, 0.05; and, agar-agar, 20.0. The salts were dissolved in distilled water, auto-

1. Universidade Federal do Rio de Janeiro, Escola de Química, Departamento de Engenharia Bioquímica.
2. Centro de Tecnologia Mineral, Serviço de Meio Ambiente e Biometalurgia, CETEM/CNPq. Rua 4, Quadra D, Cidade Universitária, Rio de Janeiro, 21949-900, Brasil.

claved, and the pH adjusted to 7.0. The biomass used in the experiments was obtained after inoculation in 200 ml of the described medium (agar-agar free), using 500 ml Erlenmeyer flasks containing glucose at 10.0 g/l. The final inoculum concentration in the medium, after growth, was around 4.0 g/l cells. The flasks were incubated for 72 h at 13000 lux, in a rotary shaker at 150 rpm and room temperature. Cell concentration was measured by a dry weight *versus* absorbance at 430nm curve.

2) Experimental procedure - The tests were conducted in duplicate, in 500 ml flasks, containing 200 ml of cadmium solution, added as chloride salt, in the range of concentrations from 8.7 to 45.0 mg/l. In the experiments, 0.09 g of cells was added to the cadmium solution, under 13000 lux, at 25°C and 150 rpm, during 5 min and 6 h. The experiments were first conducted with free cells and then with alginate immobilized cells. Blank experiments were done with alginate particles.

3) Cells immobilization - The immobilization was conducted by mixing sodium alginate (1.5%) and biomass (4.5%), stirring for 30 min followed by dropping on 0.1 M calcium chloride solution. The dropping of the polymeric matrix was done by using small capillary tubes located around 10 cm above the stirring vessel containing calcium chloride solution. This way, 3 mm diameter particles were obtained. The alginate particles were left in calcium chloride solution for 24 h and extensively washed with distilled water. In the 200 ml of cadmium solution 50 particles were added, the final polymer mass being 0.03 g and 0.09 g of biomass.

4) Quantification - The biomass concentration was monitored through an absorbance at 430 nm *versus* dry weight at 80°C curve. Cadmium concentrations were measured by an atomic absorption spectrophotometer Varian, Model AA6. In order to evaluate the accumulation capacity of the materials, the alginate particles, alginate immobilized and free cells, were dried to constant weight to express the mg cadmium/g suspended solids ratio.

RESULTS AND DISCUSSION

The ability of *Chlorella homosphaera* to accumulate cadmium was confirmed, as already stated(1). Table 1 shows the results obtained with free cells, alginate immobilized cells, and polymeric matrix, for the recovery of cadmium. It can be noted that in the uptake of the metal, free cells were more efficient than immobilized cells and the matrix, up to 26.8 mg/l, irrespective the time of contact. From a 45.0 mg/l cadmium solution, the uptake capacity was reduced from 48.0 mg/g dry cells to 13.0 mg/g, after 6 h. This observation indicated that there is a limit for the initial cadmium concentration to be efficiently treated by these free cells. Another observation is the fact that, in all cases, the absorption was enhanced by greater periods of contact between the solution and the biomass (or biomass derived matrix).

From other microbial strains similar behaviour was stated (11). Probably, the process presents two different stages: an initial and rapid uptake followed by the migration of the metals into the inner parts of the cells, through other microbial mechanisms (5). Simultaneously the sites become available for a following uptake. But, if the metal solution is strongly concentrated, the cell surface becomes readly occupied, preventing

TABLE 1 - Cadmium uptake free *C. homosphaera* cells (A), alginate matrix (B), and alginate immobilized *C. homosphaera* cells (C).

Time (min)	Initial Cadmium Concentration (mg/l)			Residual Cadmium Concentration (mg/l)			mg Cadmium/g Suspended Solids			Total Cadmium Recovered (mg)			Cadmium Recovered (%)		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
5	8.70	8.70	8.70	3.40	7.20	5.90	11.00	10.10	4.70	0.99	0.30	0.54	60.90	17.00	32.00
360	8.70	8.70	8.70	0.85	360	3.80	16.00	34.30	8.00	1.44	1.03	0.98	90.20	41.00	56.00
5	17.60	17.60	17.60	12.60	14.70	13.70	12.00	19.30	6.40	1.08	0.58	0.77	28.40	16.50	21.80
360	17.60	17.60	17.60	5.10	8.90	8.80	25.00	58.10	14.70	2.25	0.74	1.76	71.00	49.00	50.00
5	26.80	26.80	26.80	18.70	21.55	20.50	18.00	35.30	10.50	1.62	1.06	1.26	30.20	19.60	23.10
360	26.80	26.80	26.80	5.20	20.50	16.00	48.00	42.00	24.00	4.32	1.26	2.88	80.40	23.50	40.00
5	45.00	45.00	45.00	40.70	38.10	37.30	10.00	46.20	12.80	0.91	1.39	1.54	9.60	15.30	17.10
360	45.00	45.00	45.00	39.60	35.80	31.90	13.00	61.30	21.80	1.17	1.84	2.62	12.00	20.40	29.10

a rapid migration of the metal into the interior of the cells, due to ionic blockage. Consequently, the surface sites do not become available in a further step.

In these experiments, in the presence of cell-free alginate particles, it could be observed that the polymer is able to recover a great amount of ions, a fact that was not detected by other researcher in the recovery of mercury from a 500.0 mg/l solution(14). However, in this case, they used mercury as the contaminating metal, and it has a particular behaviour in this kind of process. It is volatilized by the biomass after accumulation, and due to this active process, the alginate matrix does not contribute in the uptake mechanism. The structure of alginic acid is constituted by a chain of D-mannuronic acid residues with free carbon atoms from carboxyl groups and probably responsible for cadmium uptake.

If cadmium uptake is compared in the sets of experiments tried, it can be concluded that free cells are the best materials to be used in this purpose. From Table 1 it can be extracted that the highest mg cadmium/suspended solids ratio was achieved by the cell-free polymer. If it is considered that the sodium alginate mass used (0.03 g) is less than the cell mas used (0.09 g), probably the metal has been captured on the surface and inner parts of the porous matrix. If the microorganisms are free, the metal uptake is on the surface of the cells and when immobilized in alginate they occupy the porous matrix, being their own sites responsible for the removal of the metal.

The use of particles containing high density of cells entrapped in alginate, in fixed polymeric particles, has already shown its efficiency, with metals removal ranging from 90.0 to 100.0%, from dilute and concentrated solutions (2). In this work a range of cadmium concentrations from 20.0 to 41.0 mg/l was tested, in column experiments, differently from the present system, where the operational parameter and cell density were strongly different. Beyond this fact, further natural elution of the metals was not observed; this same behaviour was shown in the work done by Watson (13), with gelatine immobilized *Micrococcus luteus*, for strontium recovery. In a following step the metals should be eluted from the polymeric particles through a wide variety of eluting solutions and reused in a new recovey system (7). In this case, alginate showed to be a good matrix in this purpose, but a neutral matrix, more economically attractive should be choosen, to evaluate the real potential of the microbial strain in the uptake of toxic polluting agents.

RESUMO

Efeito da matriz de alginato na captação de cádmio por cultura de microalgas imobilizadas

Este trabalho descreve alguns estudos de absorção de cádmio por células de *Chlorella homosphaera* livres e imobilizadas em alginato, bem como a absorção devida à matriz polimérica isenta de células em soluções de cádmio na faixa de concentrações de 8,7 a 45,0 mg/l. A captação do metal foi mais efetiva com o uso de células livres até a concentração de 26,8 mg/l do metal. Os resultados obtidos com emprego do alginato e células aprisionadas em alginato estão provavelmente associados à porosidade da matriz e à densidade celular dentro das partículas de alginato.

Palavras-chaves: absorção de cádmio, alginato, *Chlorella*.

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ASSESSMENT OF MEDIA USING β-D-GLUCURONIDASE ACTIVITY FOR THE DETECTION OF *ESCHERICHIA COLI* IN WATER

Pellizari, V.H.
Pedroso, D.M.M.
Kirschner, C.C.
Silva, L.A.G.
Martins, M.T. [1*]

SUMMARY

Four culture media containing 4-methylumbelliferyl-β-*D*-glucuronide (MUG), Lauryl Sulfate Fluorocult (Merck), ECD. Fluorocult Agar (Merck), Lauryl Tryptose Broth (Difco) and Nutrient Agar (Difco) amended with MUG (Sigma), were compared with conventional media, Lauryl Tryptose Broth, E.C. medium, M-Endo Agar LES, to assess its sensitivity for *E. coli* detection. Samples of water artificially contaminated with *E. coli* isolated from environmental or clinical specimens were analysed through the multiple tube (MPN) and membrane filtration techniques using these media. The Wilcoxon Test showed that Lauryl Sulfate Fluorocult, Lauryl Tryptose Broth-MUG and Nutrient Agar-MUG were as sensitive as Lauryl Tryptose Broth / E.C. medium and M-Endo Agar LES, and that ECDF had a poor recovery of *E. coli*. Only 6.4 % of the strains of *E. coli* isolated did not show β-D-glucuronidase activity when tested with culture media containing MUG. This method has a high potential for use in automatic techniques.

Key Words: *E. coli*, water quality, MUG, β-D-glucuronidase

INTRODUCTION

The total coliform group is the mandatory indicator for microbiological quality of drinking water. Its presence in treated water suggests either an inadequate treatment or a contamination after disinfection. The Environmental Protection Agency (EPA) and the Standard Methods for the Examination of Water and Wastewater (1) have proposed the Membrane Filter technique (MF), the Multiple Tube method (MPN) and the Presence-Absence Test (P-A) for the detection of coliforms. All of these methods require additional verification or confirmation of the presence of coliforms (5).

A new approach for drinking water quality assessment (9) requires the detection and identification of *E. coli*. Generally, this identification is attained through a series of expensive and time consuming biochemical tests (1). To overcome these constraints, enzymatic methods were developed for presumptive detection of *E. coli*. These methods are sensitive, specific and faster than the conventional ones. Various new methods are based on the utilization of chromogenic and fluo-

1. Instituto de Ciências Biomédicas - USP - Av. Prof. Lineu Prestes, 1374 - Cidade Universitária - CEP: 05508-900.
* "Corresponding author": Martins, M.T.

rogenic substrates for the detection of activities of specific enzymes. The fluorogenic substrate most frequently used for detection of *E. coli* is the 4-methylumbelliferil-β-D-glucuronide (MUG). It is hydrolyzed by the enzyme β-D-glucuronidase (GUR), releasing the 4-methylumbelliferone. When this substance is exposed to 360nm ultraviolet light (UV), a diffusible bright-bluish fluorescence appears (15, 16). It is important to emphasize that although *Pseudomonas aeruginosa* also produce fluorescence, it is a typical green one, clearly different from the blue fluorescence produced by *E. coli* (13).

There are many reports of the successful use of culture media containing MUG for the detection of *E. coli* in clinical samples (13, 23); food (10, 21); and water (4-11, 19, 20, 22). There are also reports showing high percentages of GUR-negative *E. coli* (2) and controversy about the efficiency of some minimum media containing MUG (3, 17, 18). Few reports have been published on the use of solid media containing MUG for the direct detection of *E. coli*. Heizman *et al* (1988) (13) evaluated four commercially available culture media containing MUG: Brolacin Agar, ECD Agar, MacConkey Agar and Columbia Agar supplemented with 5% sheep blood, using strains isolated from clinical samples. Mates & Shafer (19) used MUG for detection of *E. coli* from water samples analysed by MF technique. The membrane was incubated on M-Endo Agar LES and then transferred to Nutrient Agar supplemented with MUG and incubated at 35°C for 4h. These authors reported 98% recovery of *E. coli*, and did not observe false-negatives.

Before the recommendation of a new method, comparative studies with the standard methodology are necessary, especially when water and food are concerned (12). This study was conducted to compare the ECD Fluorocult Agar , Lauryl Sulfate Fluorocult, Lauryl Tryptose Broth and Nutrient Agar both amended with MUG, with the standard methods (STM) for the detection of coliforms using Lauryl Tryptose Broth, E.C. Medium and M-Endo Agar LES.

MATERIALS AND METHODS

Samples: A total of 117 samples of artificially contaminated water with *E. coli* isolated from Brazilian samples were assayed. For the preparation of these waters *E. coli* isolated from the environment were previously tested for GUR activity and those positive for this test were mixed and inoculated in buffered water which was prepared with 1,25 ml stock phosphate buffer solution (34.0 g KH_2PO_4 in 1,000 ml distilled water, pH 7.2 +/- 0.5) and 5,0 ml of $MgCl_2$ solution (81.1 g $MgCL_2.6H_2O$/1000 ml of distilled water) (1). A total of 47 artificially contaminated samples with *E. coli* from environmental sources were then prepared. Another 70 samples were prepared using GUR positive *E. coli* cultures obtained from fecal specimens and also inoculated in buffered water. These artificially contaminated waters were prepared to have an expected level of 10-100 CFU/ 100 ml of *E. coli.*

Each sample was then analysed by the MF technique using M-Endo Agar LES (M-Endo LES) (Difco), Nutrient Agar (NA) (Difco) amended with MUG and ECD-Fluorcult Agar (ECDF) (Merck); and by the MPN technique using Lauryl Tryptose Broth (LTB) (Difco), LTB amended with MUG and Lauryl Sulfate Fluorocult (LSF) (Merck).

