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SALMONELLA TYPHIMURIUM PLASMIDS SIMULTANEOUSLY CODING FOR TETRACYCLINE RESISTANCE, COLICIN PRODUCTION AND PATHOGENICITY *

Yeda Xênia Sant'Ana[1]
Geovanni Dantas Cassali[2]
Alfredo José Afonso Barbosa[3]
Tânia M.A.D. Zucchi[4]
Edmar Chartone-Souza[1]

ABSTRACT

Two wild strains of *S. typhimurium* harboring two plasmids, of 90Kb and 100 Kb, were isolated from sewage water and clinical specimens. Experiments of conjugation, curing and plasmid DNA analysis by agarose gel electrophoresis showed that the 100 Kb plasmid coded simultaneously for resistance to tetracycline (64 µg/mL), production of colicin and pathogenicity to CFW mice.The 90 Kb plasmid, on the other hand, could be considered cryptic under the experimental conditions used in this study. Curing with SDS affected only the heavier plasmid, when the bacterial strains lost the three characteristics at the same time. Conjugation experiments using *E. coli* and *S. typhimurium* showed that the transconjugants possessed either both or only the 100 Kb plasmid and that expression of tetracycline resistance and colicin production was associated with the larger plasmid. To our knowledge, this is the first report on the three properties studied being coded for simultaneously by the same plasmid in *S. typhimurium*.

Key words: *S. typhimurium*, R plasmid, colicin, pathogenicity.

INTRODUCTION

Several studies have been carried out since 1982 to establish the relationship between virulence and large-sized plasmids (between 50 and 100 Kb) among several serotypes of the genus *Salmonella* (9,10,17). Plasmids of *S. typhimurium* and *S. dublin* were handled (1) and homologous regions were found to be associated with virulence. Another study (17) concluded that the virulence plasmids of various serotypes belonged to a large plasmidial family. These observations were confirmed by other researchers (6,32,33). Further studies followed in an attempt to characterize the plasmids (7,16). Mapping of the "spv" region of the pST 100 virulence plasmid from *S. typhimurium* and the sequencing of some genes (24,27) were carried out, as well as studies on regulation (3,12). The "spv" region contains four structural genes and a regulator gene, and codes for the mouse systemic infection trait (8,19).

Considering that all the plasmids were cryptic, it was necessary to handle them genetically. Assays were also performed to establish the association between high molecular weight plasmids with virulence and drug resistance in serotypes of the

[1] Departamento de Biologia Geral, [2]Departamento de Patologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Caixa Postal 486, CEP 31270-010 - Belo Horizonte-MG - Brasil, [3]Faculdade de Medicina, Departamento de Anatomia Patológica, Av. Alfredo Balena, 190 - Universidade Federal Minas Gerais, Belo Horizonte-MG - Brasil; [4]Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade de São Paulo - São Paulo-SP - Brasil.

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** To whom correspondence should be adressed.

Salmonella genus, yet with negative results (22,23). A recently published paper (26), however, described the presence of an 80 MDa conjugative plasmid (pDEP34) in an epidemic strain of *S. typhimurium*, phage type 193 that simultaneously carried the genes for resistance to antibiotics (ampicillin; streptomycin; sulfonamide; tetracycline) and virulence in Balb/c mice. Hybridization was observed with the spv C probe, a 3,5 Kb fragment of the *spv* region of the *S. dublin* virulence plasmid.

No association between virulence and colicinogen plasmids in *Salmonella* was found by Jones *et al* (10), differently from what occurs, for instance, with plasmid Col V in invasive *E. coli* (4). Two years later, however, two strains of *S. typhimurium* serotype isolated from sewage systems in Rio de Janeiro (30) were found to harbor a 60 MDa plasmid encoding the genes for resistance to streptomycin and tetracycline and for the production of colicin Ib type. At about the same time (21), another study reported that strains of *S. typhimurium* isolated from sewage water in Belo Horizonte, State of Minas Gerais, harbored high molecular weight plasmids which concurrently bore markers for colicinogeny and resistance to tetracycline.

It is worth mentioning that investigations using cell culture or animal bioassays have always been carried out. When mice were tested *in vivo*, the organs most affected by *S. typhimurium* were the liver, the spleen and lymphnodes.

The aim of this study was to analyze the possible association between pathogenicity and high molecular weight plasmids simultaneously coding for resistance to tetracycline and colicin production (Col I) in two wild strains of *S. typhimurium* isolated from sewage water and human clinical specimens.

MATERIALS AND METHODS

Materials

The bacterial strains used, including reference strains, their phenotypic and genotypic features and origin are listed in TABLE 1.

The strains used for colicin classification were: *E. coli* K_{12} RCW - sensitive to the colicins tested; *E. coli* K_{12} Col E_1; *E. coli* K_{12} Col E_2; *E. coli* K_{12} Col I; *E. coli* K_{12} Col Ia; *E. coli* K_{12} Col Ib; *E. coli* K_{12} Row K^r; *E. coli* K_{12} V^r and *E. coli* Row B^r, producers of colicins E_1, E_2, I, Ia, Ib, K, V and B, respectively.

Twenty one days old CFW male mice were used in the *in vivo* bioassays.

The culture media (Difco) used were: EMB-Teague Agar for conjugation assays; Nutrient Agar for the production of colicin; Bacto-Pennassay

TABLE 1. Most relevant phenotypic features and source of bacterial strains

Bacterial strains	Plasmids (Kb)	Relevant features	Source
S. typhimruium MG031	90 and 100	Tc^r Col^+	Onça stream
Sn . typhimurium ED040	90 and 100	Tc^r Col^+	Hospital Clínicas FMG
S. typhimurium MG161	-	Sm^r Col^-	Onça stream
S. typhimurium MG031c	90	Tc^s Col^-	This paper
S. typhimurium ED040c	90	Tc^s Col^-	This paper
E. coli K_{12} T_1 = (*E. coli* K_{12} Sm^r x 031)	100	Tc^r Col^+ His^- Pro^- Trp^-	This paper
E. coli K_{12} T_2 = (*E. coli* K_{12} Sm^r x 040)	100	Tc^r Col^+ His^- Pro^- Trp^-	This paper
S. typhimurium MG031 T_3 = (T_1 x *S. typhimurium* MG031c)	90 e 100	Tc^r Col^+	This paper
S. typhimurium ED040 T_4 = (T_2 x *S. typhimurium* ED040c)	90 and 100	Tc^r Col^+	This paper
S. typhimurium vir	N.D.	virulent for mice	Fundação Ezequiel Dias
E. coli K_{12} Sm^r	-	Sm^r Col^- His^- Pro^- Trp^-	N. Datta
E. coli K_{12} Nx^r Rf^r	-	Nx^r Rf^r Col^-	Depto.Biofisica UFRJ
E. coli J53	pSa	Cm^r Km^r Sm^r Su^r	N. Datta
E. coli J53	pRP4	Ap^r Km^r Tc^r	N. Datta.

- : absence of plasmid; **ND:** not determined; **Col^+:** colicin producer; **Col^-:** non-colicin producer; **c:** cured strain; **r:** resistance; **s:** sensitivity; **Tc:** tetracycline; **Sm:** streptomycin; **Nx:** nalidixic acid; **Rf:** rifampicin; **Ap:** ampicillin; **Cm:** chloramphenicol; **Km:** kanamycin; **Su:** sulphonamide; **His:** histidine; **Pro:** proline; **Trp:** triptophan.

Broth for plasmid curing; media LB and TSB, for plasmid DNA extraction; Nutrient Broth, *Salmonella - Shigella* Agar, Mac Conkey Agar, and EMB - Teague Agar for the *in vitro* bioassays.

For electrophoresis, agarose type II (Sigma) was used at 0,5% or 0,8%. All the other reagents were from Merck.

Whole organs or portions were fixed in Bouin solution and stained with Eosin-Hematoxylin (EH).

Methods

Production and classification of colicins - overlay method with indicator strain (18).

Determination of resistance level for tetracycline by the dilution method in solid medium (14).

Plasmid transfer - The transfer of plasmids was carried out by conjugation (31) in Nutrient Broth (5 hours/37°C). The conjugation mixture was spread in EMB-Teague, a selective indicator medium with adequate antibiotics added. Donors were original *S. typhimurium* strains MG031 and *S. typhimurium* ED040. Receptors were *E.coli* strains K_{12} Sm^r, *E. coli* K_{12} Nx^r Rf^r and *S. typhimurium* MG161 Sm^r. A subsequent conjugation was performed using the first transconjugants obtained, that is, *E. coli* K_{12} T1 and *E. coli* K_{12} T_2, backtransferring the plasmids to the cured strains *S. typhimurium* MG031c and *S. typhimurium* ED040c. The new transconjugants produced were *S. typhimurium* MG031 T_3 and *S. typhimurium* ED040 T_4, respectively.

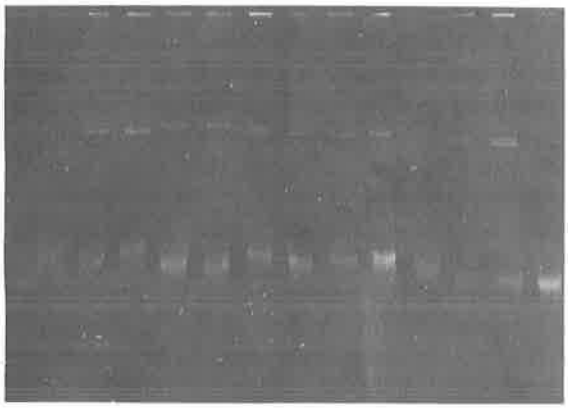
Curing of plasmid by SDS - The plasmid DNA was cured as described (28), using sodium dodecyl sulfate at final concentrations of 0,5%, 2% and 5%.

Extraction of plasmid DNA and electrophoresis in agarose gel - Alkaline extraction of plasmid DNA was carried out as described (11,20). Aliquots of 20 or 35 µl of plasmid DNA were loaded onto 0,5% or 0,8% agarose gel and were submitted to 5 V/cm. The gel was stained with ethidium bromide 0,4 µg/mL and analyzed in a Germetec transilluminator. Photographs were taken with an Eastman Kodak Corp. Panatomic Film X, 32 ASA, 16 DIN, 4 lens opening, 5 sec exposure time.

***In vivo* pathogenicity biossays** - These tests were performed according to Helmuth et al. (9), with modifications. Groups of five mice each were orally given 0,1 mL of a bacterial culture which had been incubated in Nutrient Broth for18 hours at 37°C. Separate groups were inoculated with either the original strain *S. typhimurium* ED040, its cured derivative, its transconjugant *E. coli* K_{12} T_2, the control strain *S. typhimurium* "vir" (positive control) or control strains *S. typhimurium* MG161 Sm^r, *E. coli* K_{12} Sm^r and *E. coli* K_{12} Nx^r Rf^r (negative controls). The animals were sacrificed eighteen days after oral inoculation and pieces of the liver, large intestine, small intestine, spleen and kidney were processed for histopathology. Mice that were given *S. typhimurium* MG031 were sacrificed on day 21 and submitted to the same procedure described above. Histology slides were photographed with an Olympus Vanox Photomicroscope AHB T3 using an Ilford PAN F50 ASA film.

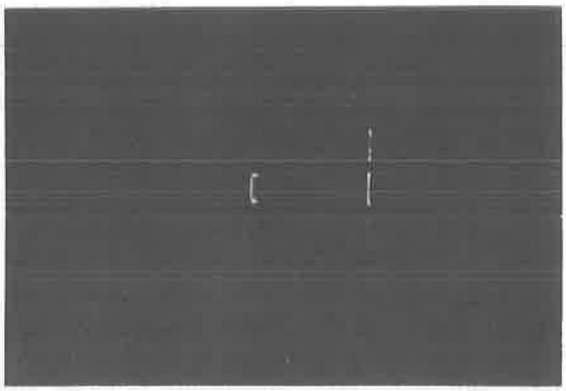
RESULTS

Wild strains *S. typhimurium* MG031 and *S. typhimurium* ED040 showed a tetracycline resistance level of 64 µg/mL and were positive for production of colicin type I. The strains harbored a plasmid of 90 Kb and a larger plasmid of 100 Kb (FIGURES 1A and 1B). When submitted to curing trials, both strains lost the larger plasmid (FIGURE 1A, lanes 6,7,11,12, and FIGURE 1B, lane 3) and no longer expressed the characteristics Tc^r Col^+ that were originally observed. When submitted to conjugation, both wild strains transferred either the larger or both plasmids to recipient strains; the transconjugants were tetracycline resistant and colicin producers with consistent electrophoretic profiles, i.e., they displayed the larger plasmid (FIGURE 1A, lanes 3,5,9,10, and FIGURE 1B, lane 2). When a second successive step was carried out (backtransferring the plasmids of *E. coli* K_{12} T_1 and *E. coli* K_{12} T_2 to the cured *S. typhimruium* MG031c and *S. typhimurium* ED040c strains that only had the smaller plasmid) the transconjugants *S. typhimurium* MG031 T_3 and *S. typhimurium* ED040 T_4 were obtained, respectively, which presented both plasmids as well as the characteristics that had been lost during curing (data not shown).



1) *E. coli* K_{12} Nx^{r}Rf^r
2) *S. typhimurium* ED040
3) *S. typhimurium* ED040 x *E.coli* K_{12} Nx^{r}Rf^r
4) *S. typhimurium* MG161
5) *S. typhimurium* ED040 x *S. typhimurium* MG161*
6) *S. typhimurium* ED040c
7) *S. typhimurium* ED040c
8) *S. typhimurium* MG031
9) *S. typhimurium* MG031 x *E. coli* K_{12} Nx^{r}Rf^r
10) *S. typhimurium* MG031 x *S. typhimurium* MG161*
11) *S. typhimurium* MG031c
12) *S.* typhimuirum MG031c
13) Plasmid standard RP4 - 34 MDa
14) Plasmid standard Sa - 23 MDa
* Receptor strains.

FIGURE 1A: Electrophoresis in agarose gel of plasmid DNA from original *Salmonella* strains and their derivatives. 0.8% agarose; 7.30 hours of migration - 80 V (Kado and Liu, 1981)



1) *S. typhimurium* ED040
2) *E. coli* K_{12} T_2
3) *S. typhimurium* ED040c

FIGURE 1 B: Electrophoresis in agarose gel of plasmid DNA from original *Salmonella* strains and their derivatives.0.5% agarose; 5.30 hours of migration - 80 V (Sambrook et alii, 1989)

In order to test whether this plasmid is associated with pathogenicity, *in vivo* tests were carried out in mice. Animals which were given wild strain *S. typhimurium* ED040 showed visible signs of disease 5 days after inoculation, including diarrhea and loss of weight, and were sacrificed on day 18. The liver and spleen presented a grayish color and reduced size. Histological analysis of the different organs revealed changes indicative of infection and alterations in the liver and the small (not shown) and large intestine, as can be observed in FIGURES 2 and 4; no histopathological changes were detected in the kidney and spleen. Mice inoculated with the cured strain and the transconjugant *E. coli* K_{12} T_2 did not show external signs of disease nor histological abnormalities (FIGURES 3, 5 and 6). A similar situation was found for animals inoculated with the cured or transconjugant strains of *S. typhimurium* MG031 and with those inoculated with the control strains, except for *S. typhimurium* vir, which induced 100% mortality on the first day of infection (data not shown). Mice that were given wild strain *S.*

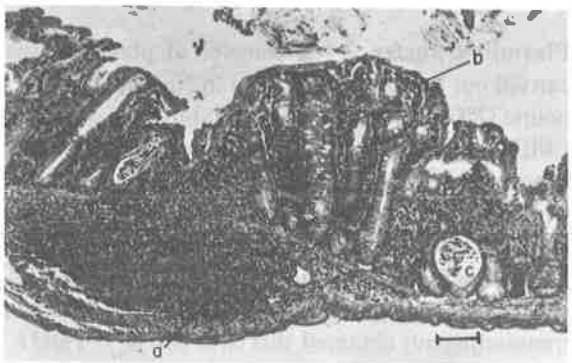


FIGURE 2: Large intestine of mouse that was given *S. typhimurium* ED040, showing diffuse colitis with follicular hyperplasia (a); neutrophils on lamina propria (b); formation of crypt abscesses (c). E.H. 1 cm = 94.35 µ.

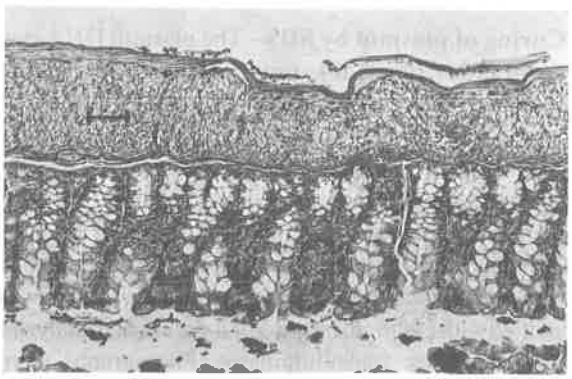


FIGURE 3: Large intestine of mouse that was given *S. typhimurium* ED040 cured. No apparent alterations can be observed. E.H. 1 cm = 46.34 µ.