Membrane Filter technique (MF): 100 ml of each sample were analysed by the MF procedure using 0.45 µm pore filters (Millipore) and incubated on M-Endo Agar LES at 35°C +/- 0.5°C for 24 h. After the incubation period, the typical metallic sheen colonies were enumerated (1). The ECDF and the NA supplemented with MUG (0.05g/l) were prepared as recommended by the manufacturer. Volumes of 100 ml of each sample were filtered and the 0.45 µm membrane were incubated on this medium at 35°C +/- 0.5°C for 18-24 h. The cultures were then exposed to 360 nm wavelength U.V. light for observation of a blue fluorescence.

Multiple Tube Technique (MPN): Samples were inoculated in volumes of 10, 1.0 and 0.1 ml in a serie of 5 tubes containing 10 ml of LTB. A double strength liquid medium was used for inovacula of 10 ml. After 24-48 h of incubation at 35°C, positive tubes (with gas) were transferred to tubes with E.C. medium which were incubated at 44.5°C +/- 0.2°C for 24-48 h. Tubes containing LSF or LTB supplemented with MUG were inoculated simultaneously and incubated at 35°C +/- 0.5°C for 24-48 h. Then the tubes were exposed to UV for observation of a blue fluorescence.

RESULTS

In Table 1 are shown the results obtained for samples contaminated with *E. coli* isolated from environmental sources, such as the minimum, maximum and geometric mean (MG) values ob-

TABLE 1 - Comparison of the results obtained by the MPN and MF techniques with LTB, M-Endo Agar LES, LSF, LTB-MUG, ECDF, and NA-MUG using artificially contamined water with *E. coli isolated* from environmental sources (n=47).

	STM		LSF	LTB-MUG	ECDF	NA-MUG
	MPN LTB-EC	MF M-ENDO LES	MPN	MPN	MF	MF
min.	<2	<1	<2	<2	<1	<1
max.	130	55	80	80	11	86
MG.	12	14	11	09	02	10

tained by the MPN technique using LTB, LSF and LTB-MUG and the number of colony forming units (CFU-MF) obtained by the MF technique using M-Endo LES, NA-MUG and ECDF. The values for samples contaminated with *E. coli* isolated from clinical samples are shown in Table 2. The results obtained by the MPN technique with LTB and LTB-MUG, the sum of all positive tubes for each sample and the results obtained by the MF technique with M-Endo LES and NA-MUG were analysed by the Wilcoxon Test. No differences between both methodologies were detected. The results obtained with M-Endo LES and ECDF when analysed by the Wilcoxon Test presented a significant difference ($p<0.0001$). No differences were detected between 24 and 48 h of incubation in the MPN method using LSF and LTB-MUG by the Wilcoxon Test.

TABLE 2 - Comparison of the results obtained by the MPN and MF techniques with LTB, M-Endo Agar LES, LSF, LTB-MUG, ECDF, and NA-MUG using artificially contamined water with *E. coli isolated* from clinical sources (n=70).

	STM		LSF	LTB-MUG	ECDF	NA-MUG
	MPN LTB-EC	MF M-ENDO LES	MPN	MPN	MF	MF
min.	<2	<2	<2	<2	<1	<1
max.	90	60	110	50	36	57
MG.	18	16	12	08	07	10

DISCUSSION AND CONCLUSIONS

The use of artificially contaminated samples to obtain a significant number of positive results for *E. coli* avoided the analysis of hundreds of samples of treated water expected to have few if any coliforms. *E. coli* counts on M-Endo Agar LES was chosen as a baseline control because it is less selective than M-FC medium. The data produced were used for assessing the efficiency of the other media on the recovery of this bacterium. The statistical analysis using Wilcoxon Test showed no differences between MPN technique with LTB-MUG/LTB and LSF/LTB. This means that, the LSF and LTB - MUG presented the same performance as the conventional media (LTB and E.C. medium) for the recovery of *E. coli* from artificially contaminated samples. These data agree with those reported by Feng & Hartman (10) when using LTB amended with MUG (100 µg/ml) for immediate confirmation of the *E. coli* presence. LTB-MUG was as sensitive as the media used in the conventional methods (LTB/E.C. media), nevertheless the fluorogenic test produced results sooner (24 h) than the conventional methods (96 h). It also confirm the presence of *E. coli* which represents another advantage of this method because the identification of this bacteria by the conventional methodology will require at least 24 h more. There was no difference between 24 h and 48 h of incubation of tubes containing LSF and LTB-MUG, then 24 h was enough for *E. coli* detection. This finding is also important in the perspective of Public Health since the answer of a test must be as fast as possible to establish preventive measures. The LTB and NA supplemented with MUG had the same efficacy as the conventional media (LTB, E.C. medium, and M-Endo Agar LES) for the recovery of *E. coli* from artificially contaminated samples as detected by the statistical analysis.

Differences were detected between data obtained with M-Endo LES and ECDF, the last one being less effective. These results are in contrast to those previously reported (13) in that out of four solid culture media, ECDF Agar had a recovery of 85.8%. However, in that study the authors worked with strains isolated from clinical samples which were previously cultivated on blood agar plates for 20 h, the colonies were suspended in saline at 10^6 CFU/ml and the Fluorocult media were inoculated with 10^4 CFU/ml. A lower level of contamination (10 - 10^2 CFU/100 ml) was used in our study. A higher concentration of bacteria and a qualitative method was used in that study so their data can not be extrapolated for aplication in clean waters and quantitative methodology.

The ECDF agar used in the MF technique recovered only 15% of the samples artificially contaminated with environmental isolates of *E. coli* and 44% of samples inoculated with strains from

clinical specimens. Another information obtained in this study is that the *E. coli* strains used for preparing artificially contaminated water were individually tested for GUR activity and 6,4% of them did not produce this enzyme. Our results do not confirm those of Chang *et al* (2) who found 34% of *E. coli* that did not produce the enzyme, but are closer to the 3.5% found by Kilan & Bulow (14). The fluorogenic media are a good alternative for simplification of the bacteriological examination of water, specially when the legislation requires the presumptive identification of *E. coli*. They also can be used in automated assays with equipment having sensors for fluorescence. However it is important to consider that other microorganisms with β-*D*-glucuronidase activity could also be detected by these culture media as *Salmonella, Shigella, Yersinia, Staphylococcus, Clostridium, Bacteroides, Corynebacterium* could present β-*D*- glucuronidase activity (16). Since these microorganisms could be found in water, it is very important to evaluate also the specificity of this culture media. LSF medium and the conventional media amended with MUG do have a good potential for application in faster methods but should be tested for specificity including with environmental samples.

RESUMO

Avaliação de meios de cultura orientados para deteção da β-*D*- Glucuronidase no isolamento de *Escherichia coli* em água

Quatro meios de cultura contendo β-*D*-glucoronide MUG (Lauril Sulfato Fluorocult, ECD Fluorocult Agar, Caldo Lauril Triptose e Agar Nutriente suplementados com MUG) foram comparados com meios convencionais (caldo Lauril Triptose,Caldo E.C. e Agar M-Endo LES) para verificar se havia diferenças entre eles na detecção de *E. coli*. As amostras de água foram contaminadas artificialmente com *E. coli* isoladas tanto do ambiente quanto de espécimes clínicos e analisadas pelas técnicas dos tubos múltiplos e de membrana filtrante usando os meios acima descritos. Entre as cepas previamente analisadas para o preparo das amostras artificialmente contaminadas, 6,4% não apresentaram atividade β-*D*-glucuronidase quando testadas com meio contendo MUG. Os resultados obtidos foram submetidos ao Teste de Wilcoxon que mostrou que Lauril Sulfato Fluorocult, caldo Lauril Triptose com MUG e Agar Nutriente com MUG foram tão sensíveis quanto o Caldo Lauril Triptose e meio E.C. e Agar M-Endo LES, e que o ECD Fluorocult Agar apresentou uma sensibilidade muito baixa. Esta técnica é de grande importância pois pode ser aplicada em métodos mais rápidos para detecção de ***E. coli*** e em automação.

Palavras-chave: *E. coli*, MUG, β-*D*- glucuronidase, qualidade da água.

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RELATION BETWEEN THE HEMAGGLUTINATION INHIBITION TEST AND THE INDIRECT ELISA IN THE SEROLOGIC MONITORING OF LAYING HENS SUBMITTED TO DIFFERENT SYSTEMS OF VACCINATION AGAINST NEWCASTLE DISEASE

Leonardo José Richtzenhain[1]
Antonio Carlos Paulillo[2]
Aramis Augusto Pinto[2]
Sergio Nascimento Kronka[2]

ABSTRACT

The relation between indirect ELISA (**IE**) and the hemagglutination (**HI**) test was studied in 240 serum samples from laying hens submitted to different systems of vaccination against Newcastle disease. A high correlation was obtained between the two serologic tests, although a wide variation in the absorbance was observed when using **IE** for sera with the same **HI** titer.

Key Words: Newcastle Disease: Hemagglutination Inhibition Test; Indirect ELISA; Serologic Monitoring.

INTRODUCTION

The humoral immune response is the major parameter for the evaluation of immunizing procedures against Newcastle Disease (**ND**) because it can be easily measured by serologic tests. The Hemagglutination Inhibition (**HI**) test is more widely used for this purpose (2). However, in view of the possibility of automatization and informatization, the Indirect ELISA (**IE**) technique has been increasingly applied to the serologic monitoring of different viral diseases in the poultry industry (7). Snyder et al. (9) and Thayer et al. (10), when processing sera from hens with an undetermined history of vaccination, observed a high correlation between **IE** and **HI**.

In contrast, Miers et al. (6), in a study of sera from hens vaccinated by the oral route and revaccinated by aerosol, found no "direct correlation" between the two tests as a function of the broad range of absorbance obtained with **IE** for sera with the same **HI** titer.

Marquardt el al. (5) demonstrated that vaccination against **ND** by the intratracheal route reduced the correlation between the **IE** and **HI** tests, a fact that was not observed in the sera from hens vaccinated by the oculo-nasal route.

According to Brown et al. (3), there is a high correlation between **IE** and **HI** in sera from hens of different ages submitted to oral vaccination.

The present study was undertaken to investigate the relation between **HI** and **IE** in the serologic monitoring of laying hens submitted to different vaccination systems against**ND**.

1. Faculdade de Medicina Veterinária e Zootecnia - USP Av. Corifeu de Azevedo Marques, 2720 CEP 05340-000 São Paulo - SP - Brasil.
2. Faculdade de Ciências Agrárias e Veterinárias - UNESP - Rodovia Carlos Tonanni, km 5 CEP 14870-000 - Jaboticabal - SP - Brasil.

MATERIAL AND METHODS

1. Experimental animals and management

A total of 408 laying hens 20 weeks old of a commercial line were divided at random into eight groups of 51 animals each, as shown in Table 1. Except for the control group, all hens been previously vaccinated against **ND** with a live virus (LaSota vaccinal strain) by the intranasal route at 10, 35 and 90 days of age.

TABLE 1 - Distribution of hens among the various experimental groups. Symbols: LSV = Live, LaSota vaccine strain; OIV = oily-inactivated vaccine, * = vaccinated only at 10, 35 and 90 days, ** = unvaccinated.

Group	Vaccine	Administration route	Revaccination by the intranasal route (LSV) (weeks)
I	OIV	Subcutaneous	
II	OIV + LSV	Subcutaneous + Intranasal	
III	OIV	Subcutaneous	42
IV	OIV	Subcutaneous	46
V	LSV	Intranasal	30, 40, 50 and 60
VI	LSV	Intranasal	28, 36, 44 and 52
VII	LSV	Intranasal	*
VIII	-	Control group	**

2. Vaccines

A live virus vaccine (LaSota strain) with EID50 = 10(7.16) - 10(7.31)/0.1 ml and an inactivated oily vaccine (PD = 56.24/0.5ml), from the same laboratory and from the same lot were used.

3. Serum collection

A total of 240 serum samples were obtained by bleeding approximately 20% of the laying hens in each group during the 29th, 43th and 58th weeks of age.

The sera were inactivated at 56°C for 30 minutes to remove nonspecific hemagglutination inhibitors and stored at -20°C until the time for processing in the serologic tests.

4. Hemagglutination Inhibition (HI) test

The **HI** test was performed on 96-well U-bottomed microplates by the method according to the technique of Beard & Wilkes (1). The antigen used was the LaSota strain of Newcastle Disease Virus (**NDV**) with 4 HA units. The titer was expressed as the reciprocal of the highest serum dilution that fully inhibited viral hemagglutination.

When expressing the titers, in order to permit the application of the $\log_2$ function to **HI** titers = 0 (null), the $\log_2$ (HI+1) artifice was used.

5. Indirect ELISA

The LaSota strain of **NDV** propagated in allantoic cavity of specific-pathogen free (SPF) embryonated eggs was used as antigen. The virus was purified by ultracentrifugation on a discontinuous sucrose gradient by the method of Snyder et al. (9). A rabbit gamma-globulin anti-chicken IgG coupled to horseradish peroxidase as described by Richtzenhain (8), was used as conjugate. The substrate was citrate-phosphate buffer, pH 5.2, containing 0.04% (w/v) ortophenilenediamine and 0.04% (v/v) 30% H_2O_2.

5.1. Procedure and data analysis

Antigen, serum, conjugate and substrate were assayed in a 100 µl volume in duplicate. Flexible, 96-well flat bottomed microplates (Hemobag - Campinas, SP, Brasil) were sensitized with an optimum antigen dose in 0.05 M sodium carbonate-bicarbonate buffer, pH 9.6, followed by incubation in a moist chamber at 4°C for 18 hs. Between the different reaction phases, the microplates were submitted to three washings of 3 minutes each with phosphate-buffered saline (PBS) containing 0.01 M PO4--/0.5 M NaCl/ 1% Tween 80. Sera and conjugate were diluted in PBS containing 10% normal rabbit serum and incubated in a moist chamber at 37°C for 30 minutes.

After addition, the substrate was maintained at room temperature for 15 minutes and then blocked with 25 µl 1 N HCl. Optimum dilutions of the different reaction components were determined by block titration. All sera were assayed in a single dilution (1:400) and results were reported in relation to the positive (hyperimmune anti-**NDV** serum produced in SPF chickens) and the negative (SPF chicken serum) controls present on each microplate. The absorbance at 490 nm was recorded using an Uniskan 1 ELISA reader (Flow-USA). The activity of each serum was calculated by the Sample/Positive (S/P) control ratio according to the formula S/P = uS - uN/uP-uN, where uS, uN and uP represent respectively the mean absorbanc-

es of the duplicates of the serum sample, the negative serum control and the positive serum control.

S/P values were later classified as ELISA levels (**EL**) from zero to 9. The maximum limit of the zero level was determined by the mean of the S/P values of the sera of unvaccinated hens from the control group + 2 standard deviations. Starting from this limit, the intervals between the other levels were arbitrarily defined by 35% increments as proposed by Wilson et al. (11).]

6. Study of the relation between IE and HI

1st, 2nd and 3rd degree polynomial regressions with the respective significance determined by the F test were calculated to establish the relations between the following parameters: S/P values X $\log_2$(**HI+1**) for all sera regardless of group: **EL** x $\log_2$(**HI+1**) for all sera regardless the group and mean **EL** x mean $\log_2$(**HI+1**) for each group.

In addition to the r correlation coefficient (calculated by 1ht degree regression), the equation best explaining the relation between parameters was considered to be that of highest degree which presented at least 5% significance, according to Gomes (4).

7. Analysis of variance

The behavior of the **EL** and $\log_2$(**HI+1**) parameters in the comparison of means for the different hen groups at each bleeding was evaluated by analysis of variance of such parameters and the Tukey test was applied at the 5% level of probability. For this procedure, **EL** was transformed to (**EL+1**) (1/2).

RESULTS AND DISCUSSION

Results presented in Table 2 show that high correlation coefficientes were obtained with significance at the 1% level both when the values for each serum (240 pairs) and the mean values for each group of 10 hens (24 pairs) were considered. There is virtually no difference in correlation coefficient when the S/P value (r = 0.9643) or its equivalent in **EL** (r = 0.9674) was considered, demonstrating that the **EL** intervals were adequately established, thus permitting these two forms of expressing **IE** to keep a good correlation with the **HI** reaction. Results obtained in the present study con-

TABLE 2 - Correlation coefficients of the different polynomial regressions studied and their respective significances calculated by the F test. Symbols: * = Significant at 5%: ** = significant at 1% NS = not significant: S/P = ELISA values expressed by the Sample / Positive ratio: EL = ELISA level expressed as a function of S/P; HI = Titer of hemagglutination Inhibition test; μ = mean; F = F test; r = Correlation Coefficient.

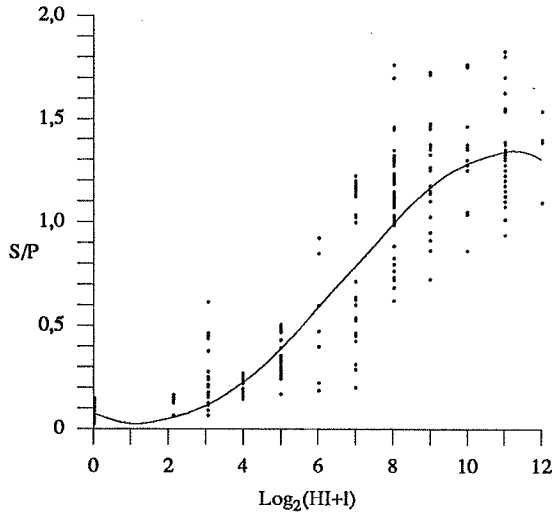
Parameters	Nº of pairs	Degree of regression	F	r
$\log_2$ (HI + 1) x S/F	240	1 st	844.08**	0.9643
		2 nd	5.44*	
		3 rd	45.85**	
$\log_2$ (HI + 1) x EL	240	1 st	1,448.27**	0.9674
		2 nd	26.11**	
		3 rd	61.77**	
μ $\log_2$ (HI + 1) x μ EL (group) (group)	24	1 st	1,416.80**	0.9911
		2 nd	NS	
		3 rd	4.29*	

TABLE 3 - Analysis of variance and Tukey test for comparison of mean EL (transformed to $(EL + 1)^{1/2}$) and HI (transformed to $\log_2(HI + 1)$ in the different groups of the three serum collections. Symbols: (1) = means in the same column followed by identical letters did not differ at 5% level of probability by the Tukey test; ** = Significant at 1%; EL = ELISA level expressed as function of S/P; HI - Titer of the hemagglutination inhibition test; F = F test; DMS = Tukey 5%; CV = Coefficient of variation.

Group	1 st Collection Mean		2 nd Collection Mean		1 st Collection Mean	
	$(EL + 1)^{1/2}$	$\log_2(HI + 1)$	$(EL + 1)^{1/2}$	$\log_2(HI + 1)$	$(EL + 1)^{1/2}$	$\log_2(HI + 1)$
VIII	1.000 C(1)	0.000 C	1.000 C	0.000 C	1.000 C	0.000 C
VII	1.801 B	2.381 B	1.124 B	0.464 C	1.124 C	0.000 C
I	3.146 A	10.302 A	2.746 A	7.711 A	3.049 A	8.805 A
II	3.114 A	9.902 A	2.693 A	7.212 A	2.999 A	9.004 A
III	3.114 A	9.703 A	3.048 A	8.008 A	3.162 A	9.404 A
IV	3.097 A	9.503 A	2.831 A	7.319 A	3.128 A	9.603 A
VI	1.898 B	3.669 B	1.880 B	3.816 B	2.437 B	5.342 B
V	2.010 B	4.373 B	1.979 B	4.187 B	2.540 B	6.521 B
DMS	0.527	2.324	0.362	1.482	0.269	1.472
F	47.80**	68.01**	93.38**	93.84**	211.47**	146.54**
CV(%)	15.77	26.73	11.99	21.95	7.93	17.33

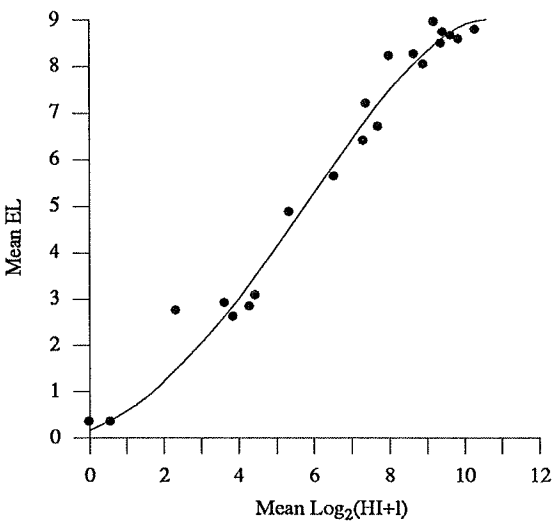
firm those reported by Snyder et al. (9) who obtained **r** =0.96 for sera from hens exposed to **NDV**, by Marquardt et al. (5) who obtained **r** =0.97 for hens vaccinated by the oculo-nasal route, by Thayer et al. (10) who, when evaluating two commercial **IE** kits, obtained **r** = 0.98 and **r** = 0.95 for sera from hens with an undetermined history of vaccination, and by Brown et al. (3) who, when using a commercial **IE** kit, detected **r** = 0.85 for sera from hens of different ages vaccinated only by the oral route. Within this context, the data presented in Table 3 show that, when using the Tukey test at the 5% level of probability in an eventual evaluation of the humoral immune response induced by different systems of vaccination against **ND**, the same conclusions are reached whether using **IE** or **HI**.

The wide variation in absorbance in the **IE** for sera with the same **HI** titer reported by Miers et al. (6) was also observed in the present study (Figure 1). However, this phenomenon did not interfere with the high correlation coefficient existing between the serologic tests. Thayer et al. (10) also pointed out this fact and recommended a minimum of 15 serum samples per sampling in order to determine good parallelism of the mean results obtained with the two tests. Thus, in the correlation of **IE** and **HI** through the mean results for 10 sera per blood collection in each vaccine group, a small dispersal was observed between **EL** and **HI** (Figure 2).



$Y = 0,0924 + 0,1233\ x + 0,0511\ x^2 - 0,0027\ x^3$
$R^2 = 0,9864\ p < 0,01$

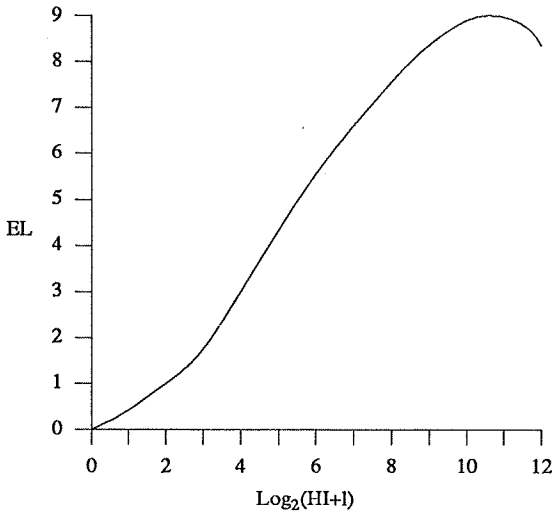
FIGURE 1 - Graphic representation of the regression equation obtained for $\log_2(HI + 1)$ and S/P. (HI = Titer of the hemagglutination inhibition test; S/P = ELISA values expressed as the Sample/Positive ratio).



$Y = 0,1284 + 0,3857\ x + 0,1291\ x^2 - 0,0079\ x^3$
$R^2 = 0,9861\ p < 0,05$

FIGURE 2 - Graphic representation of the regression equation obtained for $\log_2(HI + 1)$ for group and mean EL of the group. (HI =Titer of the hemagglutination inhibition test; EL=ELISA level expressed as function of the Sample/Positive ratio).

There was no loss of correlation between **IE** (mean **EL**) and **HI** (mean **HI**) in any vaccine group for the three blood collections regardless



$Y = 0,0550 + 0,0343\ x + 0,2345\ x^2 - 0,0149\ x^3$
$R^2 = 0,9954\ p < 0,01$

FIGURE 3 - Graphic representation of the regression equation obtained for $\log_2(HI + 1)$ and EL. (HI = Titer of the hemaggulation inhibition test; EL = ELISA level expressed as function of the Sample/Positive ratio).

of the vaccination system adopted (Table 2). The loss of correlation between the two tests reported by Marquardt el al.(5) for hens vaccinated by the intratracheal route could not be studied in the present investigation because the laying hens had been vaccinated with live and/or inactivated virus by the intranasal and subcutaneous route respectively.

Although there was a high correlation coefficient between **IE** and **HI**, the use of higher degree polynomial regressions revealed that the curve that best fitted the relation between the parameters under study appears to be sigmoidal (Table 2, Figures 1, 2 and 3).

The present results also suggest that the **IE** and **HI** tests can be equally used for the serologic monitoring of laying hens submitted to different systems of vaccination against **ND**. However, the advantages of automatization and informatization of **IE** make this test useful in the processing of large numbers of serum samples.

RESUMO

Relação entre o teste de inibição da hemoglutinação e o teste indireto de Elisa no monitoramento sorológico de galinhas poedeiras submetidas a diferentes sistemas de vacinação contra a doença de Newcastle

A relação entre os testes de ELISA indireto (**EI**) e de inibição de hemaglutinação (**HI**) foi estudada em 240 soros de poedeiras submetidas a diferentes sistemas de vacinação contra a Doença de Newcastle. Uma elevada correlação foi obtida entre as duas provas sorológicas, embora tenha sido observada uma ampla variação de absorvância no **EI** para soros do mesmo título de **HI**.

Palavras-chave: Doença de Newcastle, ELISA teste, Reação de Inibição da Hemaglutinação (HI), Monitoramento sorológico.

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QUANTITATIVE ESTIMATION OF ZOOSPORIC FUNGI AND AQUATIC HYPHOMYCETES ON LEAVES SUBMERGED IN A STREAM IN THE ATLANTIC RAINFOREST, IN THE STATE OF SÃO PAULO, BRAZIL

C.L.A. Pires-Zottarelli[1]
I.H. Schoenlein-Crusius[1]
A.I. Milanez[1]

SUMMARY

Quantification of zoosporic fungi (Chytridiomycetes) and aquatic Hyphomycetes from the leaves of *Alchornea triplinervia* (Spreng.) M. Arg., *Quercus robur* L. and *Ficus microcarpa* L.f. submerged in a stream in the "Reserva Biológica do Alto da Serra de Paranapiacaba" in Santo André, São Paulo State, Brazil, was realized from April to November 1990. Zoosporangia, for Chytridiomycetes, and conidial apparatus (conidiophores plus conidiospores), for aquatic Hyphomycetes, were taken as units of propagation: propagules-forming units per square millimeter (PFU/mm^2). Those structures were quantified in baits (corn straw, cellophane, snake skin, shrimp shells) of 1cm^2 and in leaf disks with 5 mm diameter, incubated at room temperature, in sterilized distilled water. *A. triplinervia* leaves presented the highest number of PFU/mm^2 (4799.59), followed by *F. microcarpa* (4306.73) and *Q. robur* (1800.79). The highest number of PFU/mm^2 of zoosporic fungi was obtained from *A. triplinervia* leaves, whereas for the aquatic Hyphomycetes the maximum was registered on *F. microcarpa* substrate. The zoosporic fungi may have host specificity because *Catenophlyctis variabilis* (Karling) Karling and *Polychytrium aggregatum* Ajello were the most abundant species, but the later failed to grow on *Q. robur* leaves. The total fungal PFU/mm^2 was higher at the beginning of the decomposition process, probably due to the pioneer activity of the zoosporic fungi and aquatic Hyphomycetes. Comparison of these results with the ones mentioned in the literature is difficult because of methodological differences. The results using PFU/mm^2, indicate not only the occurrences of the fungi, but also the colonization potencial of the aquatic mycota.