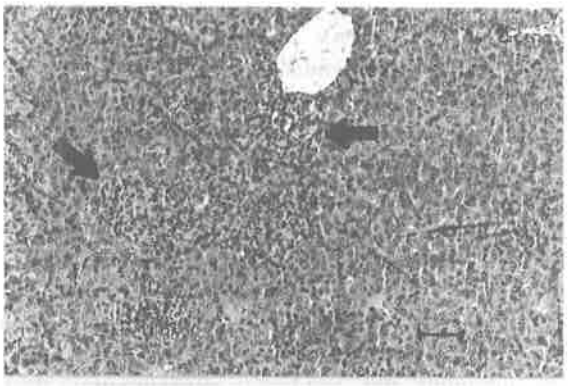


FIGURE 4: Liver of mouse that was given *S. typhimurium* ED040, showing focal inflammatory infiltrates consisting of mononuclear and polymorphnuclear cells. E.H. 1 cm = 48.92 µ.

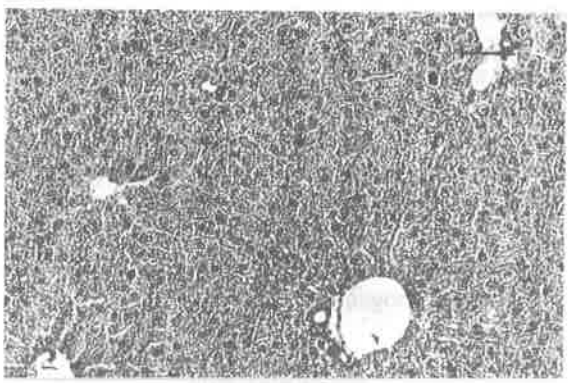


FIGURE 5: Liver of mouse that was given *S. typhimurium* ED040 cured. No apparent alterations can be observed. E.H. 1 cm = 44.03 µ.

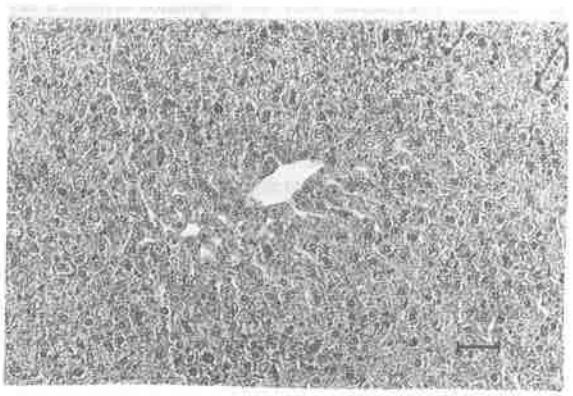


FIGURE 6: Liver of mouse that was given the transconjugant *S. typhimurium* ED040 x *E. coli* K12 Sm^r (T_2). Normal morphology observed. E.H. 1 cm = 43.75 µ.

typhimurium MG031 also did not present external signs of disease, with absence of diarrhea and apparently normal aspect and growth. Their organs, autopsied 21 days after infection, presented typical volume and color on macroscopic examination. However, histopathology data of the liver, small and large intestine showed alterations characteristic of infection (data not included).

DISCUSSION

The role played by virulence genes encoded by high molecular weight plasmids in serotypes of the genus *Salmonella* (2,6,9,17,25) is well known.

Plasmidial studies on the association between virulence and resistance to drugs in *Salmonella sp* have shown negative results (10). However, Vicente et al. (29) suggested an association between multiple-resistance and colicinogenic plasmids in an epidemic *Salmonella agona* serotype from a hospital of Rio de Janeiro, Brazil. This strain displayed two plasmids, one of 36 MDa, conjugative, responsible for multiple drug resistance and a second small one of 6.5 MDa, non curable, encoding the colicin type Ib gene that can be carried by the former.

Concerning colicin production, there is yet no reference in the literature on the coding of this property and of virulence by the same plasmid in *S. typhimurium*, as occurs with invasive *E. coli* and plasmid Col V (4). Four virulence plasmids were investigated in *S. typhimurium* (15) and it was found that they did not code for the synthesis of colicins, classical adesins, aerobactins, nor for resistance to metals.

Research on *Salmonella sp* involving plasmidial curing and transfer by conjugation and transformation has been taking place since 1980. However, because all the plasmids involved are cryptic, this type of investigation has required the use of transposon insertion (1,6,7,8). Studies involving processes of curing, production of transconjugants or transformants and pathogenicity in susceptible mice have always demonstrated that the large sized plasmids of *Salmonella sp* are associated with virulence. Some of these studies report the occurrence of infection with gastroenteritis or systemic infection without diarrhea. Gulig et al.(8) believe that the plasmids

are responsible for systemic infection, irrespective of its association with gastroenteritis. The data obtained in the present study are in agreement with the available literature. The animals that were given the cured strains as well as the transconjugants of both *S. typhimurium* MG031 and *S. typhimurium* ED040 did not present any sign of morbidity nor pathogenicity traits on histopathological examination. The 100 Kb plasmid, therefore, when transferred to the *E. coli* K_{12} did not show any activity indicating virulence. This observation is not surprising, because an interaction is probably necessary between chromosomal and plasmid genic products (19) as well as host conditions (5).

It is therefore possible to say that the 100 Kb plasmid of the two strains of *S. typhimurium* studied in this paper is apparently responsible for the simultaneous coding of the three characteristics: resistance to tetracycline, production of colicin and systemic infection followed or not by gastroenteritis. The smaller sized plasmid, however, remains cryptic under the experimental conditions used and is sometimes mobilized by the largest one.

As a conclusion, the results reported in this study are significant since, to our knowledge, they describe for the first time a virulence plasmid of two strains of *S. typhimurium* coming from natural and clinical sources (sewer water and human specimens) coding simultaneously for tetracycline resistance, colicin production, and pathogenicity in mice.

Further research will be undertaken in our laboratory in order to study the possible genetic and biochemical associations between the three phenotypic features encoded by the *S. typhimurium* virulence plasmid described herein.

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The authors are thankful to Dr. Enio Cardillo Vieira from ICB/UFMG for supplying the animals and to Andréa Reis, also from ICB/UFMG, for technical assistance.

RESUMO

Plasmídios codificando similtaneamente resistência à tetraciclina, produção de colicina e patogenicidade em *Salmonella typhimurium*

Duas linhagens selvagens de *Salmonella typhimurium* abrigando dois plasmídios de 100 e 90 Kb foram isoladas de água-esgoto e material clínico. A partir de experimentos de conjugação, cura, análise do conteúdo plasmidial através de eletroforese em gel de agarose e bioensaio com camundongos CFW observou-se que o plasmídio de 100 Kb codifica simultaneamente resistência a 64 µg/mL de tetraciclina, produção de colicina e patogenicidade, enquanto que o plasmídio de 90 Kb pode ser considerado como críptico, em ambas as linhagens, nas condições experimentais desta pesquisa. O processo de cura dessas linhagens com SDS atingiu apenas o plasmídio de peso molecular mais elevado, quando as linhagens bacterianas perderam, simultaneamente, as três características. Ao realizar os ensaios de conjugação com *E. coli* K_{12} e *S. typhimurium*, as transconjugantes receberam ambos ou apenas o plasmídio de 100 Kb e, associado a este, as propriedades de resistência à tetraciclina, produção de colicina e patogenicidade. É a primeira vez que se descrevem essas três propriedades codificadas simultaneamente pelo mesmo plasmídio em *S. typhimurium*.

Palavras-chave: *Salmonella typhimurium*, plasmídio R, colicina, patogenicidade.

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THE EFFECTS OF CHEMICAL DISINFECTANTS AND SANITIZERS ON *SALMONELLA GALLINARUM*

Angelo Berchieri Junior[1] *
P. Barrow[1]

ABSTRACT

A variety of chemical disinfectants, sanitisers and water sanitisers containing iodophor, quaternary ammonium (QAC) or phenolic compounds as active ingredients were tested for their activity against *Salmonella gallinarum* strain 9. Different test conditions were used, including some designed to mimic environmental conditions. Generally, phenol-containing compounds were the most effective, followed by QACs and lastly by iodophors. All the compounds were inactivated to some extent by wood shavings, chick fluff, chicken feces and feed. Inactivation was most marked when *S. gallinarum* was pre-dried in feed. Under this condition a glutaraldehyde-based preparation was still active. Sodium hypochlorite, at commercially recommended concentrations or higher, was readily inactivated under a variety of conditions.

Key words: Disinfectants, *Salmonella gallinarum.*

INTRODUCTION

Despite the fact that fowl typhoid, caused by *Salmonella gallinarum*, has been virtually eliminated from the national flocks of some countries, it remains a poultry disease of worldwide economic significance in countries with highly intensive industries and those that only recently began to intensify production (9).

S. gallinarum is one of the few host-adapted serotypes which produce severe systemic disease accompanied by mortality and loss of production, in this case in poultry. Other avian species also carry this microorganism. Because the reproductive tract can become infected in addition to the alimentary tract, both vertical and horizontal routes of transmission are thought to be important (9).

In countries with relatively low ambient temperatures where poultry housing is generally completely enclosed, the control measure of choice is to eliminate carrier birds by serological testing followed by slaughtering of the reactors. Schemes of this type introduced to eliminate *S. pullorum* will additionally eliminate *S. gallinarum* since both are antigenically identical, provided that improved measures of management and hygiene are also implemented. Howerer, in countries with a higher ambient temperature, however, open-sided poultry housing is common and the danger of infection from environmental sources, particularly the free-flying birds, is great. Under these conditions, the possibility of complete control is very limited. Live, attenuated vaccines may not be acceptable in many countries and extensive antibiotic usage can lead to the development of resistance (4, 5, 15).

Whatever the source of initial infection, persistence of the microorganism from one flock to the next in

[1] Division of Environmental Microbiology, Institute for Animal Health, Compton Laboratory, Compton, Newbury, Berkshire, RG20 7NN, United Kingdom.Tel: 44-1635-578411: Fax: 44-1635-577263

* Corresponding author. Current address: Faculdade de Ciências Agrárias e Veterinarias - UNESP, Rod. Carlos Tonanni, Km. 5, 14870-000 Jaboticabal - S.P., Brazil.

poorly cleaned accommodations must be important and the need for effective disinfection is obvious.

Disinfectants and sanitisers can be assessed for antibacterial activity by well recognized tests (11, 13) yet the environmental conditions under which a chemical preparation will be used are difficult to simulate precisely and with reproducibility (8). This is particularly true regarding the inclusion in the tests of potentially inactivating organic material found in poultry housing, like feces, food, wood and feathers.

We have studied a number of commercially available disinfectants and sanitisers for their activity against a strain of *S. gallinarum*. The aim of this investigation was to assess to what extent potentially inactivating organic material might reduce the antibacterial activity of the tested compounds, thus favoring *S. gallinarum* survival and consequent persistence in the environment.

MATERIALS AND METHODS

Bacterial strain

Salmonella gallinarum strain 9 was originally isolated from a case of fowl typhoid and its virulence characteristics have been well characterized (1,14). Broth cultures were made overnight in 10 mL volumes of Luria-Bertani (LB) broth (Difco) with shaking in an orbital incubator (150 revs/min) at 37°C for 24 h. Viable bacterial counts were made by dilution in phosphate buffered saline (PBS) and counting on LB agar according to the method of Miles *et al.* (7).

Experimental procedure

The procedure used was devised to minimize the time between addition of the chemical preparation to the potentially inactivating material and addition of the bacteria. In most cases, the procedure was as follows.

The potentially inactivating organic test material was weighed. Feces from adult specified-pathogen-free chickens, kept at the Institute for Animal Health, chick mash (SDS diets, Manea, Cambridgeshire, UK), chick fluff obtained from an institute incubator and wood shavings were weighed in sterile universal bottles and appropriately diluted with either water or LB broth.

Preliminary low dilutions of some disinfectants, for which the recommended working dilution was high, were made in water at room temperature. The chemical preparation was then added to the suspension of organic matter at room temperature (20-25°C) and mixed by inversion.

Bacterial cultures were diluted in distilled water so that on adding 0.1 mL of the diluted chemical preparation, the desired initial viable count was obtained. The bacterial suspension was added to the mixture of test compound with organic matter, followed by rapid mixing. Bacterial counts were performed at 0, 15 and 30 minutes.

The inactivating effect of chick mash was assessed in two ways. In the first, the above procedure was followed. In the second, 10 g of feed were ground for 5 minutes with a pestle in a mortar together with 1 mL of a ten fold dilution of broth culture in distilled water; 40 g of feed were additionally added to this mixture, further ground to homogeneity and held overnight at 4°C. The final mixture was added to the diluted chemical.

RESULTS

Results on the action of different sanitisers and disinfecting agents on *S. gallinarum* strain 9 viability are shown in TABLES 1-4. The chemicals were grouped according to the type of major active component in each preparation and were tested under a variety of conditions, including the presence of large amounts of organic material likely to be found in poultry houses or hatcheries.

The effect of several preparations based on mixed phenolic compounds on *S. gallinarum* viability is shown in TABLE 1. All preparations were highly effective in distilled water, as shown by the absence of detectable bacteria This was also the case in LB broth and 5% feces for all preparations except agent 1; in this case, a considerable proportion of added bacteria was counted immediately after addition but was no longer detectable after 15 minutes incubation. In the presence of chick fluff and wood shavings, all preparations except agent 1 effectively killed the *Salmonella* microorganisms by 15 minutes incubation. Fluff neutralized the effect of agent 1. Fluff was obviously slightly more inhibitory to phenolic compounds than were wood shavings.

TABLE 1. The antibacterial action of selected phenolic compounds on *S. gallinarum* in the presence of different organic materials

Commercial agent (concentration)	Time of incubation (mins)	Log_{10} viable counts of *S. gallinarum* after incubation for the time shown (mins) in:						
		Distilled water	LB broth	Faeces 5%	Fluff 5%	Wood shavings 5%	Mash feed	
							Method I	Method II
Agent 1 (1.500)	0	N	3.15	5.36	6.08	1.95	5.81	2.18
	15	N	N	N	6.04	N	5.78	3.46
	30	N	N	N	6.08	N	6.00	3.52
Agent 2 (1:100)	0	N	N	N	5.11	N	4.34	2.90
	15	N	N	N	N	N	3.36	2.85
	30	N	N	N	N	N	3.08	2.34
Agent 3 (1:200)	0	N	N	N	4.52	N	5.04	N
	15	N	N	N	N	N	4.53	N
	30	N	N	N	N	N	4.36	1.60
Agent 4 (1:200)	0	N	N	N	2.32	N	3.36	1.85
	15	N	N	N	N	N	1.60	N
	30	N	N	N	N	N	N	1.48
Desinfectant-free control	0	5.88	6.18	6.38	6.26	6.36	6.15	3.45
	15	5.91	6.04	6.23	6.15	6.11	6.11	3.36
	30	6.26	5.98	6.46	6.43	6.11	6.08	3.51

$N = log_{10} < 2$
Method I Test performed as with the others
Method II Bacteria premixed and stored with the feed

Chick feed had differing neutralizing effects. Of the compounds tested, agent 4 was effective after 30 minutes when method I was used. The other preparations were less effective. With method II, uneven distribution of the bacteria in feed produced variable counts but agents 3 and 4 were clearly more effective than the other two preparations. Although agent 1 was apparently less effective than the others, this may have been due to its higher dilution recommended for use.

TABLE 2 shows the action of two quaternary ammonium compounds (QAC) on *S. gallinarum*. Although they were effective in water and broth, they were almost completely neutralized by feces and fluff. In the presence of clean wood shavings, however, agent 5 resulted effective after 15 minutes incubation and agent 6 reduced the bacterial counts by 15 minutes but then presented a stable effect, suggesting an initial antibacterial activity followed by rapid inactivation of the compound.

The three iodophor compounds were all totally active against *S. gallinarum* when tested in distilled water (TABLE 3). However, all three were inactivated by chick feed, LB broth and 5% feces or fluff. Agents 7 and 9 were still active in the presence of wood shavings whereas agent 6 was inactivated by the material.

TABLE 4 shows the effect of a glutaraldehyde-based preparation. In all cases the compound was very active, since no viable bacteria were observed after 30 minutes contact under all the conditions tested. When tested in water or broth only, no *Salmonella* organisms were detectable at all, indicating the greater sensitivity of the compound when tested in the absence organic material at high concentrations. No great changes were seen in the disinfectant-free control preparations during the total period of incubation.