Key Words: fungal quantification, zoosporic fungi, aquatic Hyphomycetes, propagules-forming unit, Brazil.

INTRODUCTION

Quantification of zoosporic fungi and aquatic Hyphomycetes propagules has always been a problem, complex enough to hamper ecological studies involving these groups, mainly because reliable data are still not available (14,26).

There are many papers in the literature concerned with the quantification of conidial structures of aquatic Hyphomycetes (21,22) and Sapro-

Instituto de Botânica, Seção de Micologia e Liquenologia, Caixa Postal 4005, 01061-970 São Paulo, Brasil.

legniales (10,17,18,30,31). For each of them, some restriction has been found, involving spore viability and germination, spore counting or group limitation. For instance, they are not suitable to quantify propagules of Chytridiomycetes because of the difficulty in growing them on culture media, but a method for estimation of the most probable number of propagules of chytrids growing in pine pollen has been described by Ulken & Sparrow (28). Meanwhile, quantification studies for specific groups have been proposed. For instance, Carvalho (7) and Carvalho & Milanez (8) developed a technique to quantify the number of *Pythium splendens* Braun propagules in soil. Since this species rarely forms zoospores, they considered the sporangia as a unit of propagation.

The aquatic Hyphomycetes have been counted by filtration methods, plating techniques of water samples or direct observation of conidial structures on the surface of organic substrates (21,22).

There are many qualitative comparisons between the fungal populations of submerged leaves (2,3,19), but few informations about the intensity of the colonization of the substrates by the aquatic mycota, reflected by the quantification of fungal structures.

The aim of this paper was to estimative populations of the aquatic Hyphomycetes and Chytridiomycetes on submerged leaves, in a sequence of studies initiated with the comparison of the mycota present in submerged leaves of *Alchornea triplinervia* (Spreng.) M.Arg., *Ficus microcarpa* L. and *Quercus robur* L. (25).

MATERIAL AND METHODS

Sites characteristics and procedures for sampling have already been described in detail in an earlier paper (25).

The baits (corn straw, cellophane, snake skin, shrimp shells) dimension was maintained at 1 cm^2 to standardize the substrate size available for colonization, and couting of the fungal structures. They were added to the petri dishes containing 5 mm diameter leaf disks of each plant species and distilled sterilized water. Twenty leaf disks were randomly taken from the dishes and the zoosporangial or conidial structures were counted in a window of 4 mm^2 area drawn on the ocular (12.5 x magnification) of a Zeiss microscope. The area of the window was projected on a slide and then measured to estimative the actual size of the observation area. Values obtained from countings on twenty windows were calculated to 1 mm^2 area.

The zoosporic fungi were identified observing the sexual and asexual reproductive structures and the aquatic Hyphomycetes by observing the conidial apparati (conidiophores and conidiospores).

As mentioned earlier, the difficulties in quantifying the propagules of the zoosporic fungi and aquatic Hyphomycetes led the authors to consider the zoosporangium of Chytridiomycetes and the conidial structures (conidiophore plus conidiospores) of aquatic Hyphomycetes as propagules-forming unit per square millimeter ((PFU/mm^2) as unit for population estimation.

RESULTS AND DISCUSSION

The number of propagules-forming units (PFU/mm^2), zoosporangium of Chytridiomycetes and conidial structures of aquatic Hyphomycetes, varied according to the species of the leaves considered and time of collection (Tabs. 1-3). *A. triplinervia* showed the highest total of PFU/mm^2 (4799.59) closely followed by *F. microcarpa* (4306.73) and *Q.robur* (1800.79).

The highest number of zoosporic fungi was obtained on *A. triplinervia* leaves (4095.22 PFU/mm^2) followed by *F.microcarpa* (3378.83 PFU/mm^2) and *Q. robur* leaves (1242.41 PFU/mm^2). (Tab. 4).

On the other hand, the highest number of PFU/mm^2 of aquatic Hyphomycetes was obtained in *F. microcarpa* leaves (927.90) followed by *A. triplinervia* (704.37) and *Q.robur* (558.38), (Tab. 4). These results agree with those mentioned in a previous paper related to fungal succession on *F. microcarpa* leaves, confirming that they are an excellent substrate for aquatic mycota colonization (23).

Although *Quercus* spp. leaves have successfully been used as substrate in aquatic fungi colonization experiments (1,3,6,19,24), the present results show that they are not very atractive substrates for the decomposing mycota in the Brazilian rainforest. The success of *A. triplinervia* as a good substrate for fungi may be connected with some of its botanical characteristics. This very frequent plant of the atlantic rainforest has smooth, thin and denticulate leaves (11), easily softened by submergence.

The higher number of PFU/mm^2 of aquatic fungi in the beginning of the leaf decomposition process (Tab. 4) may be due, at least in part, to the ability of these fungi to decompose simple sugar compounds which caracterize them as pioneers in

TABLE 1 - PFU/mm² of baits and leaves of Chytridiomycetes and aquatic Hyphomycetes on leaves of *Alchornea triplinervia* submerged in a stream in the Biological Reserve of Paranapiacaba da Serra, SP.

Taxa of fungi /Month 1990	A	M	J	J	A	S	O	N	Total
Chytridiomycetes									
Catenophlyctis variabilis	1494.00	872.00	-	-	-	-	128.00	.80	2494.80
Diplophlyctis sarcoptoides	-	-	-	-	-	-	-	-	-
Karlingia rosea	-	-	27.00	30.00	48.00	-	-	.40	105.40
Nowakowskiella elegans	-	-	-	-	32.00	35.00	-	10.00	77.00
Polychytrium aggregatum	1160.00	-	59.00	130.00	-	2.00	-	12.00	1363.00
Rhizophlyctis sp.	-	-	-	-	.02	-	-	-	.02
Rhizophydium elyensis	-	-	-	-	-	-	-	55.00	55.00
Hyphomycetes									
Anguillospora longissima	-	-	-	-	-	-	-	-	-
Anguillospora sp.	.01	-	-	-	-	-	-	-	.01
Camposporium pellucidum	.20	-	-	-	-	-	-	-	.20
Dendrospora sp.	.02	-	-	-	-	-	-	-	.02
Lemmoniera aquatica	-	-	-	-	-	-	-	-	-
Lunulospora curvula	.01	-	-	-	-	-	-	-	0.1
Margaritispora sp.	-	96.00	.18	-	-	.51	-	-	96.69
Tetrachaetium elegans	.03	256.00	-	-	-	-	-	-	256.03
Tripospermum sp.	.01	160.00	.07	-	.07	.35	.25	-	160.68
Triscelophorus monosporus	.32	110.00	.03	-	40.30	40.00	-	-	190.65
Trisulcosporium acerinum	-	-	-	-	.01	-	-	-	.01
Total PFU/mm²	2654.60	1494.00	86.28	160.00	120.40	77.86	128.25	78.20	4799.52

TABLE 2 - PFU/mm² of baits and leaves of Chytridiomycetes and aquatic Hyphomycetes on leaves of *Ficus microcarpa* submerged in a stream in the Biological Reserve of Paranapiacaba da Serra, SP.

Taxa of fungi /Month 1990	A	M	J	J	A	S	O	N	Total
Chytridiomycetes									
Catenophlyctis variabilis	2260.00	-	-	-	167.00	11.00	1.50	-	2439.50
Diplophlyctis sarcoptoides	-	-	-	.22	.20	-	-	-	.42
Karlingia rosea	-	-	-	-	-	.90	-	-	.90
Nowakowskiella elegans	-	173.00	-	-	-	-	-	-	173.00
Polychytrium aggregatum	-	224.00	237.00	-	-	89.00	-	-	550.00
Rhizophlyctis sp.	-	-	-	-	-	-	.01	-	.01
Rhizophydium elyensis	-	-	-	64.00	-	-	55.00	96.00	215.00
Hyphomycetes									
Anguillospora longissima	35.00	-	-	-	-	-	-	-	35.00
Anguillospora sp.	.02	-	-	-	-	-	-	-	.02
Camposporium pellucidum	-	-	-	-	-	-	-	-	-
Dendrospora sp.	3.20	-	-	-	-	-	-	-	3.20
Lemmoniera aquatica	-	-	-	.06	-	-	-	.05	.11
Lunulospora curvula	9.60	160.00	-	-	-	-	-	-	169.60
Margaritispora sp.	-	-	.10	-	-	-	-	-	0.10
Tetrachaetium elegans	-	64.00	-	-	-	-	-	-	64.00
Tripospermum sp.	-	141.00	.53	.13	.10	30.40	.24	0.20	172.60
Triscelophorus monosporus	276.00	181.00	.02	-	-	26.00	.25	-	483.27
Trisulcosporium acerinum	-	-	-	-	-	-	-	-	-
Total PFU/mm²	2583.82	943.00	237.65	64.41	167.30	157.30	57.00	96.25	4242.73

TABLE 3 - PFU/mm² of baits and leaves of Chytridiomycetes and aquatic Hyphomycetes on leaves of *Quercus robur* submerged in a stream in the Biological Reserve of Paranapiacaba da Serra, SP.

Taxa of fungi /Month 1990	A	M	J	J	A	S	O	N	Total
Chytridiomycetes									
Catenophlyctis variabilis	507.00	295.00	22.00	.91	-	-	66.00	130.00	1020.90
Diplophlyctis sarcoptoides	-	-	-	-	-	-	-	-	1.00
Karlingia rosea	-	-	-	-	-	-	-	-	-
Nowakowskiella elegans	-	-	171.00	-	-	10.00	-	.50	181.50
Polychytrium aggregatum	-	-	-	-	-	-	-	-	-
Rhizophlyctis sp.	-	-	-	-	-	-	-	-	-
Rhizophydium elyensis	-	-	-	-	-	-	-	40.00	40.00
Hyphomycetes									
Anguillospora longissima	-	-	20.00	-	-	-	-	-	20.00
Anguillospora sp.	.01	-	-	-	-	-	-	-	.01
Camposporium pellucidum	.13	-	-	.15	-	-	-	-	.28
Dendrospora sp.	-	-	-	-	-	-	-	-	-
Lemmoniera aquatica	.02	-	-	.05	-	-	-	-	.07
Lunulospora curvula	.07	64.00	-	-	-	-	-	-	64.07
Margaritispora sp.	-	64.00	-	-	16.00	-	10.00	13.00	103.00
Tetrachaetium elegans	-	150.00	-	-	-	-	-	-	150.00
Tripospermum sp.	.11	103.00	.36	-	-	-	.02	-	103.49
Triscelophorus monosporus	.05	93.00	.31	.02	16.00	8.00	.08	-	117.46
Trisulcosporium acerinum	-	-	-	-	-	-	-	-	-
Total PFU/mm²	508.39	769.00	213.67	1.13	32.00	18.00	76.10	183.50	1801.78

TABLE 4 - PFU/mm² zoosporic fungi and aquatic Hyphomycetes according to leaf substrate submerged in the period of April to November of 1990.

	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Total
Alchornea triplinervia	2654.60	1494.00	86.28	160.00	120.40	77.86	128.25	78.20	4799.52
Zoosporic fungi	2654.00	872.00	86.00	160.00	80.02	37.00	128.00	78.20	4095.22
Aquatic Hyphomycetes	0.60	622.00	0.28	0.00	40.38	40.86	0.25	0.00	704.37
Ficus microcarpa	2583.82	943.00	237.65	64.41	167.30	157.30	57.00	96.25	4242.73
Zoosporic fungi	2260.00	397.00	237.00	64.22	167.20	100.90	56.51	96.00	3378.83
Aquatic Hyphomycetes	323.82	546.00	0.65	0.19	0.10	56.40	0.49	0.25	927.90
Quercus robur	508.39	769.00	213.67	1.13	32.00	18.00	76.10	183.50	1801.78
Zoosporic fungi	507.00	295.00	193.00	0.91	0.00	10.00	66.00	170.50	1242.41
Aquatic Hyphomycetes	1.30	474.00	20.67	0.22	32.00	8.00	10.10	13.00	559.29
Total	5746.81	3206.00	537.60	225.50	319.70	253.16	261.35	357.95	

the fungal succession, especially the pectinase-degrading aquatic Hyphomycetes (9). The decomposition of leaves in streams have been divided into three different phases: leaching, microbial colonization and invertebrate feeding (12). Leaching is based on the removal of soluble substances such as amino acids, carbohydrates and phenolic compounds (27). This process is completed within the 24-48h after the submergence of the leaves (29), attracting a rich aquatic mycota to the substrate.

The number of PFU/mm² of zoosporic fungi has been found to be much higher than the one of the aquatic Hyphomycetes (Tab. 4). Because of the data scarcity of several parameters there is no satisfactory explanation for this result as yet. However, Barlocher (2) has observed that some Oomy-

cetes and, to a lesser degree, bacteria were more common on fresh than on dried leaves during the first two weeks submergence in a stream. They were probably benefited by the initially higher concentration of soluble substances or by the scarcity of the aquatic Hyphomycetes. In general, these fungi replace terrestrial fungi immediately after the submergence of leaves (3). This may explain the higher number of PFU/mm^2 of aquatic Hyphomycetes after the second month of the leaf decomposition process.