Sodium hypochlorite was tested at the recommended concentration of 2 ppm and also at 100 ppm to ensure that the level of available free chlorine was high (TABLE 5). Complete comparisons were not carried out. The lower concentration in distilled water did not affect *S.*

TABLE 2. The effect of quaternary ammonium compounds on *S. gallinarum* viability in the presence of organic materials

Commercial agent (concentration)	Time of incubation (mins)	Log_{10} viable counts of *S. gallinarum* after incubation for the time shown (mins) in:						
		Distilled water	LB broth	Faeces 5%	Fluff 5%	Wood shavings 5%	Mash feed	
							Method I	Method II
Agent 5 (1.600)	0	N	4.00	5.85	5.93	5.88	5.95	3.00
	15	N	N	5.83	5.91	N	5.93	3.30
	30	N	N	5.60	5.99	N	5.85	3.40
Agent 6 (1:6250)	0	N	N	6.30	6.08	5.82	5.97	3.20
	15	N	N	5.30	6.15	1.95	5.96	3.30
	30	N	N	5.91	6.40	1.00	6.00	3.43
Desinfectant-free control	0	5.88	6.18	6.38	6.26	6.36	6.15	3.45
	15	5.91	6.04	6.23	6.15	6.11	6.08	3.36
	30	6.26	5.98	6.46	6.43	6.11	6.11	3.51

$N = log_{10} < 2$
Method I Test performed as with the others
Method II Bacteria premixed and stored with the feed

TABLE 3. The antibacterial action of iodophor compounds on *S. gallinarum* in the presence of organic materials

Commercial agent (concentration)	Time of incubation (mins)	Log_{10} viable counts of *S. gallinarum* after incubation for the time shown (mins) in:						
		Distilled water	LB broth	Faeces 5%	Fluff 5%	Wood shavings 5%	Mash feed	
							Method I	Method II
Agent 7 (1.200)	0	N	5.69	5.79	5.76	N	5.90	2.11
	15	N	5.81	5.26	5.76	N	5.92	3.58
	30	N	5.76	5.15	5.77	N	5.93	3.04
Agent 8 (1:200)	0	N	5.81	6.26	6.08	6.08	5.93	3.20
	15	N	5.93	6.18	6.04	6.08	5.88	3.22
	30	N	5.89	6.18	6.08	5.87	5.94	3.38
Agent 9 (1:600)	0	N	5.72	6.18	6.11	6.11	5.95	3.23
	15	N	5.74	5.85	6.00	3.04	5.94	3.43
	30	N	5.86	5.81	6.30	N	6.04	3.45
Desinfectant-free control	0	5.88	6.18	6.38	6.26	6.36	6.15	3.45
	15	5.91	6.04	6.23	6.15	6.11	6.11	3.36
	30	6.26	5.98	6.46	6.43	6.11	6.08	3.51

$N = log_{10} < 2$
Method I Test performed as with the others
Method II Bacteria premixed and stored with the feed

TABLE 4. The antibacterial action of a glutaraldehyde-based compound on *S. gallinarum* in the presence of organic materials

Commercial agent (concentration)	Time of incubation (mins)	Log_{10} viable counts of *S. gallinarum* after incubation for the time shown (mins) in:						
		Distilled water	LB broth	Faeces 5%	Fluff 5%	Wood shavings 5%	Mash feed	
							Method I	Method II
Agent 10 (1.100)	0	N	N	5.65	5.26	N	5.99	3.08
	15	N	N	N	N	N	N	N
	30	N	N	N	N	N	N	N
Desinfectant-free control	0	5.88	6.18	6.38	6.26	6.36	6.15	3.45
	15	5.91	6.04	6.23	6.15	6.11	6.11	3.36
	30	6.26	5.98	6.46	6.43	6.11	6.08	3.51

$N = log_{10} < 2$
Control = the diluent without the desinfectant
Method I Test performed as with the others
Method II Bacteria premixed and stored with the feed

TABLE 5. The antibacterial action of sodium hypochlorite on *S. gallinarum* in the presence of organic materials

Commercial agent (concentration)	Time of incubation (mins)	Log_{10} viable counts of *S. gallinarum* after incubation for the time shown (mins) in:					
		Distilled water	LB broth	Faeces 5%	Fluff 5%	Wood shavings 5%	Mash feed Method I 1%
100 ppm	0	N[1]	5.83	5.97	-	N	5.90
	15	N	6.04	6.04	-	N	6.08
	30	N	6.11	6.25	-	N	6.08
Control	0	6.43	6.18	6.38	-	6.48	6.30
	15	6.36	6.18	6.43	-	6.34	6.46
	30	6.43	6.45	6.23	-	6.26	64.1
2 ppm	0	5.63	-	5.70	5.93	5.76	5.70
	15	5.70	-	5.84	5.89	5.76	5.72
	30	5.90	-	5.98	5.83	5.81	5.86
Control	0	5.70	-	5.77	5.73	5.80	5.79
	15	5.87	-	5.80	5.91	5.80	5.81
	30	5.83	-	5.92	6.04	5.80	5.90

Method I Test performed as with the others
- = not done
[1] $N = log_{10} < 2$
[2] Feces used at 1% when the free chlorine concentration was 2 ppm

gallinarum viability whereas hypochlorite at 100 ppm was fully active against the bacterium. This higher concentration was completely inactive in LB broth and also in the presence of 5% feces and chick mash. Fluff inactivated the lower concentration of sodium hypochlorite but was not tested against the higher concentration.

DISCUSSION

In addition to vertical transmission *via* the reproductive tract and eggs (5, 6) *S. gallinarum* may be transmitted by birds which are excreting the organisms, primarily in feces. Similarly to *S. typhi* in human infections, extensive excretion of *S. gallinarum* in feces does not play a major role in the pathogenic process of fowl typhoid. However, both organisms localize in the alimentary tract as the systemic disease progresses and thus fecal shedding becomes very important to transmission by diseased hosts. As with *Salmonella* serotypes like *S. enteritidis* and *S. typhimurium*, which are more frequently associated with human food poisoning and for which gross fecal shedding is a significant aspect of their zoonotic transmission, contamination of housing through dust, cages and transport crates is likely to be very important for inter-farm transmission and also for transmission from one flock to another within the same house, after depopulation.

We have demonstrated that disinfectants and sanitisers extensively used in the poultry industry may be inactivated to different degrees by several organic materials present in poultry houses at or immediately after depopulation. With the exception of hypochlorite, all the agents assessed in this investigation were fully able to kill *S. gallinarum* when diluted in distilled water.

Phenolic agents were active under most of the conditions tested except in the presence of chick mash and, for some agents, of chick fluff. Quaternary ammonium compounds and iodophors were all greatly inactivated by most of the agents tested and only showed potential activity in the presence of wood shavings.

Among the agents tested, glutaraldehyde showed the greatest antibacterial activity in the presence of organic materials. The material with highest inactivating ability was chick mash when assayed with either suspended bacteria or with bacteria dried into the feed. Unlike many of the antibacterial agents analyzed, glutaraldehyde was able to kill *S. gallinarum* within the time tested. Recent field data have shown that formaldehyde, used either as a spray for disinfection or as a fogged disinfectant in enclosed housing, was one of the most effective agents at reducing *Salmonella* contamination in housing (2). There are, of course, specific safety problems associated with the use of aldehydes, particularly when applied by untrained personnel. However, the present data suggests that, if employed in association with cleaning and removal of excess organic material, aldehydes may be the most effective antibacterial agents.

The chlorine-releasing compound was tested at twice the concentration recommended for use with drinking water and at the level recommended for use as disinfectant. The data confirm that it is readily inactivated by feces at 1% and by chick mash also at 1%, the two materials likely to contaminate drinking water or disinfectants. There are several reports where drinking water was thought to be the main route of infection with *S. gallinarum* (3) or *S. enteritidis* (10). The addition of iodophors or chlorine-releasing agents is standard practice as recommended by Poppe and Barnum (12). The data obtained in this work indicates that such compounds are likely to be generally ineffective in the presence of organic material.

The present study indicates the importance of rigorous cleaning after depopulation and shows the danger of relying solely on the action of antibacterial agents.

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RESUMO

Avaliação de compostos desinfetantes sobre *Salmonella gallinarum*

A atividade de diferentes compostos a base de iodo, amônia quaternária (QAC), fenol, glutaraldeído e cloro, contra *Salmonella gallinarum*

foi avaliada na presença de matéria orgânica comumente encontrada em operações avícolas. De um modo geral os compostos fenólicos apresentaram melhor desempenho, seguidos pelos produtos a base de amônia quaternária que, por sua vez, apresentaram melhor desempenho que os produtos a base de iodo. Todos apresentaram algum grau de inativação quando utilizados na presença de raspa de madeira (maravalha), penugem, fezes de aves e ração para aves. A inativação do produto químico foi mais profunda quando misturou-se, previamente, a bactéria na ração. Mesmo nestas condições, o produto contendo glutaraldeído ainda manteve-se ativo. O composto contendo hipoclorito de sódio foi totalmente inibido pela presença de matéria orgânica.

Palavras-chave: Desinfetantes, *Salmonella gallinarum*.

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ANTIMICROBIAL AND MERCURY CHLORIDE RESISTANCE IN VIBRIO ISOLATES FROM MARINE FISH OF THE SOUTHEASTERN BRAZILIAN REGION.

José Cavalcante de Albuquerque Ribeiro Dias*
Djair Ernandez
Agostinho Alves de Lima e Silva
Ligia Maria Rocha Ramos de Oliveira
Sonia Ermelinda Alves da Silva

ABSTRACT

A total of 199 vibrio strains recovered from the intestinal tract of marine fishes (mullet, *Mugil* sp., and croaker, *Micropogon* sp.) were studied. Specimens were caught in the Guanabara bay and along the seashore of the city of Rio de Janeiro, Brazil. The following vibrios were isolated: *V. anguillarum*, *V. harveyi*, *V. proteolyticus*, *V.campbelli*, *V. alginolyticus*, *V. splendidus*, *V.marinus*, *V. logei* , *V. parahaemolyticus* and *Vibrio* sp (halophilic).

The antibiogram for eight drugs and the resistogram for mercury chloride (Hg) indicated a 96.4% rate of incidence of markers, being 77.0% for antimicrobial resistance and 21.8% for Hg resistance.

Multiresistance ($\geq$3 markers) was significant ($p < 0.05$) and of the order of 71.0%. A similar high incidence of the Hg^r phenotype was found in multiresistant strains (64.2%), which were isolated from croakers only. The occurrence of the Su and Ap markers, in addition to the Sm marker, was prominent in the general computation and also within the set of Hg-resistant strains. All the cultures indicated the presence of at least one marker regardless of species, except for *V.parahaemolyticus*.

Conjugation of 28 vibrio isolates with *Escherichia coli* K 12 strains revealed marker transfer in 71.4% of the experiments. Transconjugants for Su, Sm, Km and Ap were observed from donor cultures of *V. anguillarum* and *V. harveyi*. No transconjugant colonies were isolated for the Hg marker.

The results suggest an association of these two markers in vibrio isolates, probably as a consequence of the natural evolution of resistance markers in the marine bioma.

Key-words: *Vibrio*, heavy metal and antimicrobial resistance, plasmids, marine fish.

INTRODUCTION

Antimicrobial resistance has been extensively investigated among various bacteria, comprising pathogenic and potentially pathogenic species (2; 5; 9; 13). Some investigators have attempted to establish an association between antimicrobial resistance and heavy metal resistance, especially in samples of environmental origin like niches that may undergo selective pressure for certain metal ions, including collections of water (1; 6; 10; 21). It should be pointed out that bay and littoral waters

* Departamento de Bacteriologia, Instituto Oswaldo Cruz-FIOCRUZ. Av. Brasil, 4365 , Manguinhos, 21045-900 , Rio de Janeiro, RJ, Brasil, Telephone: 00.55.21.2706565, Fax: 00.55.21.2800754

constantly receive microbial or industrial discharges (3; 18) that may promote profile changes in autochthonous microorganisms.

Therefore, considering the natural occurrence of vibrios in fishes and due to some knowledge already available in our country on the distribution of genetic markers among these bacteria, the present work was undertaken to study isolates of the genus *Vibrio*, obtained from marine fish, for their antimicrobial resistance to eight drugs and susceptibility to mercury chloride. A possible association between these two types of markers was also analyzed.

MATERIALS AND METHODS

Sampling: A total of 199 vibrio strains including nine distinct species were studied (TABLE 1). All samples were isolated from the intestinal tract of mullet (*Mugil* sp.) and croaker (*Micropogon* sp.) specimens caught, respectively, in the Guanabara Bay and along the seashore of the city of Rio de Janeiro between 1986 and 1988, according to the reports by Ernandez *et al.* (7).

Antibiogram: Resistance to antimicrobial agents was assayed by the disk diffusion method as recommended by the N.C.C.L.S. (14). The inoculum consisted of bacterial growth in peptone medium with 1.0 % NaCl incubated for 18h at 37°C. When necessary, cultures were suspended in saline (0.85 % NaCl solution) so as to obtain concentrations equivalent to the 0.5 value of the MacFarland scale. The cultures were plated onto Agar Mueller-Hinton medium (Merck) containing 1.0% sodium chloride (5) and disks impregnated with eight different antimicrobial drugs (CECON) at the following concentrations (in micrograms per disk): sulfadiazine (Su): 300; tetracycline (Tc), chloramphenicol (Cm), kanamycin (Km) and nalidixic acid (Nal): 30; streptomycin (Sm), ampicillin (Ap) and gentamicin (Gm): 10. Standard cultures of *Escherichia coli* ATCC 25922 and of *Vibrio alginolyticus* ATCC 17749 were submitted to the test in parallel and readings were obtained after incubation for 18-24h at 37°C.

Test for mercury chloride (Hg) susceptibility: for the resistogram, an aliquot of aqueous mercury chloride solution (Baker Analyzed Reagent) was incorporated into Nutrient Agar medium (Merck) with 1.0 % NaCl to a final concentration of 5.0 micrograms/mL (6).

The inoculum consisted of the bacterial growth contained in a calibrated platinum loop (0.001mL) and obtained under the same conditions described above for the antibiogram. The Hg^r phenotype was assigned to cultures able to grow in medium with the heavy metal. The *E. coli* strain ATCC 25922 was used as negative control.

Conjugation experiments: using the frequency of occurrence in the sample as a criterion (7), 28 vibrio strains including the species *V. anguillarum* (16), *V. harveyi* (8), *V. splendidus* (3) and *V. proteolyticus* (1) were selected as donors. Marker transfer to the standard *E. coli* strain K 12 F- Nal^r (Cell Physiology Laboratory, Biophysics Institute, UFRJ) and K12 F- Sm^r (Department of Molecular Biology, University of Edinburgh, Scotland) was performed by previously reported methods (5).

Dilutions of the conjugant mixtures (donor + recipient) previously grown in peptone medium in 1.0 % NaCl for 18-24h at 37°C were inoculated onto plates containing Muller-Hinton agar and salt at the same concentration plus 100 mcg/mL Nal or 10 mcg/mL Sm, as well as 5 mcg/mL HgCl2 and the following drugs: 10 mcg/mL Sm, Ap and Gm; 20 mcg/mL Tc, Cm, Km and Nal, and 150 mcg/mL Su. After incubation for 24-48h at 37°C, three to five colonies per experiment were analyzed for biochemical profile, antibiogram and resistogram.

Statistical analysis: the nonparametric chi-square test with a 5% level of significance ($p < 0.05$) was applied to the results on antimicrobial resistance and the data related to the combination of Hg resistance with antimicrobial resistance.

RESULTS AND DISCUSSION

An almost absolute incidence rate of 96.4% was detected for resistance markers among the vibrio strains (TABLE 1). This percentage included mostly strains with resistance to antimicrobial agents only (77.0%), certainly indicating an intense dispersal of these markers in niches not necessarily submitted to selective antimicrobial pressure, as is the case for the hospital environment, since the bacteria studied were of marine origin. On the other hand, it is interesting to quote the resistance

TABLE 1. Distribution of resistance markers among the vibrio isolates from marine fish

Samples Samples	Resistante Studied Nº	Samples Nº	Number with markers of antimicrobial (A) and/or Hg (B) resistance											
			A	B	A + B	Sub	Sm	Tc	Cm	Km	Ap	Nal	Gm	Hg
V. anguilarum (18/34)[a]	52	52	36	0	16	49	36	7	1	23	50	8	0	16
V. harveyi (23/18)	41	39	27	1	11	23	20	2	3	11	31	11	1	12
V. proteolyticus (25/14)	39	36	33	1	2	30	27	7	2	14	30	7	4	3
V. alginolyticus (10/0)	10	10	7	0	3	3	9	2	0	10	10	2	0	3
V. campbelli (13/0)	13	13	10	0	3	9	11	7	2	10	6	5	1	3
V. splendidus (2/6)	8	8	3	0	5	7	2	5	1	2	3	1	0	5
V. marinus (2/0)	2	2	2	0	0	0	1	1	0	0	0	2	1	0
V. logei (1/0)	1	1	1	0	0	1	1	1	0	1	1	0	0	0
V. parahaemolyticus (1/0)	1	0	-	-	-	-	-	-	-	-	-	-	-	-
V. Vibrio sp. (halophilic) (15/17)	32	31	29	0	2	25	20	1	0	10	16	18	0	2
TOTAL Nº	199	192 (96.4)[c]	148 (77.0)[d]	2 (1.0)	42 (21.8)	147 (76.5)	127 (66.1)	33 (17.1)	9 (4.6)	81 (42.1)	147 (76.5)	54 (28.1)	7 (3.6)	44 (22.9)

a - Nº isolates from Croaker/Mullet.

b - Su = sulfadiazine; Sm = streptomycin; Tc = tetracycline; Cm; chloramphenicol; Km = Kanamycin; Ap = ampicillin; Nal = nalidixic acid; Gm = gentamicin and Hg = mercury chloride.

c - The percentage = Nº resistant strains/Nº total resistant strains.

d - The values of percentage = Nº resistant strains/ Nº total resistant strains.

rate of more than 85.0% obtained by Toranzo *et al.* (22) for bacterial isolates recovered from fish reared in freshwater hatcheries and tanks and submitted to treatment with antimicrobial agents.

The vibrio strains were mostly resistant to Su and Ap (76.5%) followed by Sm (66.1%), in agreement with previous results obtained for strains of non O1 *Vibrio cholerae* and *V. parahaemolyticus* from sewage water and from marine sources (5). It is worth mentioning that rates of 28.1% and 17.1% were observed, respectively, for the quinolone Nal and for Tc, an elective antibiotic used in the control of vibrioses. In contrast, the lowest rate of resistance (3.6%) was detected for the aminoglycoside Gm, an antibiotic that started to be used only more recently.

Although the numerical distribution of the strains by species was irregular, TABLE 1 shows the absence of resistance in only *V. parahaemolyticus*. Indeed, Magalhães *et al.* (12) obtained resistance just to Ap in this species.

Resistance to heavy metals, associated or not with antimicrobial resistance, has been reported for different bacteria isolated from several sources (4; 9; 15; 21). In the present study, the vibrio strains proved to be Hg^r at a rate exceeding 20% (TABLE 1). However, the 21.8% rate for the association of the two markers (antimicrobial resistance and Hg resistance) was higher than the 1.0% rate of resistance to Hg only.

Of the nine different species analyzed, only three (*V. marinus*, *V. logei* and *V. parahaemolyticus*) did not present the Hg^r phenotype.

When antimicrobial resistance was divided into marker ranges, <3 (28.9%) and ≥3 (71.0%), a significant prevalence ($p<0.05$) of multiresistance was detected for both vibrio sources. Some investigators have emphasized the occurrence of

this pattern among several enteropathogens, including *V. cholerae* (17). However, multiresistance is not an exclusive characteristic of pathogenic strains, since it appears that the bacterial microorganism, including autochthonous marine species such as members of the genus *Vibrio*, may evolutionarily acquire this resistance pattern. On the basis of the hypotheses put forward by Rusu *et al.* (19) on the origin of R plasmids in halophilic vibrio strains from the bacterial microflora of residual waters, which harbors antimicrobial resistance factors, several authors (4; 15) have reported the detection of antimicrobial resistance and/or R plasmids in *Salmonella* serotypes and in *E.coli* strains discharged by sewage treatment plants into the Guanabara Bay.

The observed multiresistance among vibrios isolated between 1986 and 1988 can also be compared to the data reported by Lopes and Moreno (11) and by Dias *et al.*(5). In the early seventies, Lopes and Moreno did not detect multiresistance ($\geq$3 markers) in strains of *E. coli* and *Shigella flexneri* recovered from salt-water fish, whereas Dias *et al.* reported a predominance of biresistant *V. parahaemolyticus* strains isolated from marine sources between 1974 and 1981. These data represent an additional argument in favor of an evolutionary process involved in antimicrobial resistance.

The significant incidence ($p<0.05$) of Hg resistance in strains multiresistant to antimicrobial agents (64.2%) is shown in TABLE 2. In this respect, Lima e Silva (9) observed this resistance pattern in *E. coli* strains from marine fishes, which were also partially the source of vibrio strains. However, in the present study, the association between the two types of markers was observed only in strains isolated from croakers, i.e. from fishes caught along the seashore of the city of Rio de Janeiro, as also reported by Lima e Silva (9). The results did not demonstrate the expected detection of this association in vibrios from mullets, caught in the Guanabara Bay where Hg has been reported to be one of the more significant pollutants (3).

A partial explanation for this fact may relate to the migration of mullets during their life cycle, as also postulated by Lima e Silva (9).

When this relationship was further analyzed, the simultaneous resistance to Hg and to the eight drugs detected revealed a prevalence of association with Su and Ap (71.4%), as reported by Dias *et al.* (5) to be prevalent for the metropolitan region of Rio de Janeiro (TABLE 4). The data also show a lack of uniformity with respect to strain source, with more frequent Hg^r / antimicrobial resistance marker associations occurring in vibrios from croakers.

With respect to marker transfer by conjugation with the standard *E. coli* K12 strain (TABLE 3), the experiments were positive in 71.4% of the cases and included the species *V. anguillarum* and *V. harveyi*. This result indicates the possible

TABLE 2. Numerical and percent distribution of vibrio strains with simultaneous resistance to antimicrobial agents and to mercury chloride according to source, resistance range and markers.

Source	No of strains resistant to the antimicrobial Agents and to Hg			Antimicrobial resistance markers							
	Total	> 3 Markers	≥ 3 Markers	Su[a]	Sm	Tc	Cm	Km	Ap	Nal	Gm
Croaker	24	5 (20.8)[b]	19 (79.1)	13 (54.1)[c]	15 (62.5)	3	2	14 (58.3)	17 (73.8)	16 (66.6)	1
Mullet	18	10 (55.5)	8 (44.4)	17 (99.4)	8 (44.4)	5	1	4	13 (72.2)	3	0
TOTAL	42	15 (35.7)	27 (64.2)	30 (71.4)	23 (71.4)	8	3	18 (42.8)	30 (71.4)	19 (45.2)	1

X - 5.40 (p = 0.05).

a - Su = sulfadiazine; Sm =- streptomycin; Tc = tetracycline; Cm = chloramphenicol; Km = kanamycin; Ap = ampicillin; Nal =\ nalidixic acid and Gm = gentamicin.

b - The values of percentage = Nº strains for the resistance marker classes/nº total strains.

c - The values of percentage = Nº resistance strain/Nº total strains.

TABLE 3. Antimicrobial resistance markers transferred in conjugation experiments

Strains	Resistance Profile Transferred	Nº	Transferred Markers Su	Sm	Nº of Positive conjugations/ Km	AP	Nº of conjugations performed'
	Su[a]	1	1	1			
	Su-Ap	1	1	1			
	Su-Sm-p	7	3	5			
	Sm-Ap-Hg	1			--[b]		
V. anguillarum	Km-Nal-Hg	1			1		13/16
	Su-Sm-Tc-Ap	1			--		(81.2)
	Su-Sm-Km-Ap	2	1	1			
	Su-Sm-Ap-Hg	2		2		1	
	Su	1	1				
	Su-Ap	2	2				
V. harveyi	Su-Sm-Ap	3	1	2			7/8
	Su-Nal-Hg	1			--		(87.5)
	Su-Sm-Km-Hg	1	1				
V. splendidus	Su-Tc	3			--		0/1
TOTAL Nº		28	11 (39.2)[c]	9 (32.1)	2 (7.1)	1 (3.5)	20/28 (71.4)

X - 5.40 (p = 0.05);

a - Su = sulfadiazine; Sm =- streptomycin; Tc = tetracycline; Km = kanamycin; Ap = ampicillin; Nal = nalidixic acid and Gm = gentamicin; Hg = mercury chloride.

b - None transferred marker

c - The values of percentage = Nº strains with transferred marker/Nº total strains.

circulation of these markers *in natura*. It should be pointed out that R plasmid transfer often occurs more effectively between members of a given taxonomic group (3). Thus, one may predict that this transfer occurs *in situ*, also involving strains that are pathogenic to man such as *Vibrio parahaemolyticus* and *Vibrio alginolyticus*.

Of the six different markers present in the vibrio strains and tested for transfer, four (Su, Sm, Km and Ap) were phenotypic expressions of the transconjugants analyzed, with emphasis on Su and Sm transfer (39.2% and 32.1%, respectively). It is important to note that the Hg marker was not transferred, regardless of the *E.coli* K 12 strain utilized, as also observed by Hayashi *et al.* (8). This result may be explained on the basis of the molecular biology of bacterial plasmid, involving not only the nature of the replicon which confers resistance to the ion, but also the mechanism of cell-to-cell transfer (20). However, even taking these points into consideration, one may postulate that marine microorganisms and especially Gram-negative bacteria such as those of the genus *Vibrio*, in addition to *Aeromonas*, may be undergoing a process of acquisition of genetic markers such as resistance to heavy metals and to antimicrobial agents. Obviously, the climax of this situation will be the stability of these markers in bacterial strains present in different marine niches, especially those located in bay waters and along the shore. In these environments, in addition to the selective pressure exerted by pollution from industrial waste containing metal ions, there is also an afflux of microflora consisting of enteric bacilli which harbor these factors, such as *E. coli* and *Salmonella* serotypes (4; 6; 15), the latter deriving especially from sewage treatment plants.

In addition to the studies by Rusu *et al.* on halophilic vibrios (19), the data obtained by Oliveira and Dias (16) on the ability of *Aeromonas hydrophila* and *Vibrio* spp to receive markers of resistance to Hg and to drugs further support this hypothesis.

Monitoring of *Vibrio* strains from marine sources is necessary for the characterization of the genetic elements involved in the circulation of

these markers. The paths of transfer from bacterium to bacterium could be better defined, and the extent of the impact of microbial and/or industrial pollution on autochthonous marine microorganisms could also be established.

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RESUMO

Resistência a Antimicrobianos e ao Cloreto de Mercúrio em Amostras de Víbrios isolados de Peixes Marinhos

Foram analisadas 199 amostras de víbrios isolados de trato intestinal de peixes marinhos, representados pela tainha (*Mugil* sp.) e corvina (*Micropogon* sp.), capturados em águas de baía e da orla litorânea da cidade do Rio de Janeiro, compreendendo *V. anguillarum*(52), *V. harveyi* (41), *V. proteolyticus* (39), *V. campbelli* (13), *V. alginolyticus* (10), *V. splendidus* (8), *V. marinus* (2), *V. logei* (1), *V. parahaemolyticus* (1) e *Vibrio* sp. - halofílico (32). O antibiograma, para oito drogas e o resistograma ao cloreto de mercúrio apontaram a taxa de 96,4% para a presença de marcadores, congregando os percentuais de 77,0% para a resistência antimicrobiana e de 21,8%, para aquela associada ao Hg.

A multirresistência ($\geq$3 marcos) foi significativa ($p<0,05$) na ordem de 71,0%, bem como a incidência de fenótipo Hg^r nas cepas multirresistentes (64,2%), englobando apenas aquelas advindas do pescado corvina. Os marcos Su e Ap, além de Sm mostraram-se em destaque no cômputo geral e também no conjunto de amostras resistentes ao Hg. Indistintamente de espécie, a totalidade das culturas revelou a presença de pelo menos um marcador, excetuando *V. parahaemolyticus*.

A conjugação de 28 amostras de víbrios com cepas de *Escherichia coli* K 12 revelou a transferência de marcadores em 71,4% dos experimentos. Transconjugantes para Su, Sm, Km e Ap foram observados a partir das culturas doadoras de *V. anguillarum* e *V. harveyi*. Não foram isoladas colônias transconjugantes para o marco Hg.

Os resultados indicam a associação entre esses dois marcadores nas amostras de víbrios, possivelmente, em conseqüência do processo de evolução dos marcadores de resistência no bioma marinho.

Palavras-chave: *Vibrio*, resistência a antimicrobianos e a metais pesados, plasmídios, peixes marinhos.

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CONSTRUCTION OF A HYBRID PLASMID CODING FOR ADHERENCE ANTIGEN K88AB AS WELL AS THE B SUBUNIT OF *ESCHERICHIA COLI* THERMO-LABILE ENTEROTOXIN AND MOUSE IMMUNE RESPONSE TO THE ENCODED ANTIGENS

Maria Aparecida V. Paiva e Brito[1]*
Christopher R. Stokes[2]
Peter M. Bennett[3]

ABSTRACT

A hybrid plasmid (pUB 3744) encoding the adherence antigen K88ab and the B subunit of *Escherichia coli* heat-labile enterotoxin (LT-B) was prepared by linking plasmids pFM 205 (K88ab$^+$) and pUB 1844 (LT-B$^+$). The three plasmids were subsequently transferred to the porcine *E. coli* strain O45:K$^-$, producing variants K88ab$^+$, LT-B$^+$ and K88ab$^+$/LT-B$^+$. These variants were orally given to distinct groups of mice on five consecutive days. The isotype-specific antibody response to K88ab, LT-B and to sonicated bacterial antigen was measured after a further five days in serum and scrapings of intestinal mucosa. Excretion of the inoculated bacteria was monitored by culturing fecal samples. Strain LT-B$^+$ persisted longer in the intestine and induced a local immune response to LT-B (IgA, IgG and IgM). No antibody to K88ab was found in mice inoculated with the strain producing only K88ab, but mice inoculated with strain K88ab$^+$/LT-B$^+$ showed an increase in intestinal antibody levels to K88ab. All mice inoculated with the O45:K$^-$ variants had an increase in serum IgA levels to sonicated bacterial antigens, which were higher in animals fed with variant K88ab$^+$/LT-B$^+$.

Key words: Thermo-labile enterotoxin, K88, ETEC, intestinal immune response.

INTRODUCTION

Two families of plasmid-mediated enterotoxins, namely the thermo-labile and the thermo-stable enterotoxins (1), are associated with secretory diarrhoea in piglets. Thermo-labile enterotoxin (LTp-I) is composed of one A subunit and five B subunits. Subunit B is non toxic and is involved in binding of the toxin to receptors on susceptible cells. Subunit A has the ability to stimulate adenylate cyclase and is responsible for the diarrhoeagenic effect (18).

The LT DNA region of P307, a plasmid derived from an *E. coli* strain isolated from pig, was cloned in plasmid EWD299 (5). Sanchez et al. (15) cloned the DNA fragment of EWD299, which expressed the B subunit in plasmid pUB1844.

The DNA fragment coding for adherence antigen K88ab was isolated from a K88ab$^+$ plasmid by Mooi *et al.* (12) and cloned into pBR322 to form plasmid pFM205. Since the cloning vectors of pUB1844 and pFM205 are compatible (3), we constructed a hybrid plasmid by linkage of pFM205 and pUB1844. Next, pFM205, pUB1844 and the composite plasmid were transferred to a porcine

1 EMBRAPA/CNPGL - Rodov. MG 133, km 42 - 36155-000 Coronel Pacheco, MG.
* Corresponding author.
2 University of Bristol, Dept. of Veterinary Medicine, Langford House, Langford, Bristol BS 18 7DU.
3 University of Bristol, Dept. of Microbiology, Medical School, University Walk, Bristol BS8 1TD.

strain of *E. coli*, O45:K⁻. Variants expressing K88ab, LT-B and K88ab/LT-B were obtained. Groups of mice were orally inoculated with these three variants. Mouse inoculation was carried out to verify whether the antigens would be expressed by the vehicle strain in an *in vivo* system and, additionally, whether they would be able to stimulate a humoral immune response.

MATERIALS AND METHODS

Bacterial strains and plasmids - The *E. coli* strains used were the K-12 derivative C600 Lac⁻, Thr⁻, Leu⁻ and Thi⁻ and the porcine wild type O45:K⁻, isolated from piglet. Plasmid pFM 205 (K88ab⁺, ampicillin^r, kindly supplied by F. R. Mooi) (12) is a 7.0 Md plasmid; it contains the 4.3 Md fragment with the K88ab genes inserted into the tetracycline resistance (Tet^r) region of pBR322. Plasmid pUB 1844 (LT-B⁺, chloramphenicol ^{r}) (15) is a 3.2 Md plasmid; it contains the LT-B DNA region of EWD299 (5) cloned into the *Hind*III site of the Tet^r region of pACYC 184 (3). Plasmid pUB3744 was made by linking pUB1844 and pFM205 by their unique *Bam*HI cleavage site, which is located outside the K88ab and LT-B regions. The two plasmids were individually digested with *Bam* HI and ligated with Ligaid (P and S Biochemicals Ltd). The ligated mixture was used to transform C600 (16); transformants were selected on nutrient agar plates containing carbenicillin (100 μg/mL) and chloramphenicol (25 μg/mL). The new plasmid was submitted to restriction with *Eco*RI, *Bam*HI and *Hind*III. Analysis of the fragments obtained showed that pUB1844 and pFM205 were linked. pUB3744, pUB1844 and pFM205 were subsequently transferred to the porcine wild type strain 045:K⁻ (16). Spontaneus choromosomal mutants of nalidixic acid-resistant (Nal^r) O45:K⁻ pUB3744, O45:K⁻ pFM205 and rifampicin-resistant (Rif^r) O45:K⁻ pUB1844 were isolated and used for experimental infections in mice.

Plasmid isolation - The isolation of plasmids was carried out according to the method of Birnboim and Doly (2).

Agarose Gel Electrophoresis - Electrophoresis was carried out in a vertical slab gel apparatus according to Thomas (20). The agarose concentration of gels was 0.7% for isolated plasmids or 1.0% for cleaved plasmids after restriction endonuclease digestions. The electrophoresis buffer used was 89mM Tris base, 89mM boric acid, 2.5mM EDTA (pH 8.2). Bands were developed by incubation of the gels in ethidium bromide and fluorescence visualization under UV light.

Expression of K88ab and LT-B - Production of K88ab was evaluated by mannose-resistant haemagglutination of guinea pig red blood cells (10). LT-B production was tested by G_{M1}-ELISA in whole cell lysates prepared from bacteria cultured in 100 mL of CAYE broth according to Clements and Finkelstein (4). ELISA was carried out as described by Sack *et al.* (14), on microplates coated with 5 μg mL^{-1} bovine type III ganglioside in phosphate buffered saline (PBS), pH 7.4. Binding of LT-B was visualized by addition of 100 μg of rabbit anti-cholera toxin serum to each well. Antiserum dilution was done with PBS containing 0.05% Tween 20, 0.02% sodium azide and 1% foetal calf serum (PBSTFN). Sheep anti rabbit IgG-alkaline phosphatase conjugate diluted to 1:600 with PBSTFN was added and absorbance readings were taken at 405 nm. A standard curve was prepared with the B subnit of cholera toxin (CT-B) and used to estimate the relative concentrations of LT-B.