The activity of the zoosporic fungi as pioneers in the decomposition process of the leaves is reinforced by the fact that the most frequent species, *Catenophlyctis variabilis* (Karling) Karling and *Polychytrium aggregatum* Ajello were usually isolated from chitinic and keratinic baits, while *Nowakowskiella elegans* (Nowak.) Schroeter and *Karlingia rosea* (De Bary & Woronin) Johanson were abundantly isolated from cellulosic substrates.

Some of the zoosporic fungi seem to be more host especific than the aquatic Hyphomycetes. Host specificity tendency has been found at specific level in both groups. *Diplophlyctis sarcoptoides* did not grow in leaves of *A. triplinervia*, as well as, *Lemoniera aquatica* and *Anguillospora longissima* (Tab. 1). *Polychytrium aggregatum*, *Karlingia rosea* and *Rhizophlyctis* sp., *Dendrospora* sp. and *Trisulcosporium acerinum* were not observed in *Quercus robur* leaves (Tab. 3). *Camposporium pellucidum* and *Trisulcosporium acerinum* were the only ones that did not grow in *F. microcarpa* leaves (Tab. 2). It is not possible at the moment to explain the reasons for these results, but some morphological features (16) linked with the release of inhibitory substances may act against the development of the aquatic mycota, as has been demonstrated for conifer needles (4,5), pine and oak wood (15). The decomposition rate of the substrate may be an important parameter for some fungi, as has been mentioned by Nilsson (20) for *Alnus glutinosa* and *Acer platanoides* leaves.

These results may not be compared with the ones obtained by Willoughby & Collins (31) because these authors used other group of zoosporic fungi (Saprolegniales) with a very different type of thallus and the zoospores being counted as propagules. Dick (13) also used saprolegniaceous fungi but counted colonies growing in hemp seed agar blocks in plates with distilled water. The disadvantage is that not all zoospores germinate to form colonies. Ulken & Sparrow (28) used monocentric chytrids but the substrate used was pine pollen and the zoospores being the propagules. In this paper the organisms used for quantitative study were monocentric and polycentric chytrids and aquatic Hyhomycetes. Due to the difficulty in having most of them growing in culture, the extremely variable thalli characteristics, the zoosporangium of the chytrids and the conidiophore with conidiospores of the aquatic Hyphomycetes, the propagules-forming units were chosen as unit of propagation (propagules), as mentioned earlier. We are aware that the zoosporangia form zoospores, the final propagules, but the zoosporangium, besides being a real unit of propagation, also gives a measure of colonization.

There is still much to be done concerning quantitative studies or seasonality of the aquatic Hyphomycetes and zoosporic fungi. According to Sparrow (26) "for the bulk of the aquatic fungi few reliable quantitative data exist, a lacuna which needs immediate attention". Dick (14) extends this point of view for all ecological data concerning the zoosporic fungi.

RESUMO

Quantificação de fungos zoospóricos e Hyphomycetes aquáticos em folhas submersas em um riacho na mata atlântica

O objetivo do presente trabalho foi estimar populações de fungos zoospóricos (Chytridiomycetes) e de Hyphomycetes aquáticos em folhas submersas de *Alchornea triplinervia* (Spreng.) M. Arg., *Ficus microcarpa* L.f. e *Quercus robur* L., submersas em um riacho na Reserva Biológica do alto da Serra de Paranapiacaba, Santo André, SP, Brasil, de abril a novembro de 1990.

Zoosporângio de Chytridiomycetes e o aparelho conidial (conidióforo mais conídiosporos) de Hyphomycetes aquáticos, respectivamente, foram usados como unidades de propagação e quantificado da seguinte forma: unidade formadora de propágulos por milímetro quadrado (UFP/mm^2). A quantificação dessas estruturas foi efetuada em iscas (palha de milho, ecdise de cobra, celofane e exoesqueleto de camarão) de 1 cm^2 e em discos de folhas de 5mm de diâmetro incubados àtemperatura ambiente, em água destilada esterilizada. As folhas de *Alchornea triplinervia* (Spreng.) M. Arg. apresentaram o maior número de UFP/mm^2 (4799.59), seguido de *Ficus microcarpa* L.f. (4306.73) e *Quercus robur* L.(1800.79). O maior número de UFP/mm^2 de fungos zoospóricos foi obtido em folhas de *A. triplinervia*, enquanto os Hyphomycetes aquáticos foram mais abundantes nas folhas de *F. microcarpa*.

Os fungos zoospóricos parecem possuir maior especificidade quanto ao tipo de folha do que os Hyphomycetes, porque *Catenophlyctis variabilis* (Karling) Karling e *Polychytrium aggregatum* Ajello, foram os fungos zoospóricos mais abundantes no presente estudo, porém o último não foi isolado de folhas de *Q. robur*. A quantidade de PFU/mm^2 apresentou-se maior no início da decomposição das folhas, possivelmente devido à atividade pioneira da micota. Comparações entre os resultados obtidos neste estudo e os existentes na literatura são difíceis, em decorrência de diferenças metodológicas. A quantificação de fungos neste estudo indicou não somente a ocorrência, mas também o potencial de colonização dos fungos aquáticos em substratos submersos.

Palavras-chaves: quantificação de fungos, fungos zoospóricos, Hyphomycetes aquáticos, unidade formadora de propágulos, Brasil.

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AIRBORNE FUNGI ISOLATED FROM NATAL, STATE OF RIO GRANDE DO NORTE - BRAZIL*

Maria Tereza Barreto de Oliveira
Regina de Fátima dos Santos Braz
Maria Auxiliadora Guerra Ribeiro

SUMMARY

The airborne fungi of Natal, State of Rio Grande do Norte, Brazil, were studied during a one year period. One hundred and twenty Petri dishes with culture medium were exposed at five different locations in the city. Of the thirty one fungi genera identified the most frequent were: *Aspergillus* (78%), *Penicillium* (60%), *Fusarium* (42%), *Cladosporium* (21%), *Curvularia* (19%), *Rhizopus* (17%) and *Rhodotorula* (13%). These genera appeared in all collection sites and the frequency of occurrence of *Neurospora* and *Drechslera* was higher in Natal than in other Brazilian cities. The genus *Cladosporium* was more frequent in the summer. There was no difference in the number of fungi genera, number of sporulating colonies and number of non-sporulating colonies btween the five collection sites. The data suggest a similar level of pollution among Natal's suburbs. Detected air pollution level was low, apparently because Natal is not industrialized.

Key Words: Natal, airborne, fungi, pollution.

INTRODUCTION

The interest in airborne fungi flora appeared with Blackley's investigation (5) on the influence of fungi in respiratory allergies. Ancona apud Costa (7) observed that fungal spores were the cause of an asthma epidemic in Italy.

Data on fungi flora have been collected in different countries (1,2,4,8,11,16,23,25,26,27,31). In Brazil, data on airborne fungi are available for major cities such as Presidente Prudente (6), Belém (7), Belo Horizonte (10), Sao Paulo (12,19), Porto Alegre (17), Baixada Santista (19,29), Rio de Janeiro (20), Recife (21), Piracicaba (28) and Curitiba (15).

Even though abiotic factors such as wind velocity, barometric pressure, relative humidity, solar radiaton, temperature and rainfall have been related to the frequency of the anemophilous fungal flora (9,13,14), this relationship appears to be unclear. Knowledge about anemophilous fungal flora, as well as other aspects of the ecosystem in the various regions of the country, becomes fundamental for the relation between the fungal flora and allergic processes to be understood (7,10,24). The relative frequency of fungi in the air also appear to be an important indicator of the presence of environmental pollution (29,33).

Althoug certain genera of fungi are common among most cities, those that are specific to cities or regions may be important in terms of epidemiology and therapeutics (21). For the diagnosis of allergies and hyposensitizing treatments it is nec-

* Depto. de Microbiologia e Parasitologia - Centro de Biociências-UFRN - Campus Universitário - Lagoa Nova - 59072-970, Natal - RN, Brazil.

essary to know the anemophilous mycotic flora and the antigenic differences between them. The objective of this study was to determine the anemophilous flora of the city of Natal, adding information to the current knowledge of fungal distribution in the country and abroad.

MATERIALS AND METHODS

The city of Natal is located on the East coast of the State of Rio Grande do Norte, Brazil, at latitude 5°45'42" South and longitude 35°12'04" West. According to Koppens' classification, Natal's climate is tropical humid, with an average annual temperature between 26° C and 28° C. Typically there are two seasons: rainy and dry.

The rainy season extends from March through August, with slightly lower temperatures. During the rainy season of the year of the study (1988), the following averages were recorded: precipitation, 1952 milimeters; barometric pressure, 1007.8 milibars; relative humidity, 80.6 percent; wind velocity, 3.9 meters/second and temperature, 26.6°C. During the dry season, which extends from September to February, the following averages were recorded: rainfall, 366.6 milimeters; barometric pressure, 1007.5 milibars; relative humidity, 76.2 percent; wind velocity, 4.9 meters/second and temperature 27.6°C.

Five locations of the city were selected for data collection: Ponta Negra (South), Cidade Esperança (West), Tirol (East), Santos Reis (North) and Lagoa Sêca (downtown). Every two weeks during the year of 1988, spores were collected using Petri dishes containing a medium of agar Sabouraud. With the exception of rainy days, these were exposed to the air for 15 minutes, at 1.2 mers above the ground. A total of 120 dishes were exposed.

After four days at ambient temperature colonies were isolated in the same medium for the observation of formation of macrocolonies. Micromorphology was observed after microcultivation, following Riddel (32) and the fungal strains were identified using the classifications of Barnett & Hunter (3), Kreger-van Rij (18) and McGinnis et al (22). Wirtz staining was used for the identification of asci. Biochemical tests, such as urease enzyme research and saccharose fermentation, were also used for the identification of different genera.

Non-sporulating fungi were transfered to agar-potatoes medium and kept at room temperature, under white light, bween 7 to 10 days, to sporulate.

RESULTS

The 120 dishes exposed yielded 1190 colonies of 30 genera. Non-sporulating strains were grouped in the genus *Mycelia sterilia*. Table 1 shows the relative frequency of each fungal genera in the city of Natal and the five collection sites. The totals for Natal represent the sum of all five locations. Seven genera, isolated in all five locations, showed a relative frequency greater than 10%: *Aspergillus* (65%); *Penicillium* (50%); and *Fusarium* (20.8%), *Clodosporium* (17.5%); *Curvularia* (15,8%); *Rhizopus* (14.1%); *Rhodotorula* (10.8%). *Verticillium, Phoma, Nectria, Nigrospora*, and *Brachysporium* were only solated in Ponta Negra; *Geotrichum. Alternaria* and *Stemphylium* in Santos Reis; *Cephalosporium, Seimatosporium* and *Bipolaris* in Cidade da Esperanca; *Pestalotia* in Tirol and *Mucor* in Lagoa Seca. The genera only isolated in a single location were also present in one or two collections.

The most frequent genera, *Aspergillus*, *Penicillium* and *M.sterilia*, were found during all monts of the year (Table 2). *Cladosporium* were collected from July to December; *Fusarium* from March to November, and *Neurospora* from August to November. The remaining genera were randomly present throughout the year.

Table 2 also shows the seasonal distribuition of each genera. Among the genera with a relative frequency greater than 10% *Aspergillus*; *Penicillium*; *Cladosporium*; *Rhodotorula* and *Neurospora* were more frequent during the dry season. *Fusarium*; *Curvularia*; *Rhizopus* and *Drechslera* were more frequent during the rainy season.

Table 3 shows the number of genera and the number of sporulating and non-sporulating colonies in the five collecion sites. The comparison between fungi collected in Natal with the information available for other cities, indicates that only *Neurospora* and *Drechslera* were not predominant in the majority of cities where similar studies have been conducted (Table 4).

DISCUSSION

Most of the data on fungi collected in Brazil originate from the South and Southeastern regions of the country, where the climatic conditions differ greatly from the Northeast. Because of the climatic differences it becomes difficult to make seasonal comparison of the fungi genera common to different

TABLE 1 - Relative frequency of the airborne fungi genera isolated in the city of Natal and in the five collection sites within it.

Genera	Relative frequency					
	Natal	Ponta Negra	Santos Reis	Cidade da Esperança	Tirol	Lagoa Seca
Aspergillus	65.0	50.0	62	62	66	83
Penicillium	50.0	50.0	29	54	54	62
Fusarium	20.8	20.8	8	25	37	12
Cladosporium	17.5	20.8	4	20.8	16.6	25
Curvularia	15.8	4	20.8	25	12	16
Rhizopus	14.1	8	20.8	12	8	20.8
Rhodotorula	10.8	12	16	4	8	12
Neurospora	7.5	16	12	8	-	-
Drechslera	6.6	8	4	16	16	-
Aureobasidium	5.0	–	4	4	4	12
Trichoderma	3.3	–	8	4	-	-
Cunninghamela	2.5	8	-	-	-	4
Periconia	2.5	4	-	-	-	-
Syncephalastrum	2.5	4	4	-	-	4
Absidia	1.6	–	-	4	4	-
Paecilomyces	1.6	–	-	4	4	4
Mucor	1.6	–	-	-	-	8
Pestalotia	1.6	–	-	-	-	-
Cephalosporium	0.8	–	-	4	4	-
Verticillum	0.8	4	-	-	-	-
Seimatosporium	0.8	–	-	4	4	-
Geotrichum	0.8	–	4	-	-	-
Phoma	0.8	4	-	-	-	-
Nectria	0.8	4	-	-	-	-
Nigrospora	0.8	4	-	-	-	-
Alternaria	0.8	–	4	-	-	-
Stemphylium	0.8	–	4	-	-	-
Brachysporium	0.8	4	-	-	-	-
Bipolaris	0.8	-	-	4	4	-
Mycelia sterilia	43.3	29	41	45	62	37

regions. Purchio al. (29), for example, observed a predominance of *Cladosporium* in the Baixada Santista area during the cold months. In Natal this genus was most common during the dry season. (Table 2). It is likely that the presence of this genus is associated with low rainfall, common to the dry Northeastern season and the Southeastern winter.