In vivo experiments - Thirty-two 6 weeks old BALB/c mice from the colony maintained at the Veterinary School, University of Bristol were used. The animals were divided in four groups of eight mice: control group (non-inoculated); K88ab group (inoculated with Rif^r *E.coli* O45:K⁻ pFM205); LT-B group (inoculated with Rif^r *E.coli* O45:K⁻ pUB1844) and K88/LT-B group (inoculated with Nal^r *E. coli* O45:K⁻ pUB3744). The mice were orally given 0.2 mL of bacterial suspension containing 2.0 x 10^9 colony forming unit (CFU) mL^{-1} on five consecutive days. Five days after the last dose, all mice were sacrificed for sampling of blood and intestinal secretion.

Collection of serum and intestinal secretion - Intestinal secretions were obtained from scrapings of the intestinal mucosa (19). The entire small intestine was opened longitudinally and the mucosa gently scraped with a glass slide. Scrapings were collected in 0.5 mL of ice-cold PBS containing protease inhibitors (1 mM PMSF, 1 mM iodoacetate, 10 mM EDTA, 0.1% trypsin

inhibitor and 0.2% sodium azide), kept on ice and sonicated for two minutes. The supernatant was separated by centrifugation (50,000 x g; 4°C; 30 minutes) and stored at -70°C.

Antibody assays- Mouse antibody concentrations in serum and in intestinal scrapings were determined by ELISA. Antibodies to LT-B were evaluated by G_{M1}-ELISA (14). Microplate wells were pre-coated with 100 µg of a 5.0 µg mL^{-1} solution of bovine type III ganglioside in PBS for two hours at 37°C, following addition of a solution of 100 ng mL^{-1} of cholera toxin B subunit in PBSTFN. Antibacterial and anti-K88ab antibody were determined using sonicated bacteria (21) and purified K88ab as solid phase antigens, respectively. The bacterial antigen was prepared from overnight cultures of *E. coli* O45:K^- on nutrient agar at 37°C. The cells were harvested in PBS and formalin was added at a final concentration of 0.2% for microbial inactivation. The bacterial suspension was pelleted and washed in PBS. The pellet was resuspended in 2.0 mL of PBS and the cells disrupted by sonication. The preparation was centrifuged at 100,000 x g for two hours at 4°C; volumes of 100 µl of the supernatant diluted at 1:300 in 50 mM carbonate-bicarbonate buffer (pH 9.6) were used to coat the wells of the ELISA microplates. Purified K88ab antigen was extracted from *E. coli* strain C600 carrying the plasmid pFM205 and purified as described by Mooi and De Graaf (13). The fractions obtained were tested by gel diffusion against antiserum to K88ab and K88ac (sera kindly supplied by the Ministry of Agriculture Food and Fishery Department, Langford, UK). Precipitation reactions were detected only against K88ab antiserum. The purified antigen was also tested by ELISA against the specific antiserum and used to coat the microplates at a concentration of 5.0 µg mL^{-1} in 50 mM carbonate-bicarbonate buffer (pH 9.6).

Assay procedure: Following the coating step, the ELISA microplates were washed. Samples (serum or intestinal scrapings) were then added to the wells, double diluted in PBSTFN and incubated at 37°C for three hours. After another washing step, goat anti-mouse immunoglobulins (IgA, IgG and IgM)-alkaline phosphatase conjugate in PBSTFN was added to the wells; incubation was carried out overnight at 4°C. The substrate p-nitrophenyl phosphate was then added diluted at 1.0 mg/mL in carbonate-bicarbonate buffer. After a final incubation for 100 minutes at 37°C, the microplates were read.

The immune responses of the different groups of mice were compared by the Mann-Whitney *U*-test. P values less than 0.05 were considered significant.

Shedding of the inoculum strain- Shedding of the inoculum strains was examined by culturing one gram of feces per group of mice, collected on days 3, 6, 7 and 9 post inoculation. Each experimental group was transferred to a clean cage and the excreted feces were then weighed and suspended in 1.0 mL of sterile PBS. Serial $\log_{10}$ dilutions of the fecal samples were prepared in sterile PBS and cultured on MacConkey agar containing selective agents (nalidixic acid or rifampicin for the bacterial strains; carbenicillin for plasmid pFM205; chloramphenicol for plasmid pUB1844; carbenicillin and chloramphenicol for plasmid pUB3744).

RESULTS

Digestion of pUB3744 with *Bam*HI and *Hind*III + *Eco*RI showed that the sizes of the fragments produced (FIGURE 1) corresponded to those obtained with pFM205 and pUB1844 after treatment with the same enzymes. This indicates

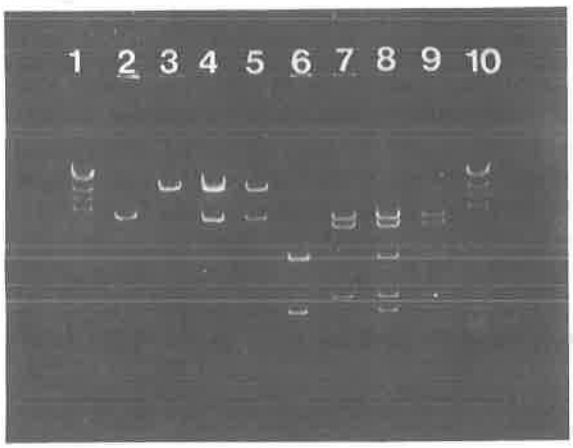

FIGURE 1: Digestion of pFM205; pUB1844 and pUB3744 with restriction endonucleases. Lanes 1 and 10: mixture of lambda DNA digested with *Eco*RI and *Bgl* I. Lanes 2 to 5 correspond to *Bam*HI digestion of pUB1844 (2); pFM205 (3); pUB3744 isolated from C600 (4) and from C600 that was transformed with pUB3744 isolated from O45:K^- (5). Lanes 6 to 9: *Eco*RI + *Hind*III digestion of pUB1844 (6); pFM205 (7); pUB3744 isolated from C600 (8) and from C600 that was transformed with pUB3744 isolated from 045:K^- (9).

that pFM205 and pUB1844 were completely maintained in pUB3744. The physical map is shown in FIGURE 2.

Antigens K88ab and LT-B were produced by the variants of porcine strain O45:K$^-$ Haemagglutination titers did not differ between the bacterial strains (TABLE 1). Negative strains carrying pUB1844 or not carrying recombinant plasmids were included as controls. The concentrations of LT-B were slightly higher for wild type strains.

Shedding of the bacterial strains varied between the experimental groups of mice. Variant O45:K$^-$ pUB1844 (LT-B$^+$) was recovered from feces on all four days sampled (TABLE 2). Variant K88ab$^+$/

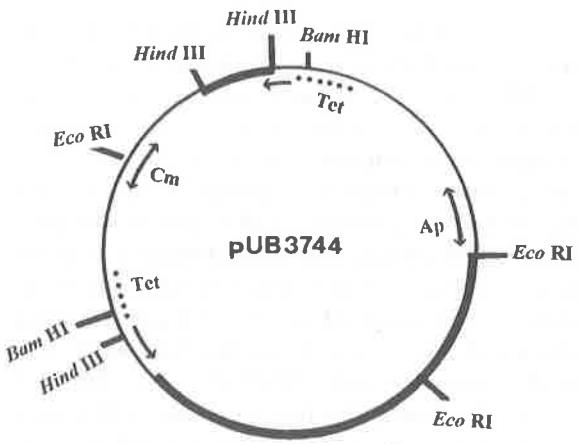

FIGURE 2: Physical map of pUB3744. Bold lines show the LT-B DNA region (pUB 1844) and the K88ab DNA region (pFM205). Single headed arrows indicate the direction of transcription of LT-B and K88ab. Dotted lines refer to the tetracycline resistance region homologous in both cloning vehicles and inactivated by insertion into the *Hind* III sites.

TABLE 1. Estimated concentrations of produced LT-B and titers of mannose-resistant haemagglutination of guinea pig erythrocytes for the bacterial strains used as inoculants in Balb/c mice.

Bacterial strains	LT-B concentrations (μg/ml)	Haemagglutination titers
O45:K$^-$	-	negative
C600	-	negative
O45:K$^-$ pUB3744	3,2	128
O45:K$^-$ pUB1844	3,2	negative
C600 pFM205	-	128
O45:K$^-$ pFM205	-	128
C600 pUB 1844	2,7	negative

TABLE 2. Counts of the inoculated strains in one gram of excreted feces sampled on different days post-inoculation.

Sampling days (post-inoculation)	Groups of mice			
	Control	K88ab	K88ab/LT-B	LT-B
3	0	3.2×10^6	6.6×10^5	1.7×10^7
6	0	1.7×10^7	4.0×10^7	2.4×10^4
7	0	5.0×10^4	0	5.0×10^4
9	0	0	0	4.0×10^2

LT-B$^+$ was recovered only immediately after the last inoculation, whereas variant K88ab$^+$ was eliminated for only two days following the last inoculation dose. None of the bacterial strains was recovered from control mice.

Serum antibody levels (IgA, IgG or IgM) to antigens K88ab and LT-B did not differ significantly between inoculated and control mice. All mice had similar high titers of IgG against the bacterial antigen in serum. Conversely, differences in serum levels of IgA against the bacterial antigen were detected between inoculated and control animals, which were statistically significant at $p<0.001$ for mice exposed to K88ab or K88ab/LT-B and at $p<0.03$ for mice exposed to LT-B (FIGURE 3).

The antibody response to LT-B in the intestinal mucosa is presented in FIGURE 4. Mice inoculated with the LT-B-producing bacterial strain showed a

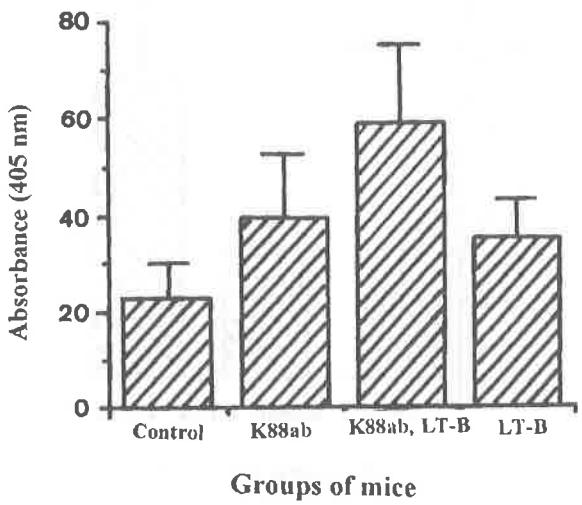

FIGURE 3: Mouse serum IgA responses to the bacterial sonicated antigen five days after the last oral inoculation. The serum samples were diluted 1/8; results are expressed as the mean ñ SD.

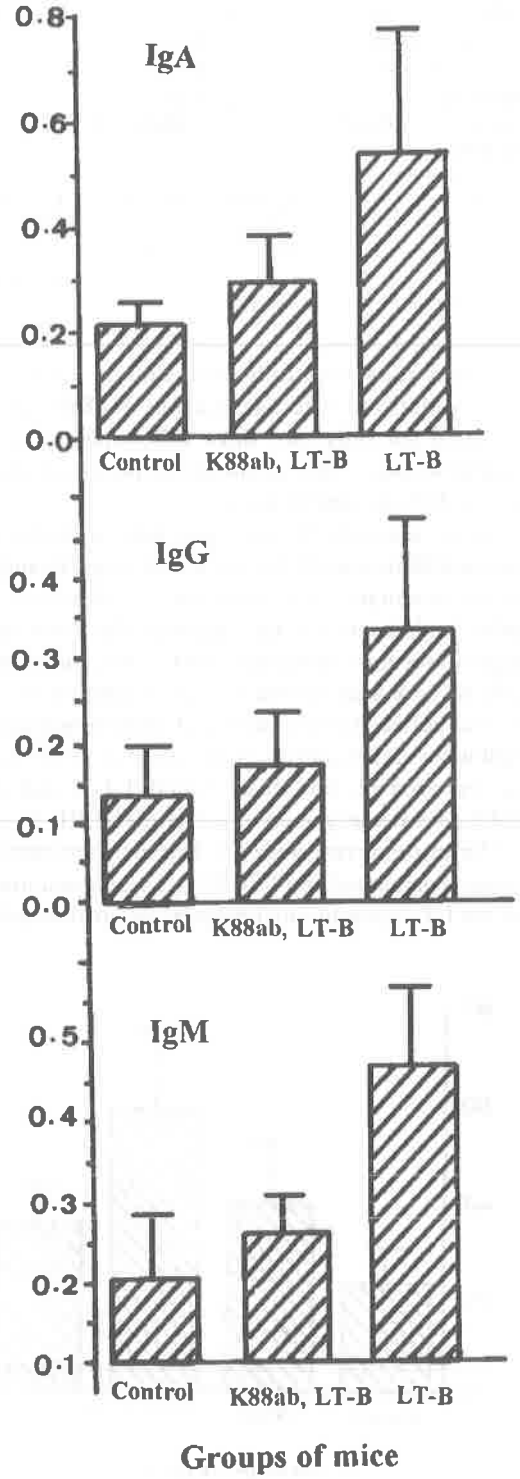

FIGURE 4: Mouse antibody responses to LT-B in intestinal mucosa five days after the last oral inoculation with LT-B-producing *E.coli*. Intestinal scraping were diluted 1/5; results are expressed as the mean ñ SD.

significant increase in IgA, IgG and IgM titers compared with control mice ($p<0.05$). The anti-LT-B response of animals inoculated with the strain that produced both K88ab and LT-B was lower than that obtained with mice inoculated with the LT-B-producing strain, and was not statistically different from control levels ($p>0.05$). No marked production of antibodies against K88ab or the sonicated bacterial antigen was detected in the intestinal mucosa. Mice inoculated with the K88ab$^+$/LT-B$^+$ strain showed a 1.5 fold increase in IgA and IgG levels to K88ab compared with control mice, yet this difference was not statistically significant.

DISCUSSION

The patterns of variant excretion of the distinct groups of mice showed that the bacterial strain that produced only LT-B$^+$ was able to colonize the intestinal tract for a longer period, a feature that probably determined the local response to LT-B. It has been shown that the colonization capacity of a bacterial strain correlates with its ability to induce an intestinal immune response. Hohmann *et al.* (8) inoculated mice with avirulent strains of *Salmonella sp* and *E. coli* or hybrid strains and found that those which did not persist in the intestine were only weakly immunogenic. Similarly, Horsfall and Rowley (9) showed that the magnitude and duration of a local immune response to *Vibrio cholerae* in mice was dependent on both the oral dose and the persistence of the organism in the intestine. The anti LT-B response obtained in this work indicates that the cloned LT-B determinant in pUB1844 was expressed *in vivo*. Although the intestinal immune response to LT-B in mice inoculated with the K88ab$^+$/LT-B$^+$ plasmid did not differ significantly from that of control animals, the titers found were higher than control levels, suggesting that LT-B was also expressed by pUB3744.

In contrast, a secretory response to K88ab was not detected in mice inoculated with the K88ab-producing variants. Nonetheless, mice fed with the K88ab$^+$/LT-B$^+$ strain showed an increase in anti K88ab IgA and IgG titers. Although this difference was not statistically significant, it suggests that the presence of LT-B may have promoted the response to K88ab. Different factors could have influenced the immune response to K88ab. The surface

antigens which confer adhesive properties to *E. coli* have been shown to be host specific, adherence to the intestinal epithelium occurring in a very limited number of host species (7). Likewise, host specificity may have influenced intestinal colonization by the K88$^+$ strains and the weaker response observed with the K88ab$^+$/LT-B$^+$ strains. Although K88 has not been associated with spontaneous disease in species other than swine (1), Laux *et al.* (11) identified and characterized a receptor for K88ab in the small intestine of CD-1 mice. In another study, a recombinant plasmid encoding K88ac in a carrier *Salmonella* strain was fed to BALB/c mice (6) and a serum response was found. In this case the response may have been influenced by the carrier organism.

In summary, the results presented show that the constructed hybrid plasmid expressed the cloned K88ab and LT-B determinants *in vitro* and that LT-B was expressed *in vivo*. LT-B was able to induce an intestinal immune response in mice inoculated with the bacterial strain that carried only this determinant. Mice inoculated with the variant that expressed both K88ab and LT-B presented increased intestinal levels of antibody to LT-B compared to controls, but the difference was not statistically significant. No immune response to K88ab was found. This may have been influenced by the lower intestinal persistence of K88ab$^+$ strains and by the lack of receptors for K88ab in the animals. The vehicle strain was effective as a carrier of the recombinant plasmids, since the variants recovered from mouse feces carried the plasmids resistance markers. The *in vivo* system was effective in evaluating bacteria that produced LT-B only. However, probably because of the lack of K88 receptors in the mice tested, the K88 antigen would be better evaluated in a animal species that naturally expresses the specific binding sites.

RESUMO

Construção de um plasmídio híbrido codificando para K88ab e para a subunidade B da enterotoxina termo-lábil de *Escherichia coli* e a resposta imune de camundongos aos antígenos codificados

Um plasmídio híbrido (pUB3744) codificando para o antígeno de aderência K88ab e para a subunidade B da enterotoxina termo-lábil (LT-B) de *Escherichia coli* foi construído pela ligação dos plasmídios pFM205 (K88ab$^+$) e pUB1844 (LT-B$^+$). Estes três plasmídios foram subseqüentemente transferidos para a amostra de *E. coli* O45:K$^-$, isolada de suíno, obtendo-se variantes K88ab$^+$, LT-B$^+$ e K88ab$^+$/LT-B$^+$. Estas variantes foram inoculadas por via oral em grupos de camundongos durante cinco dias consecutivos. A resposta de anticorpos isotipo-específica para K88ab, LT-B e para antígeno de bactérias sonicadas foi medida no soro e em raspados da mucosa intestinal cinco dias após a última inoculação. A excreção da bactéria foi avaliada cultivando-se amostras de fezes. A bactéria LT-B$^+$ foi eliminada por mais tempo pelos camundongos e induziu uma resposta imune local para LT-B (IgA, IgG e IgM). Não foram detectados anticorpos para K88ab nos camundongos inoculados com a bactéria produzindo somente K88ab, mas o grupo de camundongos inoculado com a bactéria K88ab$^+$/LT-B$^+$ apresentou um aumento de anticorpos para K88ab nos raspados da mucosa intestinal. Todos os camundongos inoculados com as variantes O45:K$^-$ apresentaram um aumento de anticorpos séricos para o antigeno de bactérias sonicadas e os níveis foram mais elevados no grupo inoculado com a variante K88ab$^+$/LT-B$^+$.

Palavras-chave: Enterotoxina termo-lábil, K88, ETEC, resposta imune intestinal.

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VIRULENCE FACTORS OF *ESCHERICHIA COLI* STRAINS ISOLATED FROM DIARRHEIC CHILDREN: INFLUENCE OF STORAGE CONDITIONS.

Loreny G. Giugliano[1]
Andrea N. Araujo[1]
Iriane C. Piva[1]
Terezinha M. Inham[2]
Lucia R. Ferraz[2]
Beatriz C. Guth[3]

ABSTRACT

The frequency of isolation of enterotoxigenic (ETEC), verotoxigenic (VTEC) and necrotizing *Escherichia coli* (NTEC) was determined in 167 children with diarrhea from a central area of Brazil. *E.coli* strains producing verotoxin (VT), cytotoxic necrotizing factor (CNF) and thermolabile enterotoxin (LT) were found in 3%, 3.6% and 7.2% of the cases, respectively. The *E.coli* strains were also assayed for their ability to induce mannose resistant haemagglutination (MRHA) of human and bovine erythrocytes. MRHA+ strains were observed in 42% of the cases and colonization factor antigens I and II were identified in 32% of the MRHA+ cases. After long term storage at room temperature, the stability of virulence factors produced by 28 selected isolates was evaluated. Production of cytotoxins was maintained in 3 of the 5 VT+ and in 5 of the 6 CNF+ isolates. Expression of LT was maintained in 9 of the 12 LT+ isolates. Of the 23 CFA+ strains, only 7 maintained the haemagglutination pattern. Expression of ST was detected in 4 strains and, besides those, 4 further ST+ strains were recognized by colony hybridization. The LT genotype was found in 11 of the selected isolates. In conclusion, by studying strains of *E.coli* isolated from children's diarrhea, NTEC strains were identified for the first time in Brazil. This study also confirms the great importance of storage conditions for the detection of virulence factors of ETEC and shows that VTEC and NTEC may lose its cytotoxin producing ability after prolonged storage at room temperature.

Key words: virulence factors, enterotoxins, cytotoxins, cytotoxic necrotizing factor (CNF).

INTRODUCTION

Diarrheal diseases are an important cause of mortality and morbidity among infants in developing countries. An assessment of the relative importance of several possible pathogens in different areas is feasible through the identification of etiologic agents. Although it has been known for more than

1 To to whom all correspondence should be sent, at the address: Laboratório de Microbiologia, Dept. de Biologia Celular, Instituto de Biologia, Universidade de Brasília, CEP 70910-900, Brasília, DF, Brazil.
Phone: (+55-61) 348-2176. Fax: (+55-61) 272-1497.

2 Instituto de Saúde do Distrito Federal, Gerência de Biologia Médica, SGAN Q601 lotes O e P, CEP 70830-010, Brasília, DF, Brazil

3 Disciplina de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina, Rua Botucatu 862, CEP 04023-062, São Paulo, Brazil.

40 years that some strains of *E.coli* were associated with infant diarrhea (1,13,33), only recently it became clear that diarrheagenic *E.coli* includes heterogeneous group of enteric pathogens which can be responsible for a great proportion of diarrheal diseases (3,34,40) These groups of *E.coli* present distinct pathogenic mechanisms, comprising production of enterotoxins and cytotoxins, invasiveness and characteristic adhesion to the intestinal epithelium (36,39).

Enterotoxigenic *E.coli* (ETEC) strains cause diarrhea by producing heat labile (LT) and /or heat stable (ST) enterotoxins. Added to the production of enterotoxins, ETEC strains also possess colonization factor antigens (CFAs). These are serologically distinct fimbriae or fimbria like structures that enable ETEC strains to attach and colonize the intestinal epithelium (36,39). Although several CFAs or putative CFAs (PCFs) have already been described, the most studied and frequently isolated CFAs of human ETEC strains are CFA/I and CFA/II (7,18,41). In general, the production of CFAs are plasmid mediated (22). Genes coding for CFA/I and ST production have been found on the same plasmid (52), while genes coding for CFA/II production have been associated with plasmids encoding both LT and ST enterotoxins (46). It has been shown that these genes may be lost during laboratory storage and passage of the strains (2,20).

Mannose resistant haemagglutination of different species of erythrocytes has been largely used for screening CFA production after the demonstration that CFA/I-carrying strains agglutinate human type A, bovine and chicken erythrocytes, whereas CFA/II-carrying strains agglutinate bovine and chicken but not human erythrocytes (23). The presence of CFA/I or CFA/II must however be confirmed with specific CFA antisera (24).

Verotoxin producing *E.coli* (VTEC) strains were first identified by Konowalchuk *et al.* (38) and subsequently associated with diarrhea, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (35,37,42). VTEC of serotype O157:H7 has been most frequently isolated from HC and HUS (17,51). However, other serotypes of VTEC strains have been isolated and associated with infant diarrhea (12,37,42). Two main antigenically distinct verotoxins (VT1 and VT2) active on Vero cells have been described (45), and the genes coding for their production have been found in temperate bacteriophages (45).

Necrotizing *E.coli* (NTEC) strains are able to produce a cytotoxic necrotizing factor (CNF) that was first recognized in *E.coli* strains isolated from diarrhea (15). Subsequently, the factor was also found in strains causing urinary tract infections and bacteremia in humans (9,16). This cytotoxin induces enlargement and multinucleation of Vero and HeLa cells, necrosis in rabbit skin and is also lethal to adult mice (10,15). Most of the NTEC isolated from humans were shown to produce haemolysin (Hly), another *E.coli* toxin whose role in pathogenesis is not clear. Studies by Falbo *et al.* (29) showed the linkage of genes encoding the production of CNF and Hly on the chromosome of extraintestinal NTEC isolates. Additionally, it has been suggested that NTEC strains might be opportunistic pathogens causing diarrheal disease in humans (5,8).

The present study reports the frequency of isolation of ETEC, VTEC and NTEC from children's diarrhea in a central area of Brazil, and evaluates the stability of virulence factors produced by these pathogenic groups after long-term storage.

MATERIAL AND METHODS

Bacterial strains and storage conditions

E.coli strains were isolated from fecal specimens of 167 diarrheic children, aged 0 to 36 months, which attended the out-patient clinics of two local hospitals within the area of Brasília, DF. An average of three *E.coli* isolates per child was obtained by standard procedure (26). The strains were maintained in liquid media with 15% glycerol at -20°C until tested for LT , VT, CNF and colonization factor antigens CFA/I and CFA/II. The strains were then stored at room temperature in nutrient agar for 30 months and subsequently retested for the same virulence factors mentioned above and also for ST.

Toxin assay

For detection of LT, VT and CNF, Vero cells freshly seeded in 96 well microtitration plates were used as previously described (31). In all assays,

control reference strains were included, namely: H296 (LT+) provided by Dr. H.W. Smith (Houghton Poultry Research station, Huntingdon, Cambridgeshire, UK); 933 (VT+) provided by Dr. A. O'Brien (Uniformed Services University of the Health Science, Bethesda, Maryland, USA); and ISS-2 (CNF+) provided by Dr. A. Caprioli (Istituto Superiore di Sanitá, Rome, Italy).

Production of ST was studied in 21 cases positive for CFA/I or CFA/II antigens (17 CFA/I+ and 4 CFA/II+ cases) using the infant mouse assay described by Dean et al (19). The test was carried out 30 months after CFA detection.

Haemagglutination.

All *E.coli* strains were assayed for their ability to induce haemagglutination of human group A (H) and bovine (B) erythrocytes. The erythrocytes were washed in phosphate buffered saline (PBS) and suspended to 5%. Haemagglutination activities were examined by mixing on a glass slide one drop of erythrocytes suspension with or without D-mannose (1%) and bacterial growth, obtained after 18 hours incubation at 37°C in CFA medium and harvested with a sterile wooden stick as described by Evans and Evans (25).

Agglutination with anti-colonization factor antigens (CFA) sera:

Specific polyclonal CFA antisera were prepared in rabbits by the procedure of Evans *et al.* (22) using *E.coli* strain TR50-3 (CFA/I^+) and PB176 (CFA/II^+). Crude antisera were absorbed with live cells of a segregant strain TR50-3P (CFA/I^-) and of PB 176P (CFA/II^-) obtained by growing PB176 at 18°C.

Haemolysis test

The strains were tested on 5% rabbit blood agar plates, and haemolysin production was recorded after incubation at 37°C for 18 hours.

Serotyping and neutralization of the VT producing E.coli.

The VT producing *E.coli* strains were tested by slide agglutination with O157:H7 antiserum (Probac do Brasil). Cytotoxin neutralization assays were performed in Vero cells as previously described (43) using anti VT1 and VT2 antisera provided by Dr. A. O'Brien.

DNA probes and colony hybridization

The DNA sequences used as probes for *E.coli* enterotoxins were kindly provided by Dr. Nataro (Center for Vaccine Development, University of Maryland., School of Medicine , Baltimore, Maryland , USA) and were as follows: the 1200bp HincII fragment of pCVD403 (29) for detection of LT; the 216bp Eco R1 fragment of pCVD427 and the 157bp Pst I fragment of pCVD 426 for detection of STaH and STaP, respectively. DNA fragments were labelled by random priming with (a32P) dATP (3000Ci/mmol, Amershan International plc, UK) and a random primers DNA labelling kit (New England Biolabs, USA). Colony blots were prepared, processed and hybridized under high stringent conditions as previously described (49). The *E.coli* strains H 10407 and HS, also provided by Dr. Nataro, were used as positive and negative controls, respectively.

RESULTS

Expression of virulence factors before storage at room temperature

E.coli strains producing VT, CNF and LT toxins were correspondingly found in 5 (3%), 6 (3.6%) and 12 (7.2%) of the 167 cases studied. Production of haemolysin was detected in all CNF-producing strains.

Mannose resistant haemagglutination (MRHA+) was observed in 71 (42%) of the 167 cases. Agglutination of human and bovine (HB+) erythrocytes was the pattern most frequently found (62%), while agglutination of only human (H+) or bovine (B+) erythrocytes occurred in 15 (21%) and 12 (17%) of the MRHA+ cases, respectively (TABLE 1).

CFAs were identified in 23 (32%) of the 71 MRHA+ strains: CFA/I occurred in 19 of the 44 HB+ cases and CFA/II in 4 of the 12 B+ ones. Production of LT toxin was observed in 8 (11%) of the 71 MRHA+ stains, 7 being positive for CFAs (TABLE 1).

TABLE 1. Haemagglutination pattern and production of CFA/I, CFA/II and LT enterotoxin in *E.coli* strains isolated from diarrheic cases.

Haemagglutination pattern[a]	Nº of cases (n= 167)	Nº of cases positive for CFA/I	CFA/II	LT
MRHA+ -H	15	nt[b]	nt	1
-B	12	nt	4	1
-HB	44	19	nt	6
MRHA-	96	nt	nt	4

[a] MRHA+, mannose resistant haemagglutination; H, human erythrocytes; B, bovine erythrocytes; HB, human and bovine erythrocytes; MRHA-, no haemagglutination.

[b] nt, not tested.

TABLE 2. Detection of virulence factors in 28 selected *E.coli* strains after storage.

Haemagglutination pattern *a*	N° of strains (n = 28)	N° of strains presenting Expression of LT	ST *b*	Genes for LT	ST
MRHA+ -H	7	1	0	2	1
-B	1	0	0	0	0
-HB	6	2	3	3	3
MRHA-	14	6	1	6	4

[a] MRHA+, mannose resistant haemagglutination; H, human erythrocytes; B, bovine erythrocytes; HB, human and bovine erythrocytes; MRHA-, no haemagglutination.

[b] assayed in 21 strains.

Expression of virulence factors after 30 months of storage at room temperature

The 11 cytotoxin-producing *E.coli* strains were retested with the Vero cells assay. The ability to induce cytotoxic effects was maintained in 3 of the 5 VT+ *E.coli* isolates and in 5 of the 6 CNF+ isolates. CNF-producing *E.coli* strains maintained their ability to produce haemolysin, but this characteristic was not observed in the strain that lost CNF.

None of the VT-producing *E.coli* isolates belonged to serotype O157:H7. Neutralization assays with specific antisera showed that the remaining VT+ strains belonged to one of the toxigenic phenotypes VT 1, VT 2 or VT 1 / VT 2.

Twenty-eight selected strains comprising 19 CFA/I+, 4 CFA/II+ and 5 LT/MRHA- isolates were retested with the Vero cells assay and MRHA. Expression of LT was maintained in only 9 of the 12 LT+ *E.coli* strains after storage. The MRHA assays showed that 9 strains became negative for haemagglutination of human and/or bovine erythrocytes during storage, whereas 7 strains changed their haemagglutination response from HB+ to H+. Of the 23 CFA+ strains, only 7 maintained their original haemagglutination pattern (TABLE 2).

After storage, 21 strains (17 CFA/I+ , 4 CFA/II+) were assayed for ST production with the infant mouse assay. Expression of ST was detected in 4 cases, 2 of which had been classified as CFA/I+ and 2 CFA/I+ / LT+ before storage (TABLE 2).

Colony hybridization of the 28 selected strains showed that, besides the 4 strains found to express ST, 4 more ST+ isolates were present, of which 3 had not been tested for ST expression and 1 had not been detected as ST+ by the infant mouse assay. In addition to the 9 cases which maintained the expression of LT after storage, 2 more strains where no expression of LT had been detected before or after storage appeared LT+ by colony hybridization.

DISCUSSION

Some studies suggest that NTEC might be opportunistic pathogens in diarrheal diseases of humans. However, very few investigations have determined the incidence of NTEC strains in infant diarrhea. In the present study, we isolated NTEC strains at a lower frequency (3.6%) than that reported in Italy (6%) (5) or Spain (20%) (11). The differences could be explained by geographical variation, but further studies should be carried out to confirm this assumption. To our knowledge, this is the first report on isolation of NTEC from cases of diarrhea in our country.

Non O157:H7 VTEC have been isolated from sporadic cases of diarrhea at a low frequency. Several studies in Brazil, North America and Europe reported the occurrence of non O157:H7 VTEC in unselected cases of children's diarrhea at frequencies varying from 0.1% to 1.1% (12,14,30,47,48). However, Lopez *et al.* (42) found non O157:H7 VTEC in 7% of diarrheic Argentinian children and suggested a relationship between the high incidence of non O157:H7 VTEC and the high

incidence of HUS in Argentina (28). This is consistent with the observation in other countries of a low incidence of HUS concomitant with a low incidence of VTEC associated diarrhea (50). We found non O157:H7 VTEC in 3% of the cases of diarrhea in the present study, a value higher than that recorded in developed countries but lower than the incidence observed in Argentina. Since, to our knowledge, there are no reports on the incidence of HUS in the studied area, further research is necessary to characterize the role of non O157:H7 VTEC in local diarrheal cases and its possible association with HUS, now recognized as a complication of infection caused by VTEC.

Storage of VTEC and NTEC at room temperature led to loss of cytotoxicity in some strains, demonstrating the importance of storage conditions for the expression of these cytotoxins.

The frequency of isolation of ETEC from cases of diarrhea in developing countries has been shown to vary between 6% and 27%. In general, ST producing strains are more frequent than the other toxigenic phenotype (3,4,6,21,32,34,40,41,44). In this study, LT producing strains were found in 7% of the cases of diarrhea when analysed before storage; however, at that time, isolates expressing CFA/I or CFA/II antigens were identified at a frequency of 14%. Since ST was not assayed before storage and neither CFA/I nor CFA/II antigens have been demonstrated in non-ETEC strains (4,18), all the CFA+ strains negative for LT could have been ST producers.