Nevertheless, the genera most frequently found in Natal were the same identified in other cities of Brazil: *Aspergillus; Penicillium; Fusarium; Curvularia: Rhizopus; Rhodotorula and Aureobasidium* (Table 4). Recife and Natal are close and climatically identical cities. Interestingly, *Rhizopus* and *Rhodotorula* were more frequent in Natal while *Phoma* was more frequent in Recife (21).

No significant differences were observed among the five collection sites in the number of fungi colonies sporulating and those not sporulating (Table 3). These results differ from those found by Purchio el al. (29) in the Baixada Santista. They found an increased rate in the number of colonies and the relative frequency of non-sporulating fungi, in the air (29) and relative frequency of non-sporulating fungi, in the air (29) and sea (30), suggesting that the existance of chemical pollutants inhibit the sporulation. Depending on the standardization of the techiques, the index of non-sporulating mold could be used as a measure of pollution, in the air and sea.

Therefore, the absence of a significant differences in the number of colonies of non-sporulating fungi among the five collection sites in Natal, suggests a low and homogeneous level of air pollution. This is probably linked to the small number of industries in the city and the fact those that do exist are of the non-polluting types.

RESUMO

Fungos anemófilos isolados de Natal, Estado do Rio Grande do Norte - Brasil

A flora fúngica anemófila foi estudada em Natal, Estado do Rio Grande do Norte, Brasil, durante

TABLE 2 -Seasonal and monthly relative frequency of airbone fungi isolated in Natal between January and December of 1988.

Genera	Months												Dry season	Rainy season
	Jan	Fev	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec		
Aspergillus	77	100	90	50	40	46	40	70	70	80	80	70	77.7	55.3
Penicillium	44	60	40	30	30	53	40	40	50	90	80	50	62.9	40.0
Fusarium	-	-	20	10	10	13	40	60	20	60	10	-	16.6	44.6
Cladosporium	-	-	-	-	-	-	40	30	40	40	40	20	25.9	10.7
Curvularia	22	-	30	40	20	20	-	10	-	-	20	20	11.1	20.0
Rhizopus	11	-	-	-	30	20	-	50	30	20	-	-	11.1	16.9
Rhodotorula	-	-	-	-	-	13	20	20	-	-	50	20	12.9	9.2
Neurospora	-	-	-	-	-	-	-	20	40	20	10	-	12.9	3.0
Drechslera	-	-	-	20	20	6.6	-	20	-	10	-	-	1.8	10.7
Aureobasidium	-	-	-	20	10	-	-	-	10	-	20	-	5.5	4.0
Trichoderma	-	-	-	-	-	-	30	10	-	-	-	-	-	6.1
Cunninghamela	-	20	-	-	-	13	-	-	-	-	-	-	1.8	3.0
Periconia	-	-	20	10	-	-	-	-	-	-	-	-	-	4.6
Syncephalastrum	-	-	-	10	-	6.6	-	-	-	10	-	-	1.8	3.0
Absidia	-	20	10	-	-	-	-	-	-	-	-	-	3.7	1.5
Paecilomyces	-	-	-	-	-	-	-	10	-	10	-	-	1.8	1.5
Mucor	-	20	-	-	10	-	-	-	-	-	-	-	1.8	1.5
Pestalotia	-	-	-	-	-	-	10	-	-	-	-	10	1.8	1.5
Cephalosporium	-	-	-	-	-	-	-	-	-	10	-	-	1.8	-
Verticillum	-	-	-	-	-	-	-	-	-	-	10	-	1.8	-
Seimatosporium	-	-	-	-	-	-	10	-	-	-	-	-	-	1.5
Geotrichum	-	-	-	-	-	-	-	-	-	-	10	-	1.8	-
Phoma	-	-	-	10	-	-	-	-	-	-	-	-	-	1.5
Nectria	-	-	-	-	-	-	-	-	-	-	10	-	1.8	-
Nigrospora	-	-	-	-	-	-	-	-	-	-	-	10	1.8	-
Alternaria	-	-	-	-	10	-	-	-	-	-	-	-	-	1.5
Stemphylium	-	-	-	-	10	-	-	-	-	-	-	-	-	1.5
Brachysporium	-	-	-	-	-	6.6	-	-	-	-	-	-	-	1.5
Bipolaris	-	-	-	-	10	-	-	-	-	-	-	-	-	1.5
Mycelia sterilia	55	40	40	30	60	46	50	40	30	40	40	50	38.8	44.6

TABLE 3 - Number of genera and colonies of sporulating and non-sporulating fungi in the five collection sites within Natal.

Sites of collection	Number of genera	Number of colonies of sporulating fungi	NSF*
Ponta Negra	18	159	26
Santos Reis	16	223	16
Cidade da Esperança	17	266	35
Tirol	14	163	40
Lagoa Seca	13	239	23

*NSF: Number of non sporulating fungi colonies.

um ano, em cinco pontos da cidade, através da exposição de 120 placas de Petri, contendo o meio agar-Sabouraud. Foram isolados 31 gêneros. Destes, os mais freqüentes foram: *Aspergillus* (78%); *Penicillium* (60%); *Fusarium* (42%); *Cladosporium* (21%); *Curvularia* (19%); *Rhizopus* (17%) e *Rhodotorula* (13%). Estes gêneros apareceram em todos os locais de coleta. *Neurospora* e *Drechslera* foram isolados em Natal com freqüência relativa superior às de outras cidades brasileiras. O gênero *Cladosporium* foi mais freqüente no verão. Não foi observada diferença significativa entre o número de gêneros, número de colônias de fungos esporulados e não esporulados, nos cinco locais de coleta. Como tem sido sugerido por outros autores, estes dados indicariam uma poluição homogênea entre os bairros de Natal e seria de baixo nivel, pois Natal não e uma cidade industrializada.

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TABLE 4 - Prevalent fungi isolated in some Brazilian cities. (*)

Genera	Cities (*)										
	NA	RE	BE	RJ	BH	SP	PP	BS	PI	CR	PA
Aspergillus	65.0	58.8	63.8	10.0	58.6	23.3	55.8	23.7	43.7	43.4	46.6
Penicillium	50.0	41.4	36.6	20.0	64.7	41.7	17.9	51.1	62.3	43.1	-
Fusarium	20.8	19.6	14.5	-	24.7	14.0	26.9	17.4	-	-	-
Cladosporium	17.5	20.8	17.5	15.0	90.3	64.8	74.3	48.8	50.0	32.9	-
Curvularia	15.8	18.9	68.3	-	33.3	-	10.2	10.5	-	-	-
Rhizopus	14.1	-	-	-	-	-	26.9	10.1	18.1	10.5	-
Rhodotorula	10.8	-	-	10.0	28.0	48.9	22.4	-	12.5	14.5	-
Neurospora	7.5	-	-	-	-	-	35.9	-	-	-	-
Drechslera	6.6	-	-	-	-	-	-	-	-	-	-
Aureobasidium	5.0	10.8	-	-	31.9	19.9	37.2	15.5	-	-	-

* NA = Natal (present study); RE = Recife (21); BE = Belém (7); RJ = Rio de Janeiro (20); BH = Belo Horizonte (10); SP = São Paulo (12); PP = Presidente Prudente (6); BS = Baixada Santista (29); PI = Piracicaba (28); CR = Curitiba (15) e PA = Porto Alegre (17).

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AEROMONAS SPECIES ISOLATED FROM HUMAN URINE: BIOLOGICAL CHARACTERIZATION AND ANTIBIOTIC SUSCEPTIBILITY

Angela Corrêa de Freitas[1]
Marly Paiva Nunes[1]
Ilvan Delgado Ricciardi[1]

SHORT COMMUNICATION

SUMMARY

Two strains of *Aeromonas hydrophila* and four strains of *A. caviae* were isolated from five hundred urine samples of human origin. One strain of *A. hydrophila* was associated with urinary infection and presented well defined virulence factors in contrast with the other five isolates.

All the strains were susceptible to aminoglycosides, nalidix acid, chloramphenicol, nitrofurantoin, colistin and polymyxin B.

Key Words: *Aeromonas* , urine, biological characterization.

Aeromonas has been isolated with increasing frequency from clinical sources. A variety of clinical syndromes are well documented in man including gastroenteritis, wounds, meningitis, endocarditis and urinary infections (Altweeg, M. & Geiss, H.K. Crit. Rev. Microbiol, *16:* 253-286, 1989).

This organism could also be isolated from the environment, food and animal (Krieg, N.R. & Holt, J.G. (eds.) Bergey's Manual of Systematic Bacteriology, 1984). The production of enterotoxin as well as cytotoxic proteins, hemolysin and extracellular enzymes are some of the virulence factors associated with the bacterial pathogenic potential (Cahill, M.M.; J. Appl. Bacteriol, *69:* 1-16, 1990). Susceptibility to antimicrobial agents is variable but these organisms are usually susceptible to chloramphenicol, tetracycline, aminoglycosides and trimethoprim-sulphamethoxazole and resistant to penicillins, erythromycin and cephalosporin. The aim of this study was to investigate the presence of *Aeromonas* in human urine and to study some virulence factors and antibiotics susceptibility in the isolates.

Five hundred urine samples from adult patients of a General Hospital in Rio de Janeiro city, Brazil, were examined for the presence of *Aeromonas* spp. The samples of urine were transported and analysed immediately at the laboratory. Urine samples were plated on Deoxycholate citrate agar-BBL (Neves, M.S. & Cols. Rev. Bras. Pat. Clin., *25:* 56-59, 1989). After 24 h of incubation at 28°C, colorless colonies (lactose negative) and redish colonies (lactose positive) were inspected for oxidase test, fermentation of glucose, motility and sensitivity to the vibriostatic agent (2,4-diamino-6,7-diisopropyl pteridine) in 150 µg disks. Isolates were then classified to the species level, using the biochemical tests proposed by Hickman-Brenner, F.W. & Cols. (J. Clin. Microbiol; *25:* 900-906, 1987) and Popoff and Véron (J. Gen. Microbiol, *94*: 11-22, 1976).

Hemolysin activity was determined by the demonstration of zones of ß-hemolysis around col-

1. Laboratório de Zoonoses - Instituto de Microbiologia da UFRJ - CCS - Bloco I, Rio de Janeiro 21941 - RJ - Brasil.

onies on sheep blood agar containing 5% (v/v) erythrocytes. The strains were also tested for the production of hemolysin by Hugh's method (Pacini. Int. Bull. Bacteriol. Nomencl. Taxon, *15*:13-24, 1965). Protease, lipase and lecithinase activities were detected by the plate assay method described for *Pseudomonas aeruginosa* protease (Janda, J.M. & Bottone, E.J., J. Clin. Microbiol. *14*:55-60, 1981). Staphylolytic activity was assayed by the method of Satta, G. & Cols. (Infect. Immun., *16*: 37-42,1977) and the autoagglutination test for self-pelleting and precipitation after boiling was determined as described by Janda,J .M & Cols. (Infect Immun., *55*: 3070-3077, 1987). The suckling mouse test system (Dean, A.G. & Cols., J. Infect. Dis, *125*: 407-411, 1972) was used for the detection of enterotoxin. Cytotoxin production was performed in Vero cells according to the description of Donta and Shaffer (J. Infect. Dis., *141*: 219-222,1980). Antibiotic susceptibility tests were performed by the agar-disk diffusion method.

It was possible to isolate and identificate 2 strains of *Aeromonas hydrophila* and 4 strains of *A. caviae* from six adult women representing an isolation rate of 1.2%. One patient (51 years old, presenting lombar pain) had a high total count ($1.5x10^7$ CFU/ml). *A. hydrophila* ($2.0x10^5$ CFU/ml) was isolated in mixed culture with *Escherichia coli* ($148x10^5$ CFU/ml). The other five patients presenting *Aeromonas* species in the urine had not urinary infection ($<10^5$ CFU/ml).

The isolation of *Aeromonas* spp. from urinary infection is an unusual occurrence. Few reports in the literature have documented the presence of this microorganism in urine. Mc Cracken and Barkley (J. Clin. Path., *25*: 970-975, 1972) reported *Aeromonas* infection of the urinary tract in two patient from whom the organism was isolated in pure culture with significant counts. One patient (72 years old) was catheterized and probably that was the source of infection. In the second case (a five month-old child) the source was not apparent, although the use of ampicillin may have favoured the selection of *Aeromonas* as the causing agent. Urinary infection by *Aeromonas* associated with mixed culture was found by Freij (Experientia, *43*: 359-360, 1987) and Graevenitz & Mensch (New Engl. J. Med., *278*: 245-249, 1964). Another cases showed a previous urinary pathology, particularly chronic infection of the urinary tract (Freij, B. Pediatr. Infect. Dis., *3*: 164-175, 1984), nephorostomy for obstructive uropathy (Freij, B. Experientia, *43*: 359-360, 1987) and nasocomial infection (Washington,J. Ann. Intern. Med. *76*: 611-614, 1972). Batolomé,R.M & Cols. (Infection, *17*:172-173, 1989) reported the isolation of A. *hydrophila* in pure culture in a newborn with bladder and bilateral renal dilation, suggestive of urethral valve involvement. The stool culture was also positive for this organism but the ethiology of the gastroenteric process was not clear. The immaturity of the immune system and the urological pathology of the patient may have been the cause of the urinary infection.