After a long period of storage at room temperature, CFA/I+ and CFA/II+ strains were retested for enterotoxin production and presence of genes for LT and ST. Not only enterotoxin detection was negative for most of these strains but the majority had also changed their haemagglutination pattern, indicating that they had lost the ability to produce CFAs. It has been shown that, in general, the loss of CFA/I and CFA/II production is associated with the loss of sequences encoding for ST (20), thus these results may be explained by loss of plasmid or of sequences of DNA encoding for CFA production.

In contrasts with data from most surveys, where ST+ strains are described as the most frequent among ETEC (3,4,6,21,32,34,40,41,44), the frequency of phenotype ST+ observed in the present study was lower than that for LT+. This also suggests that the CFA/I+ and CFA/II+ isolates detected before storage may have lost, besides the ability to produce CFAs, also the ability to produce ST.

On the other hand, the fact that some of the strains that still bore the genes for production of enterotoxins after storage changed their haemagglutination pattern indicates that, in some cases, the loss of enterotoxin production and CFA expression may occur independently.

Even considering all the strains that presented some of the ETEC virulence factor assayed, which account for 14% of the diarrheic cases, the data may be a underestimate of the frequency of ETEC in this survey, since detection of ST-producing strains expressing CFAs distinct from CFA/I and CFA/II was excluded from the tests.

By studying *E.coli* strains from diarrheic children of a central area of Brazil, NTEC were identified for the first time in our country. Moreover, this study confirms the great importance of storage conditions for the detection of virulence factors in ETEC and shows that VTEC and NTEC can also lose their cytotoxin producing ability after prolonged storage at room temperature.

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RESUMO

Fatores de virulência de cepas de *Escherichia coli* isoladas de crianças diarreicas: influência das condições de estocagem

A freqüência de isolamento de *Escherichia coli* enterotoxigênica (ETEC), verotoxigênica (VTEC) e necrotizante (NTEC) foi determinada em 167 crianças com diarréia de uma área central do Brasil. Cepas de *E.coli* produtoras de verotoxina (VT), fator citotóxico necrotizante (CNF) e enterotoxina termo lábil (LT) foram encontradas, respectivamente, em 3%, 3,6% e 7,2% dos casos. As cepas de *E.coli* foram também testadas quanto à capacidade de induzir hemaglutinação manose

resistente (MRHA) de eritrócitos humanos e bovinos. Cepas MRHA+ foram observadas em 42% dos casos e os fatores de colonização I e II foram identificados em 32% dos casos MRHA+. Após longo período de estocagem a temperatura ambiente, a estabilidade dos fatores de virulência produzidos por 28 cepas selecionadas foram reavaliados. A produção de citotoxinas foi mantida em 3 de 5 cepas VT+ e em 5 de 6 cepas CNF+ . A expressão de LT foi mantida em 9 de 12 cepas LT+. De 23 cepas CFA+ somente 7 mantiveram o padrão de hemaglutinação. Expressão de ST foi detectada em 4 cepas. Usando o método de hibridização de colônias além das 4 cepas que expressaram ST, outras 4 cepas ST+ foram detectadas. O genótipo LT foi encontrado em 11 das cepas selecionadas. Em conclusão, através do estudo de cepas de *E.coli* isoladas de crianças diarreica, cepas NTEC foram identificadas pela primeira vez no Brasil. Este estudo também confirma a grande importância das condições de estocagem para a detecção de fatores de virulência de ETEC e ainda mostra que VTEC e NTEC podem perder a capacidade de produzir citotoxinas após longos períodos de estocagem.

Palavras-chave: fatores de virulência, enterotoxinas, citotoxinas, fator citotóxico e necrotizante (CNF).

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CHARACTERIZATION OF NECROTIZING *ESCHERICHIA COLI* STRAINS ISOLATED FROM DIARRHEIC CHILDREN IN BRAZIL

Loreny G. Giugliano[1]*
Jorge Blanco[2]
Miguel Blanco[2]
Jesus E. Blanco[2]
Rodolfo Giugliano[3]

ABSTRACT

Seventeen strains of *Escherichia coli* were isolated during etiologic studies of diarrheic children in Brazil. Culture supernatants of these strains induced morphological changes in Vero cells similar to the alterations elicited by the cytotoxic necrotizing factor (CNF). Since CNF is considered a cell associated toxin normally undetectable in culture supernatants, we undertook this study to further characterize these *Escherichia coli* isolates. The strains were assayed for the presence of CNF in culture supernatants and sonic extracts, necrotizing activity, haemolysin production, mannose resistant haemagglutination (MRHA), cell surface hydrophobicity and serogrouping. All the isolates caused necrosis in rabbit skin and typical CNF1-induced morphological changes that could be observed assaying both the sonic extracts and culture supernatants. Haemolysin and hydrophobicity was found in 76% and 54% of the strains, respectively. Haemagglutination tests with erythrocytes of 6 distinct species showed that all the strains belonged to MRHA type III or IVa. Serogrouping showed that most of the strains (14/17) belonged to serogroup O6, two to serogroup O14 and one to serogroup O4. To conclude, we have shown that NTEC strains can be isolated from children with diarrhea in Brazil. These isolates produce CNF1 detectable in both culture supernatants and cell extracts and possess characteristics very similar to those of NTEC strains isolated in Europe.

Key words: necrotizing *Escherichia coli* , virulence factor, cytotoxic necrotizing factor (CNF)

INTRODUCTION

The ability of certain strains of *E.coli* to produce cytotoxic necrotizing factor (CNF), a toxin that causes multinucleation and enlargement in cultured cells and necrosis in rabbit skin, was first described by Caprioli *et al.*(12) in strains isolated from diarrheic children. Subsequently, CNF-producing *E.coli*, now known as necrotizing *E.coli* (NTEC), was isolated from animal enteritis (5,15) and from human extraintestinal infections (2,13).

NTEC may produce 2 types of CNF (CNF1 or CNF2) that can be distinguished by morphological alterations induced in HeLa cells, cross-neutralization assay (17),

1 Departamento de Biologia Celular , Universidade de Brasília, Brasília, DF, Brasil.
* To whom all correspondence should be sent, at the address: Laboratório de Microbiologia. Dept. de Biologia Celular, Instituto de Biologia, Universidade de Brasília, Brasília 70910-900, DF, Brazil. Phone: (061) 348 2176. Fax: (061) 272 14 97
2 Departamento de Microbiologia , Universidade de Santiago de Compostela, Lugo ,Spain.
3 Departamento de Nutrição, Universidade de Brasília, Brasília , DF, Brasil.

specific necrotizing activity of CNF2 in mouse footpads (16) and other virulence factors associated with NTEC (17,6,8). Furthermore, CNF1 is chromosomally encoded (18) whereas CNF2 is encoded by plasmid (22). Both necrotizing toxins are considered cell-associated products which can only be detected when the bacteria are disrupted by sonication or when they are grown in the presence of mitomycin C (12,7).

In Europe, most of the NTEC producing CNF1 were isolated from human extraintestinal infections and belonged mainly to serogroups O2, O4, O6, O14, O22, O75 and O83, whereas the NTEC producing CNF2 were principally obtained from diarrheic calves or septicemia and the majority belonged to serogroups O1, O3, O15, O88 and O123 (9).

The present work reports the characteristics of NTEC strains isolated during etiologic studies of sporadic cases of diarrhea in two regions of Brazil (North and Mid West).

MATERIALS AND METHODS

Bacterial strains

Seventeen toxigenic *E.coli* strains isolated from diarrheic children from Manaus (North of Brazil) and Brasília (Mid West) were characterized. The strains were stored at room temperature in nutrient agar (Difco).

Production and detection of CNF

Culture supernatants, filtrates of cultures treated with mitomycin C and sonic extracts of each strain grown in Tryptone Soya Broth (Oxoid) for 20h at 37oC were assayed on Vero and HeLa cell monolayers as previously described (5,7). Briefly, 2.4x104 cells per well of the Vero or HeLa cell lines were seeded on a microtiter plate and incubated at 37oC, to obtain a monolayer. One hundred microliters of each *E.coli* strain preparation were then added to a distinct monolayer and the cultures were incubated at 37oC in an atmosphere of 5% CO2. Morphological changes induced in the Vero or HeLa cells were observed under a phase contrast inverted microscope 24h and 48h after inoculation. Reference CNF1+ strains ISS2 and ISS51 from Caprioli *et al.* (12) and CNF2+ strain S5 described by Blanco *et al.* (10) were used as positive controls.

Haemolysin production

Haemolysin production was detected after 24h of growth on blood agar base medium (Merck, FRG) containing 5% (v/v) of washed erythrocytes. The reference CNF1+ strains ISS2 and ISS51 from Caprioli *et al.* (12) previously described as Hly+ were used as positive controls.

Necrotizing activity

In vivo necrotizing activity was assayed by the rabbit skin test using filtered sonic extracts as previously described (5). Briefly, the backs of New Zealand albino rabbits were shaved and 0.1mL of each sonic extract was injected intradermally. Forty-eight hours after inoculation, the presence of necrotic reactions was assessed by inspection of ulceration and induration at the injection sites. Reference CNF1+ strains ISS2 and ISS51 from Caprioli *et al.* (12) and CNF2+ strain. S5 described by Blanco *et al.* (10) were used as positive controls.

Serogrouping

Serogrouping was carried out with the microtechnique described by Guinée et al.(20) using 101 antisera, selected on the basis of their reported association with pathogenic *E.coli* that cause infections in humans and animals (21). The antisera were obtained from the National Institute of Public Health and Environmental Protection (Bilthoven, The Netherlands) and Difco Laboratories (USA).

Haemagglutination test

Mannose-resistant haemagglutination (MRHA) of the strains grown on CFA medium (19) was determined by the rocked-tile method at 4oC and at room temperature, using human group A, calf, guinea-pig, adult chicken, sheep and pig erythrocytes, as previously described (6). The strains were classified according to their MRHA pattern as established by Blanco *et al.*(3,4).

Cell surface hydrophobicity

Relative cell surface hydrophobicity was measured by the improved salt aggregation test (ISAT) with suspensions (5x109cfu/mL in 0.02M

phosphate buffer, pH 6.8) of bacterial growth on CFA medium.(19). The final molar ammonium sulfate concentrations used were 2.0, 1.4, 1.0, 0.4, 0.1, 0.06 and 0.02M. Strains were considered hydrophobic when they aggregated in ò1.4M ammonium sulfate concentrations.

RESULTS AND DISCUSSION

During etiologic studies of diarrheic children in Brazil, we isolated 17 *E.coli* strains whose culture supernatants induced morphological changes in Vero cells similar to the CNF-elicited alterations described by Caprioli *et al.*(12). The isolates caused morphological changes typically induced by CNF1. CNF1 was detected in sonic extracts, filtrates of cultures treated with mitomycin C and in culture supernatants. Culture supernatants of the reference strains ISS2 and ISS51 used as positive controls also caused the same effect. The detection of CNF1 in culture supernatants of the NTEC isolates might be due to cell lysis during normal bacterial growth. A greater susceptibility of the cell lines utilized in the tissue culture assay could also explain this result. Further studies will be necessary to verify these hypotheses.

A study of NTEC strains isolated in Spain (9) showed that NTEC from humans usually produce CNF1, whereas strains obtained from animals generally produce CNF2. Our data, which relate to isolates of human origin, are in agreement with these findings.

TABLE 1 summarizes the results obtained with the rabbit skin test, serogrouping, production of haemolysin and cell surface hydrophobicity assay. The 17 isolates induced necrosis in rabbit skin and thus fulfill the criterion for definition of the NTEC group.

Production of haemolysin has been shown to be a common feature of CNF1 producing strains (13,9). In this study we found that 13 of the 17 NTEC isolates were hemolytic.

It has been reported that high levels of cell surface hydrophobicity facilitate bacterial adhesion (1,19). Since the adhesion ability is an important phenotype for colonization, the cell surface hydrophobicity of the NTEC isolates was analyzed. The results shown in TABLE 1 reveal that most of these strains were hydrophobic at varying levels.

TABLE 1. Necrotizing activity, production of haemolysin, relative cell surface hydrophobicity and serogroups of the NTEC strains isolated in Brazil

Strain	RST	Hly	ISAT (M)	Serogroup
SJ8	+	+	0.02	O6
SJ175	+	-	2.0	O14
SJ218	+	+	>2.0	O6
SJ285	+	-	nt	O6
SJ286	+	-	0.4	O6
SJ298	+	+	0.4	O6
SJ310	+	+	1.4	O6
35F/1	+	+	>2.0	O6
V11/1	+	+	>2.0	O6
V11/2	+	+	1.4	O4
V22/1	+	+	nt	O6
09F/4	+	+	0.4	O6
4HMT	+	+	nt	O14
49/4	+	+	0.4	O6
50/2	+	+	aa	O6
54/1	+	-	0.4	O6
264/1	+	+	>2.0	O6

RST = rabbit skin test assay
Hly = production of haemolysin
ISAT = improved salt aggregation test ; M = molar

TABLE 2 - Haemagglutination pattern and MRHA types of the NTEC strains isolated in Brazil

Strain	Erythrocytes						MRHA type
	H	B	GP	Ck	Sh	Pg	
SJ8	+	+*	+*	+	+	+	IVa
SJ175	+	+*	+*	+	+	+	IVa
SJ218	+	-	+*	+	+	+	III
SJ285	nt	nt	nt	nt	nt	nt	nt
SJ286	+*	-	-	-	+*	+	III
SJ298	+	+*	+*	+	+	+	IVa
SJ310	+*	+*	-	-	+*	+	III
35F/1	+*	-	-	-	+	+*	III
V11/1	+	+*	+*	+*	+	+	IVa
V11/2	+	-	-	+	+	+	IVa
V22/1	nt	nt	nt	nt	nt	nt	nt
V22/2	+	-	+*	+	+	+	IVa
09F/4	+	+*	+*	+	+	+	IVa
04HMT	nt	nt	nt	nt	nt	nt	nt
49/4	-	-	-	-	-	-	-
50/2	+	+*	+*	+*	+	+	IVa
54/1	+	+*	+*	+	+	+	IVa
264/1	-	-	-	-	-	-	-

H = human group A; B= bovine; GP= guinea-pig; Ck= chicken; Sh= sheep; Pg= pig
+* haemagglutination only after incubation at 4oC.
+ haemagglutination at room temperature
- no haemagglutination
nt not tested

Serogrouping of the 17 NTEC isolates revealed that 14 belong to serogroup O6, two to serogroup O14 and one to serogroup O4. Serogrouping of NTEC strains isolated from humans in Italy (14) and Spain (9) showed that these toxigenic *E.coli* generally belong to serogroups O2, O4, O6, O14, O22, O75 and O83, serogroup O6 being the most frequently found. Therefore, our results confirm that CNF1 strains of human origin usually belong to a restrict number of serogroups, type O6 being the most common.

The MRHA pattern with the six erythrocyte species used is presented in TABLE 2. All the MRHA+ strains belonged to MRHA type III or type IVa established by Blanco et al. (3,4). This is in agreement with a previous study (11) which showed that most of the NTEC CNF1+ can be classified as type III or IVa.

In conclusion, we have shown that the NTEC strains isolated from diarrheic children in Brazil produce CNF1 detectable in culture supernatants and possess characteristic very similar to those of toxigenic *E.coli* isolates from Europe.

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RESUMO

Caracterização de cepas de *Escherichia coli* necrotizantes isoladas de crianças com diarréia no Brasil

Durante estudos epidemiológicos de casos de diarréia em crianças de duas regiões do Brasil, 17 cepas de *Escherichia coli* foram isoladas cujos sobrenadantes induziram alterações morfológicas em células Vero semelhantes àquelas induzidas pelo fator citotóxico e necrotizante (CNF). Uma vez que tem sido descrito que CNF não é detectado no sobrenadante de culturas este estudo foi desenvolvido para melhor caracterizar estas cepas. Todas as amostras foram analisadas quanto à presença de CNF nos sobrenadantes e nos extratos sonicados; atividade necrotizante; produção de hemolisina; hemaglutinação manose-resistente; hidrofobicidade superficial; e sorogrupo. Os resultados mostraram que todas as cepas são capazes de causar necrose em pele de coelho e induzir alterações morfológicas típicas de CNF1, as quais foram observadas tanto nos extratos sonicados quanto nos sobrenadantes das culturas. Produção de hemolisina e hidrofobicidade superficial foi encontrada em respectivamente 76% e 54% das cepas analisadas. Os resultados das hemaglutinações com 6 espécies de eritrócitos mostrou que todas as cepas pertenciam aos tipos III ou IVa de MRHA. A análise dos sorogrupos mostrou que a maioria das cepas (14/17) pertenciam ao sorogrupo O6, duas cepas eram O14 e uma pertencia ao sorogrupo O4. Em conclusão, nós demonstramos que cepas de NTEC podem ser isoladas de fezes de crianças com diarréia no Brasil. Estas cepas produzem CNF1 que pode ser detectado tanto nos sobrenadantes como nos extratos celulares e possuem características muito semelhantes às cepas NTEC isoladas na Europa.

Palavras-chave: *Escherichia coli* necrotizante, fatores de virulência, fator citotóxico e necrotizante (CNF).