Results regarding the presence of possible virulence factors in the six strains of *Aeromonas* spp. are listed in Table 1. The strain of *A. hydrophila*

TABLE 1 - Production of virulence factors by Aeromonas hydrophila and A. caviae isolated from urine specimens.

Species	Virulence factors							
	Cytotoxin	Heat-stable enterotoxin	Hemolysin	Autoagglutination	Staphylolysin	Protease	Lecithinase	Lypase
A. hydrophila (patient 1)	+	-	+	-	+	+	+	+
A. hydrophila (patient 2)	-	-	+	-	+	-	-	-
A. caviae (patient 3)	-	-	-	-	-	+	-	-
A. caviae (patient 4)	-	-	-	-	-	-	-	-
A. caviae (patient 5)	-	-	-	-	-	-	-	-
A. caviae (patient 6)	-	-	-	-	-	+	+	+

TABLE 2 - Susceptibility profiles of *Aeromonas* species isolated from urine specimens.

Species	Antimicrobials																	
	CO	NA	NT	NOR	TT	SFT	GN	AMI	KN	TOB	AP	AMO	CR	CL	PL	CFE	CF	CT
A. hydrophila (patient 1)	S	S	S	R	S	S	S	S	S	S	R	R	R	S	S	R	R	S
A. hydrophila (patient 2)	S	S	S	S	S	R	S	S	S	S	R	R	S	S	S	R	R	R
A. caviae (patient 3)	S	S	S	S	S	S	S	S	S	S	R	R	R	S	S	R	R	S
A. caviae (patient 4)	S	S	S	S	R	S	S	S	S	S	R	R	R	S	S	R	R	S
A. caviae (patient 5)	S	S	S	S	S	S	S	S	S	S	R	R	R	S	S	R	S	S
A. caviae (patient 6)	S	S	S	S	S	R	S	S	S	S	R	R	R	S	S	R	R	S

S - susceptible strain
R - resistent strain
CR - Carbenicillin (100 μg)
TT - Tetracycline (30 μg)
CL - Colistin (10 μg)
SFT - Sulfazothrim (25 μg)
PL - Polymyxin B (300 U)
CO - Chloramphenicol (30 μg)
GN - Gentamicin (10 μg)
CFE - Cephalexin (30 μg)
AMI - Amikacin (30 μg)
CF - Cephalothin (30 μg)
KN - Kanamycin (30 μg)
CT - Cefoxitin (30 μg)
NOR - Norfloxacin (10 μg)
TOB - Tobramycin (10 μg)
NA - Nalidixic acid (30 μg)
AP - Ampicillin (10 μg)
NT - Nitrofurantoin (300 μg)
AMO - Amoxicillin (10 μg)

isolated from patient 1 presenting urinary infection produced complete hemolysis on sheep blood, was cytotoxic to Vero cells and positive for the production of extracellular enzymes. The other five strains of *Aeromonas* presented negative results for the assays of enterotoxin, cytotoxin, autoagglutination and variable results on production of extracellular enzymes.

Most of the considered virulence factor produced by *Aeromonas* species have been associated with possible mechanisms of the pathogenicity for human or animal infections caused by aeromonads (Altwegg, M. & Geiss, H.K. Crit. Rev. Microbiol. *16*: 253-286, 1989).

Enterotoxin, cytotoxin and hemolysin have been studied to elucidate the mechanism by which *Aeromonas* species produce intestinal infection (Janda, J. M. Clin. Microbiol. Rev., *4:* 397-410, 1991). *A. hydrophila* and *A. sobria* are accepted as enteric pathogens that are capable of causing gastroenteritis, in addition to a variety of extraintestinal infections (Altwegg, M. & Geiss, H.K. Crit. Rev. Microbiol., *16*: 253-256, 1989). *A. caviae* produces diarrhea in children (Moyer, N. P. J. Clin. Microbiol., *25*: 2044-2048, 1987) and adult patients (George, N.L. & Cols. J. Clin. Microbiol., *23*: 1026-1029, 1986) but it is considered less virulent than *A. hydrophila* or *A. sobria*.

In our study only one strain of *A. hydrophila* caused urinary infection. The strain produced most of the virulent factors investigated.

The results of antibiotic sensitivity tests are summarized in Table 2. All the strains were resistant to ampicillin and consistently susceptible to chloramphenicol, nalidixic acid, nitrofurantoin, polymyxin B, colistin and aminoglycosides (amikacin, gentamicin, kanamycin and tobramycin) which confirm the findings of these authors (Fainstein, V. & Cols. Antimicrob. Agents Chemother., *22*: 513-514, 1982; Kuijper, E.J. & Cols. Eur. J. Clin. Microbiol. Infect. Dis., *8*: 248-250, 1989). Cephalosporinas (cephalexin, cephalotin and cefoxitin) showed variable results. The six strains were resistant to cephalexin, and one strain (patient 5) was susceptible to cephalotin. Cefoxitin was the most active antibiotic among the cephalosporins.

This report presents the isolation of *Aeromonas* species from uroculture of six patients, one of which had quantitative counts above 10^5 CFU/ml. The strain isolated from this patient showed the presence of some virulence markers. Our results and other listed above suggest that aeromonads should be considered among Gram-negatives involved in urinary infection. The extent of such participation deserves further investigations.

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RESUMO

Espécies de *Aeromonas* isoladas de urina humana, caracterização biológica e susceptibilidade antimicrobiana

Duas amostras de *Aeromonas hydrophila* e 4 de *Aeromonas caviae* foram isoladas de 500 amostras de urina de origem humana. Uma amostra de *A. hydrophila* estava associada a infecção urinária e apresentou fatores de virulência bem definidos, em contraste com as outras isoladas.

Todas as amostras foram sensíveis aos aminoglicosídeos, ácido nalidíxico, cloranfenicol, nitrofurantoína, colistina e polimixina B.

Palavras chaves: *Aeromonas*, urina, caracterização biológica.

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SURVIVAL OF FUNGI PRESERVED BY LYOPHILIZATION AFTER 49 YEARS

I. H. Schoenlein-Crusius
L. K. Okino
C. M. M. Lucon

SUMMARY

The recovery of seventy five filamentous fungal strains (Deuteromycotina), Ascomycotina and Zygomycotina) and ten yeast cultures which have been preserved for 49 years in a lyophilized state was examinated. The cultures donated to the Instituto de Botânica in São Paulo, in 1980, were processed in, Northern Regional Research Laboratories (NRRL) in Peoria. Illinois, in the United States in 1943 by Dr. E. Duprat under the direction of Dr. K. Raper, Dr. D. Fennel and Dr. S. Wicherham. For the revival of the cultures, the lyophil tubes were broken, the contents dissolved in distilled sterilized water, plated in potato dextrose agar petri dishes and incubated during one week at 20 C. Morphological and taxonomical features were observed for every culture examinating colonies growth and microscopical structures to check the identification. From the total 85 lyophilized isolates, 49 (55%), divided in 43 filamentous fungal strains and six yeast cultures were recovered by this procedure. The results were compared and discussed in terms of longevity and viabilit of the lyophil process for fungal preservation.

Key Words: preservation, lyophilization, longevity.

INTRODUCTION

Lyophilization has been one of the most employed method to preserve fungal cultures, and maintaining the physiological and morphological original characteristics (6,7). The efficiency of the preservation of fungal cultures by lyophyl process depends on many factors such as the type of equipment used, freezing temperature, moisture and processing time (6). Experiments concerned the influence of growth conditions, cooling rate and suspending media on the viability of yeast cells revealed that an appropriate combination of cultivation conditions, cryoprotectant type and coolings rates may result in higher levels of cell recovery (18). The recovery rates of lyophil cultures seems to be specific for each taxon, as in the case of *Paracoccidioides brasiliensis*, for which a special preservation method is recommended to maintain its pathological characteristics (8).

A preservation method may be selected according to the laboratory facilities, equipments, fungal longevity and genetic stability of the strains (15).

Raper & Alexander (13) tested the viability of many strains processed by a three pieces lyophil apparatus method (20).

Viability tests were conducted with fungi of industrial importance, cultures with difficulty to maintain by conventional methods of periodic recultivation on agar slants and cultures characterized by particularly different morphological and cultural features. It was concluded that "the lyophil techniques provides a convenient means of preserving a

Instituto de Botânica, Seção de Micologia e Linquenologia, Caixa Postal 4005, 01061-970 São Paulo, Brasil.

large number of replicate cultures, which can be used as seed material for standard cultures or to set series of fermentations over an extended period of time".

Seventy five filamentous fungal strains and ten yeast cultures were donated by Dr. Enrique Duprat to the Culture Collection of the "Instituto de Botânica" in October of 1980. The fungi were lyophilized in 1943 in the Northern Regional Research Laboratory in Peoria, Illinois, USA during his training period under the direction of Dr. Kenneth Raper, Dr. Dorothy Fennel and Dr. S. Wicherham.

The aim of this paper is to report data about the recovery of the lyophilized strains and discuss about the longevity of lyophil cultures.

MATERIAL AND METHODS

The lyophil method employed to preserve the donated fungi was described by Raper & Alexander (13) for the preservation of many isolates of the Culture Collection of the Northern Regional Research Laboratories (NRRL) in Illinois. They processed the cultures in a three pieces lyophil apparatus. Cultures were grow n on Czapek or malt agar media during one week or until a sufficient quantity of conidia was produced. About 0.25 cc of esterile beef serum was placed in an agglutination tube and spores or conidia were added from the culture until the solution was dense. Approximately 0.05 cc of this suspension was introduced in sterile lyophil tubes, that were attached to the manifold inserting them in the rubber sleeves. The end of the tubes were submerged in a bath of dry ice and methyl cellosolve at a temperature of -40 to -50 C. As the samples were frozen, air was retained by a vacuum pump and the tubes were sealed.

The 85 liophyzed cultures used in this study were dissolved in distilled sterilized water and plated directly on potato-dextrose-agar petri dishes. The fungi were incubated at 22° C during one week. Colonies growth and microscopical structures were observed to check the original identifications. Current literature was consulted for Deuteromycotina (2,3), especially for *Aspergillus* (14) and *Penicillium* (12), Zygomycotina (17) and Ascomycotina (4). The names of the yeasts were also confirmed (9,10). The recovered strains will be lyophilized again and will also be maintained in distilled sterilized water (Castellani's Method).

RESULT AND DISCUSSION

Table 1 shows the original name, the corresponding NRRL Culture Collection number, isolation source and recovery of the fungi. In 1945, twenty nine isolates of the same strains tested here, were evaluated for their viability (13), being interesting to compare the results. Unfortunately it was not possible to conduct cell viability tests with the donated fungi. A tested fungi was considered to be recovered if its colonies were able to grow on the culture media. From the 89 lyophylized isolates, 49 (55%) of them were recovered. The morphological and taxonomical features of the recovered fungi were compatible with those mentioned in the literature.

From the 49 Deuteromycotina strains, 21 were *Aspergillus* species, from which fourteen isolates were recovered. Except by *A. flaviceps* (NRRL 286), which colonies did not grow, the data are the same as mentioned by Raper & Alexander (13) for the viability tests of 1-2 years old cultures of *Aspergillus repens* (NRRL 17), *A. fumigatus* (NRRL 163), *A. nidulans* (NRRL 187), *A. versicolor* (NRRL 226), *A. sydowi* (NRRL 247) *A. terreus* (NRRL 255) and *A. flaviceps* (NRRL 286) with excellent results. The viability of *A. echinulatus* (NRRL 131) and *A. itaconicus* (NRRL 161) was also very low as mentioned by Raper & Alexander (13).

Six of the ten *Penicillium* species were now recovered. The results are according to those mentioned by Raper & Alexander (13), with excellent recovering rates for *Penicillium duclauxii* (NRRL 1030), *P. expansum* (NRRL 976), *P. purpurogenum* (NRRL 1061), *P. stoloniferum* (NRRL 859), (= *P. brevicompactum*) and low viability for *P. vinaceum* (NRRL 739). The last culture was tested in the past because of its particularly different morphological features. The gluconic acid and penicilin producer *P. chrysogenum* (NRRL 811), that showed a high recovering rate in 1945 could not be isolated in the present.

Verticillium albo-atrum (NRRL 1204) showed high recovering, according with the results mentioned before (13). *Fusarium moniliforme* (NRRL 1675) and *Helminthosporium* sp (NRRL 1680) showed a low recovering rate after a period of one year of lyophilization (13) and did not show any growth now. In a same way, *Cladosporium fulvum* (NRRL 1671) and *Trichothecium roseum* (NRRL 1588) could not be recovered after 49 years of preservation. Concerning the Zygomycotina, within the nine *Mucor* species, five strains were recovered at this opportunity. Raper & Alexander (13) tested *Mucor rouxianus* (NRRL 1429) and *Blackeslea trispora* (NRRL 1718) as cultures difficult to

TABLE 1 - List of the names, corresponding NRRL Culture Collection numbers, growth responses to media plating (+ positive; - negative; +/- fairly growth), isolation sources of the donated fungi; comparison of the growth responses reported by Raper & Alexander (13).

FUNGI	NRRL number	growth	sources	Raper & Alexander, 1945
Alternaria sp.	1284	+	-	
Aspergillus amstelodami Mangin	90	+	-	
A. candidus Link	311	+	-	
A. echinulatus = *echinulatum* Delacr.	31	-	-	-
A. effusus = *A. oryzae* Ahlburg	506	+	milk	
A. flaviceps Bain & Sart.	286	-	-	+
A. flavus Link	482	-	-	
A. fumigatus Fres	163	+	lug	+
A. itaconicus Kinoshita	161	-	-	
A. nidulans Eidam (Winter)	187	+	-	+
A. ochraceus = *A. allutaceus*	398	+	-	
A. oryzae Ahlburg	447	+	-	
A. oryzae Ahlburg	1808	-	-	
A. repens (de Bary) Fischer	17	+	-	+
A. restrictus G. Smith	147	-	-	
A. sulphures = *A. fresenii*	- - - -	+	-	
A. sydowi (Bain. & Sart.)	247	+	-	+
A. tamarii Kita	429	+	soya sauce	
A. terreus Thom apud Thom & Church	255	+	-	+
A. ustus (Bain.) Thom & Church	278	+	sugar	
A. versicolor (Vuill.) Tiraboschi	- - - -	+	soil	
A. versicolor (Vuill.) Tiraboschi	226	+	human mamma	+
A. wentii Wehmer	377	-	coconut	
Botrytis cinerea	1648	+	Camellia leaves	
Cephalosporium sp.	1866	-	cotton	
Cladosporium fulvum	1671	-	tomato	+/-
Fusarium moniliforme	1675	-	-	+/-
Helminthosporium sp.	1680	-	-	+/-
Hormodendrum sp. = *Cladosporium* sp.	1873	+	cotton	
Macrosporium sp. = *Alternaria* sp.	1683	-	air	
Metharhizium sp.	1874	-	cotton	
Paecilomyces variotii	1122	-	-	
Penicillium chrysogenum Thom	811	-	penicillin	+
P. citrinum Thom	1842	-	citrinine	
P. duclauxii Delacroix	1030	+	-	+
P. expansum = *Coremium alphitopus*	976	+	apple	+
P. luteum Zukal	- - - -	-	soil	
P. puberulum	845	+	-	
P. purpurogenum Stoll	1061	+	-	+
P. spinulosum = *P. purpurescens*	724	-	-	
P. stoloniferum Thom	859	+	soil	+
P. vinaceum	739	-	soil	-
P. wortmanni	- - - -	+	soil	
Scopulariopsis brevicaulis	1096	-	-	
Trichoderma lignorum	1762	+	soil	
Trichothecium roseum	1588	-	-	+
Stachybotrys atra	1877	-	-	
Stemphyllum sp. = *Monodictys nigrosperma*	1621	+	dung	
Verticillium albo-atrum	1204	+	-	+
Absidia sp.	1807	-	soil	
Absidia coerulea	1312	+	soil	
Absidia coerulea	1315	+	-	
Blakeslea trispora	1718	-	-	-
Circinella spinosa	1360	-	chestnut	
Cunninghamella echinulata	1384	-	soil	
Cunninghamella echinulata	1386	-	-	
Helycostylum sp.	1396	-	-	
Morkierella isabellina	1757	-	soil	

Continuação da **TABLE 1**

FUNGI	NRRL number	growth	sources	Raper & Alexander, 1945
Mucor sp.	1400	-	nut	
M. genevensis	1407	+	-	
M. mucedo	1424	+	-	+
M. mucedo	1425	+	-	+
M. racemosus	1427	-	-	
M. racemosus	1428	+	-	
M. ramannianus = *Mortierella ramanniana*	1559	-	-	+/-
M. rouxianus	1430	-	-	
Phycomyces blakesleeanus	1554	+	-	+
Rhizopus japonicus = *R. oryzae*	1895	-	-	
R. nigricans	1487	-	-	+
R. nigricans	1488	+	-	+
Syncephalastrum sp.	1487	+	chestnut	
Syncephalastrum sp.	1488	+	chestnut	
Thamnidium elegans = *Helycostylum* sp.	1613	+	-	
Byssochlamys fulva	1125	+	-	
Chaetomium globosum	1669	-	-	-
Monoascus purpureos	1596	+	-	+
Neurospora sp.	1669	+	-	
Candida albicans	477	+	faeces	
Endomycopsis fibuliger	25	-	-	
Hansenula anomala	11	-	-	
Mycoderma vini	33	-	-	
Rhodotorula mucilaginosa = *R. rubra*	843	+	-	
Saccharomyces carlsbergensis var. mandshuricus = *S. uvarum*	379	+	-	
Saccharomyces cerevisae	567	+	-	
Schizosaccharomyces octosporus	854	-	-	
S. versatilis = *S. japonicus*	1026	+	-	
Schwanniomyces occidentalis	10	+	-	

maintain by conventional methods of periodic recultivation on agar slants.

The recovering of those strains was no longer possible. The results were the same for *Mucor ramannianus* (NRRL 1559), now *Mortierella ramanniana*. However, *Phycomyces blakesleeanus* (NRRL 1554), considered a problematic fungi by Raper & Alexander (13) showed vigorous growth after 49 years of lyophylization. The Zygomycotina strains tested here showed a good revival after long preservation time, according with positive results mentioned by Fennell (7) for *Mucor* species.

Within the Ascomycotina, *Chaetomium globosum* (NRRL 1669) did not grow on the culture media, whereas *Monoascus purpureos* (NRRL 1596) showed a vigorous development.

Six of the ten yeasts were recovered by plating method. In a similar experiment, 55% of pathological yeasts, including *Saccharomyces cerevisae* and *Candida albicans* were recovered after 34 years of lyophil preservation (1). Although the lyophil process resulted in high cell losses, the viability level of the remaining *Saccharomyces* cells was stable during the storage period (19).

It is interesting to note, that *Aspergillus oryzae* (NRRL 447) survived the preservation time, whereas *A. oryzae* (NRRL 1808) fail to grow on the culture media. In a same way, *Rhizopus nigricans* (NRRL 1488) were completely recovered, whereas *R. nigricans* (NRRL 1487) was not able to grow on the media. *Helicostylum* sp.(NRRL 1396) died, while its synonym species, *Thamnidium elegans* (NRRL 1613) showed a vigorous development. It seems that the recovering of fungal cultures may be specific for each strain.

Cultures that showed a low recovering capacity in the past, after a short time preservation (13), were difficult to revitalizate at the present. To warrant the recovery of fungal cultures after a long time, it may be interesting to maintain replicates by alternative efficient conservation methods, such as mineral oil preservation (5,16), clay soil (16) and destilled water submergence (Castellani's Method), (5). The continuous improvement of the lyophil process stages, optimizing viability rates (11) and the combination with alternative methods may be the way maintain preserved fungal cultures for long periods of time.

RESUMO

Sobrevivência de fungos preservados por liofilização após 49 anos

A recuperação de setenta e cinco linhagens de fungos filamentosos (Deuteromycotina, Ascomycotina e Zygomycotina) e dez culturas de leveduras mantidas em estado liofilizado durante 49 anos foi examinada. Estas culturas foram doadas ao Instituto de Botânica em São Paulo, em 1980, tendo sido processadas no "Northern Regional Research Laboratories" (NRRL) em Peoria, Illinis nos Estados Unidos em 1943 pelo Dr. Enrique Duprat durante um período de treinamento sob a orientação dos Dr. K. Raper, Dr. D. Fennel e Dr. S. Wickerham. Para a revitalização das culturas, os tubos liofilizados foram rompidos, o conteúdo dissolvido em água destilada esterilizada, vertido sobre meio de batata-dextrose-agar e incubado durante uma semana a 20 C. Características morfológicas e taxonômicas foram observadas para cada cultura, examinando o crescimento das colônias e estruturas microscópicas para confirmar a identificação original. Dos 85 isolados liofilizados, 49 (55%), divididos em 43 linhagens de fungos filamentosos e seis de leveduras foram recuperadas. Os resultados foram comparados e discutidos em termos da longevidade e viabilidade do processo de liofilização para a preservação de fungos.

Palavras-chaves: preservação, liofilização, longevidade.

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DETECTION OF SALMONELLA TYPHIMURIUM IN A BROILER CHICKEN FLOCK

A. Berchieri Junior [1]
A.M. De Carvalho [2]
S.A. Fernandes [3]
A.M. Iba [1]

SHORT COMMUNICATION

SUMMARY

An outbreak of *Salmonella typhimurium* in a commercial broiler chicken flock is reported. The signs of the disease started on the 5th day-old. The symptoms, the gross alterations and the damage to the birds and to the farm are discussed.

Key Words: *Salmonella typhimurium*, avian salmonellosis, paratyphoid infection.

INTRODUCTION

Paratyphoid infections of poultry consist of avian disease caused by *Salmonella* organisms other than *Salmonella gallinarum* and *Salmonella pullorum*. Out of the 2,000 *Salmonella* serotypes described, S.typhimurium is the most frequent among those capable of infecting fowls. Young birds are more easily infected, excrete the bacteria for longer periods and present the disease in a more severe form, while birds older than 3-4 weeks are more resistant to the infection and to manifestation of the disease (1,3). Poultry is considered the main source of foodborne salmonellosis to human beings (2). This report describes the occurence of avian paratyphoid infection in a commercial broiler farm caused by *Salmonella typhimurium*.

Description of the case

The one-day-old chicks were housed in the farm according to the usual sanitary and hygienic technical procedures adopted throughout the regional poultry industry.

At 5 days of age, some birds were examined as their generic appearence did not correspond to age expectance. From 13 sacrified birds, 7 showed omphalitis. Bacteriological exams were made from macerated liver and bile, vitelline sac contents (yolk), and cecal contents. The bacteriological analysis consisted of inoculation of the material into tetrathionate-novobiocin broth (TN) incubated at 37C for 24 hours. Next, the TN culture was plated on brilliant green agar, incubated at 37°C/24 hours. The non-lactose fermenting bacteria colonies were transferred to IAL medium (Adolfo Lutz Institute) which was maintained at 37°C for 24 hours. The confirmation of the *Salmonella* genus was obtained through serological tests by slide agglutination using polyvalent antiserum against Salmonella somatic-antigen (0) and polyvalent serum against Salmonella flagellar-antigen (H). The serotyping was made by Adolfo Lutz Institute in São Paulo, Brazil. *Salmonella typhimuri-*

1. FCAVJ-UNESP. Rodovia Carlos Tonanni, Km 5. 14870.000 - Jaboticabal/SP.
2. J.O Labortorio, São José do Rio Pardo.
3. Instituto Adolfo Lutz, São Paulo-Capital.

um was isolated from macerated liver and bile, and yolk.

Although the birds were treated orally with ampicillin for 4 days, mortality continued to occur, reaching the level of 4.4% of the flock until the third week of age. At the 22nd day of age examined birds showed severe aerosaculitis, brownish liver with pinpoint necrotic foci on the surface, peritonitis, yolk retention and ascitis. Another bacteriological exam from macerated liver, heart and bile showed *Salmonella typhimurium*. Once again treatment was tried, this time with chloramphenicol during 4 days followed by furazolidone until the flock achieved market age. Even then, total mortality and stunting were up to 8.28%.

DISCUSSION

The presence of paratyphoid salmonellae in broiler chicken flocks, producing the disease with evidence of clinical signs and anatomic-pathological alterations has been most frequent in young birds, although the disease can occur in adult birds (3). Bacteriological findings showed the presence of *S. typhimurium*, having this serotype been historically considered the most frequent, concerning paratyphoid avian infections (1,3). With exception of the ocular lesions, alterations found are similar to those described (4), showing that lots affected by *S. typhimurium* present great risk to the farm concerning economic losses such as mortality, stunting, medication expenses and final lot performance. On the other side, there still will be the risk of contamination of personnel in contact with the birds and carcasses destined the human consumption.

There are few diagnosed cases of paratyphoid infections in commercial broilers. However, this does not necessarily means that the occurence is irrevelant. The poultry industry is based on fast solutions of possible accomplishments and measures are adopted in function of a quick answer considering mainly immediate economic aspects. Therefore it becomes more attractive and practical the adoption of generic measures without trying a confirmative diagnosis. These measures include indications of antibacterial drug administration to newly-hatched chicks or previous treatment relying only on clinical signs.

RESUMO

Detecção de *Salmonella typhimurium* em um lote de frangos de corte

Um surto de paratifo aviário, causado por *Salmonella typhimurium*, acometeu um lote de frangos de corte de uma granja comercial. Os sintomas começaram a aparecer a partir do 5º dia de vida das aves. As alterações sofridas culminaram com perdas, devido a mortalidade e refugagem, acima do esperado.

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ERRATUM

Proticine typing of *Proteus mirabilis* strains

Tavechio, A. T.; Vaz, T. M. I.; Buschinelli, S. S. O.; Fernandes, S. A.; Calzada, C. T. Irino, K.

Secção de Bacteriologia - Instituto Adolfo Lutz - Av. Dr. Arnaldo, 351 - 9º andar - São Paulo - CEP 01246.902

Volume 24, Nº 2, p. 91, Table 3. The table bellow substitute table 3 in the original paper.

TABLE 3 - Frequency of induced proticine production according to the source of *P. mirabilis* strains.

Source of strains	Number of strains	Number of strains with procticine production	
		Negative	Positive
Urine	90	17 (18.89%)	73 (81.11%)
C.S.F.*	15	4 (26.67%)	11 (73.33%)
Blood	8	3 (37.50%)	5 (62.50%)
Total	113	24 (21.23%)	89 (78.76%)

* Cerebrospinal fluid.

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