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CANDIDA ALBICANS ISOLATES FROM THE ORAL MUCOSA OF HEALTHY CARRIERS

Fernando R. X. da Silveira[1]
Claudete R. Paula[2]
Esther G. Birman[1]
Walderez Gambale[2]

ABSTRACT

Thirty *Candida albicans* strains isolated from the oral mucosa of 150 healthy carriers, dentate, non prosthesis wearers, smokers and non smokers, were evaluated for colony morphology, production of chlamydospores and germ-tubes, serotyping and proteinase and phospholipase activity. Biotyping of the strains was based on this set of characteristics. The most frequent type was 11114 (36.7 %), followed by 11124 (13.4 %), 22114 (13.4 %), 21114 (10.0 %) and 12114 (6.7 %). The remaining types detected showed a frequency of 3.3 %.

Key words: *Candida albicans*, oral mucosa; healthy carriers, virulence, proteinase, phospholipase.

INTRODUCTION

Candida albicans is a common component of the human oral flora. Worldwide studies have demonstrated that the presence of this yeast in the oral cavity of healthy carriers falls within a variable detection range depending on different sampling sites, sample collection techniques, age, sex and race, as well as the methodology employed to quantify and/or qualify the host-yeast interaction.

All these factors have raised some controversies about the actual condition of *Candida albicans* carriage in the oral mucosa of man. Many host-derived or yeast-derived characteristics are mentioned as features predisposing to candidal hostage (1, 8, 14). Several studies from different countries have emphasized the importance of human factors such as aging, drug therapy (with antibiotics, corticosteroids or cytostatic and immunosuppressive drugs, among others), certain diseases (mainly innate and acquired immunodeficiencies or endocrine-metabolic disturbances) and tobacco smoking to *Candida albicans* carriage, some of them strongly influencing this man-yeast association (2, 8, 22).

On the other hand, microbial characteristics such as antigenic structure (serotypes) and enzyme production and activity (proteinases and phospholipases) apparently modulate the virulence and invasive capacity of *Candida albicans* (5, 24).

The aim of this study was to verify the prevalence of *Candida albicans* in the oral cavity of healthy individuals, dentate, non prosthesis wearers, smokers and non smokers, and discuss some aspects of the interaction on the basis of the more prevalent biotypes recovered.

MATERIALS AND METHODS

Swabs of the jugal mucosa were taken from 150 healthy individuals. All the subjects were

[1] Faculdade de Odontologia, Departamento de Estomatologia, USP, São Paulo, Brazil.
[2] Instituto de Ciências Biomédicas, Departamento de Microbiologia, USP, São Paulo, Brazil.
Address for correspondence: Fernando R. Xavier da Silveira, Departamento de Estomatologia, Faculdade de Odontologia, Universidade de São Paulo, Av. Prof. Lineu Prestes, 2227, Cidade Universitária, Butantã.

dentate, non prosthesis wearers and included 69 smokers and 81 non smokers, the youngest being 15 years of age. Samples were cultivated on Sabouraud dextrose agar plates supplemented with chloramphenicol (1mg/mL) at 25° C for 7 to 15 days. Yeast strains were identified according to Kreger-van Rij (12).

Morphological tests

- Morphology of colonies: cultivation on Sabouraud-dextrose agar plates at 25° C for 24 to 48 hours to establish the surface characteristics of formed colonies.
- Chlamydospore formation: cultivation on corn-meal agar with Tween-80 at 25° C for 96 hours to determine the presence or absence of chlamydospores.
- Production of germ-tubes: 24 to 48 hr cultures were inoculated into tubes containing bovine fetal serum (10^5 cells/mL suspension), and cultivated at 37° C for three hours, to observe germ-tube presence or absence.

Physiological tests

- Diazonium blue B (DBB) color test (on solid media): cultures of 24 to 48 hours incubation were cultivated on Sabouraud-dextrose agar 4 % plates supplemented with 0.5% yeast extract. The plates were incubated at 25°C for seven days and then at 55°/ 60° C for 16 hours. Two drops of a freshly prepared chilled DBB reagent were applied directly to the surface of colonies to observe positive or negative reaction (12).
- Assimilation of carbon substances (dextrose, soluble starch, sucrose, innositol, erythritol, raffinose, maltose, galactose, lactose, celobiose) and nitrogen substances (KNO_3, peptone) was evaluated according to the method of Beyjerinck (12).
- Fermentation: One microliter of a suspension of each yeast isolate was cultivated in basic medium (5 g yeast extract; 7,5g peptone; 1000 mL distilled water with 2% dextrose, sucrose or maltose) for 15 days at 25° C, to observe fermentation.
- Enzyme production:
  proteinase: cultivation for 48 hours at 37° on basic medium containing vitamins b o v i n e serum albumin (Fraction V - Sigma 4503) as sole nitrogen source (21).
  phospholipase: the activity of the same strains was assayed by cultivation for 48 hours at 37° C on basic medium supplemented with sterile chicken egg yolk (20).
- Enzymatic activity: The ratio between the diameter of the colony and the total diameter of colony plus zone of enzymatic precipitation was employed to evaluate the activity of the yeast isolates regarding enzyme production, according to the formula described by Price et al (20). A modification was introduced by us in order to rank the strains by their activity indexes (zones of production) for proteinase (Prz) and phospolipase (Pz) according to the following ranges (23):
  Not producers (Prz and/or Pz > 0.99).
  Low producers (Prz and/or Pz > 0.69 and £ 0.99).
  Intermediate producers (Prz and/or Pz >0.29 and £ 0.69).
  High producers (Prz and/or Pz < 0.30).
- Serological test: Antisera to *Candida albicans* serotype A were raised in New Zeland male rabbits, which were inoculated with suspensions of strain ICB 12 . The agglutination titer of pooled sera against ICB 12 was determined by means of slide agglutination. Specific serotype A antiserum was prepared according to Hasenclaver and Mitchell (13); the procedure was repeated until the absorbed pooled antisera did not produce any agglutination reaction against *Candida albicans* serotype B (ICB 56) (10, 11).

RESULTS

Forty one strains of yeasts (27.3%) were isolated from the 150 swabs studied, of which 30 were identified as *Candida albicans* (present in 20% of the samples, with a prevalence of 73.1% over other fungi). A higher frequency was observed among the younger subjects up to the 30 years old and no differences were found between smokers and non smokers (15 strains recovered from each group).

The morphological study revealed that 18 (60%) C. albicans strains were smooth and 12 (40%) were rough. Chlamydospores were produced by all the

30 strains whereas germ-tubes were produced by 23 (76.7%), as shown in TABLE 1. None of the strains were positive for DBB.

The carbon auxanographic tests indicated that all the isolates were positive for dextrose, soluble starch, sucrose, maltose and galactose. Only one strain was positive for celobiose (3.3%) and all were negative for innositol, raffinose, lactose and erythritol. Regarding assimilation of N substances, all the strains were positive for peptone and negative for KNO_3.

Testing of carbohydrate fermentation revealed that the totality of the isolates used dextrose and maltose as substrates, only one strain (3.3%) being positive for sucrose.

Concerning enzyme production, 27 strains(90%) were positive for proteinase and 23 (76%) for phospholipase. Twenty one strains (70%) were able to produce both enzymes and only one (3.3%) did not produce any.

The analysis of proteinase activity showed that 20 (66.6%) strains had an intermediate activity level, five (16.6%) were highly active, two (6.6%) were low producers and only three (10.0%) were non-producers. With respect to phospholipase, 14 strains presented an intermediate level of activity (46.6%), nine (30%) were low producers, seven (23.3%) were non-producers and none was highly active. The average intermediate activity level was 0.47 for Prz and 0.57 for Pz (TABLE 2).

Serotyping revealed that type A was more frequent (21 strains = 66.7%) than type B (9 strains = 33.3%).

No significant association was found between serotype A and smoking, 12 strains resulting from smokers (57%) and 9 from non smokers (43%). Serotype B was more frequent among strains from non-smokers (6 = 66.7%) than from smokers (3 = 33.3%), as shown in FIGURE 1.

The findings on the 30 isolates of *Candida albicans* analyzed in this study enabled their classification into 11 different types according to the proposed parameters, as shown in TABLES 3 and 4.

TABLE 1. *C.albicans* biotypes according to surface morphology of colonies and production of germ tubes and chlamydospores.

Surface of colonies *				Germ tube production **				Chlamydospores production ***			
Rough		Smouth		Rough		Smouth		Rough		Smouth	
Nu.	%	Nu.	%	Nu.	%	Nu.	%	Nu.	%	Nu.	%
12	40.0	18	60.0	23	76.7	07	23.3	30	100	-	-

* Sabouraud dextrose agar, 12 days, 25°C.
** Bovine foetal serum, 3 hours, 37°C.
*** Corn meal agar added with Tween-80, 96 hours, 25°C.

DISCUSSION

There are no established rules for the biotyping of *Candida albicans*. Most of the criteria employed include the evaluation of a wide range of

TABLE 2. Proteinase and Phospholipase activity of 30 strains of *C.albicans* isolated from the oral mucosa of healthy carriers.

Serotypes[1]	A								B								Totals (A + B)							
Level of activity[2]	N		L		I		H		N		L		I		H		N		L		I		H	
Number / %	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Proteinase	2	6.6	-	-	17	56.6	2	6.6	1	3.3	2	6.6	3	10.0	3	10.0	2	6.6	20	66.6	5	66.6		
Phospholipase	6	20.0	4	13.3	12	40.0	-	-	1	3.3	5	16.6	2	6.6	-	-	7	23.3	9	30.0	14	46.6	-	-

1 According to Hasenclever and Mitchell (1961).
2 According to Price et ali (1982) (Modified by the authors):
N = Not producer (Prz and Pz = 1.00);
L = Low producer (Prz and Pz > 0.69 < 0.91);
I = Intermediate producer (Prz and Pz > 0.29 < 0.70);
H = High producer (Prz and Pz < 0.30)

TABLE 3. Morphological and physiological characteristics of the 30 *C.albicans* isolates used for ranking of biotypes.

Digit order	Studied Characteristic	Possible codes
1	Serotypes: A and B	1 or 2
2	Colony surface morphology Smooth (S) or Rough (R)	S = 1 R = 2
3	Germ Tube Production: (GT^+ or GT^-)	GT^+ = 1or GT^- = 2
4	Enzyme's activity (Proteinase = P) (Phospholipase = Ph)	
	P^+ and Ph^+	1
	P^+ and Ph^-	2
	P^- and Ph^+	3
	P^- and Ph^-	4
5	Sucrose Fermentation (SF) and Celobiose Assimilation (CA):	
	SF^+ and CA^+	1
	SF^+ and CA^-	2
	SF^- and CA^+	3
	SF^- and CA^-	4

TABLE 4. Biotypes of 30 strains of *C.albicans* based on the criteria listed in TABLE 3.

Biotypes	Number of Strains	Percentage
11114	11	36.7
11124	4	13.4
22114	4	13.4
21114	3	10.0
12114	2	6.7
11224	1	3.3
12114	1	3.3
12144	1	3.3
12214	1	3.3
22221	1	3.3
22234	1	3.3
Totals	30	100.0

characteristics and properties in order to obtain a set of strains with the same phenotypic and/ or genotypic behavior (12-19).

Our study analyzed morphological and physiological features in association with serotyping and activity levels for proteinase and phospholipase. The quantitative production of the enzymes was also introduced as criterion for classification because the strains were isolated from the mouth of healthy carriers, dentate, smokers and non smokers. This characteristic is therefore important since the data obtained showed a predominance of intermediate levels of proteinase and phospholipase activity, which have to be compared to the activity of isolates from patients with different types of candidosis. Our definition of eleven biotypes allows us to suggest a compatible pattern of saprophytic interaction. Nevertheless, further studies have to be carried out bearing in mind that the pathological mechanisms involved in candidosis relate not only to the factors presently studied but also to other important features such as adherence, susceptibility to antimycotics and host immune responses, among others (5-8, 20, 21, 24).

Our data show that in a healthy carrier, without disease, the enzymatic activities are not so low as predictable. Many other factors already described may act as modulators of the microorganism's activity. The local conditions of the mouth, including saliva, state of mucosa and presence of other components of the buccal flora are very relevant to the maintenance of a state of equilibrium that sustains the condition of normality (2, 17, 22, 23).

Tobacco as a modifying factor in candidal hostage is still controversial (2, 3, 4, 23). In this respect, our data showed a prevalence of serotype B *Candida albicans* strains among non smokers as compared to isolates from smokers.

The present investigation contributes to a broader understanding of smoking habits in healthy carriers of *Candida albicans*. Further and more comprehensive studies are needed to establish whether smoking is indeed a contributing factor to the qualitative and/or quantitative aspects of *Candida albicans* occurrence in the human mouth.

Basic research on the role of *Candida albicans* in the mouth of healthy carriers is not easily available. Our finding of different types of strains in healthy carriers contrasts with some reports of an identical behavior between isolates recovered from homologous sites of non healthy subjects (6, 8, 18).

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RESUMO

Candida albicans isolada da mucosa bucal de portadores sadios

Foram estudadas 30 amostras de *Candida albicans*, isoladas da mucosa bucal de portadores saudáveis, dentados, fumantes e não fumantes. O estudo compreendeu tanto características morfo-fisiológicas (padrão, clássico), como algumas propriedades tidas como fatores de virulência "*in vitro*" da levedura (produção de proteinase e fosfolipase). A partir do perfil estudado, foi possível compor biotipos, que foram discutidos, à luz da interação da levedura com o meio bucal humano. Dentre os 11 biotipos encontrados, o mais frequente foi 11114 (36,7 %), seguido de 11124 e 22114, ambos com 13,4 %, e 2114 com 10,0 %. Todos os demais figuraram com frequencia de 3,3 %.

Palavras-chave: *Candida albicans*; mucosa bucal, portadores, virulência, proteinase, fosfolipase.

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EVALUATION IN MICE OF A SUBUNIT VACCINE PRODUCED WITH THE BARTHA STRAIN OF AUJESZKY'S DISEASE VIRUS

Leonardo José Richtzenhain[1]
Marcos Bryan Heinemann[1]
José Antonio Jerez[1]
José Roberto Barbosa[2]
Julio Guilherme Gubel[2]

ABSTRACT

The protective action of a subunit vaccine prepared with the Bartha strain of Aujeszky's Disease Virus was tested in mice. The vaccine was obtained by detergent solubilization of viral envelope glycoproteins with 1% Triton X-100, removal of nucleocapsids by ultracentrifugation and addition of Avridine* as adjuvant. In challenge studies, 83% of the vaccinated mice were protected from lethal viral infection after two immunization doses containing 500 mg of protein. Humoral immune responses to different protein doses were assayed by indirect ELISA. If applied to swine, this subunit vaccine should additionally permit the serological differentiation between vaccinated and infected animals, due to the deletion of glycoprotein gI in the Bartha strain.

Key words: Aujeszky's disease, subunit vaccine, challenge of mice, humoral immune response.

INTRODUCTION

Aujeszky's Disease (AD) is one of the most important viral diseases in the swine industry. AD affects a large number of mammalian species that usually die, with the exception of adult pigs for which mortality is low. As with other *Herpesviruses*, Aujeszky's Disease Virus (ADV) can persist in a latent state in infected pigs that recovered from disease, independently of the degree of immunity of the animals. Some stimuli like stress and immunosupressants can reactivate the latent virus and cause its excretion. Immunization of pigs with inactivated or attenuated ADV vaccines does not prevent neither infection with the virus nor its subsequent latency or spread within a herd. Nonetheless, vaccination is practiced in many countries to reduce or eliminate clinical disease and consequent economic losses (13)

ADV envelope glycoproteins are thought to be the target antigens of protective immune responses in the host. The following structural glycoproteins were identified in the ADV envelope: gI, gII, gIII, gp50, gp63 and gH. Another glycoprotein, namely gX, is released by infected cells into the medium in large amounts, yet it is not found in the virion. Some of these glycoproteins, like gI, gIII, gp63 and gX, were shown to be nonessential for viral replication. On the other hand, the genes that code for glycoproteins gII and gp50, and most likely also

* Pfizer Laboratories

1 Laboratory of Applied Virology and Immunology - Faculty of Veterinary Medicine and Zootechny of the University of São Paulo-São Paulo- Brazil.

2 Laboratory of Vaccine Production - Ministry of Agriculture -LARA - Campinas- São Paulo - Brazil.

those that code for gH, cannot be deleted from the viral genome without affecting virus viability and are thus considered essential. Antibodies against gp50 and gIII are the most potent in neutralizing ADV, and gIII is a major target antigen of murine and swine virus-specific cytotoxic T-lymphocytes (3).

ADV strains with a serologically identifiable marker deletion involving the gene of either glycoprotein gI (11), gIII (1) or gX (2) have been introduced for vaccine production. This creates a privileged situation because vaccinated animals can now be differentiated from field virus infected animals, as they will not exhibit antibodies against the deleted marker protein when tested by ELISA. The Bartha strain of ADV, with deletions in gI and gp63, is one of the strains used for this purpose.

Since ADV envelope glycoproteins are pointed as targets of host protective immune responses, they have been recommended as the viral antigens of choice for the production of subunits vaccines (SUVs) (4). An important advantage of SUVs is the absence of most of the viral proteins apparently not involved in protective immunity that, if present, might deviate the focus of a mounting immune response and lower the effectiveness of vaccination. Furthermore, SUVs can minimize the risk of adventitious agents. It is also worth mentioning that, in the case of feline herpesvirus, it is the viral nucleocapsid proteins but apparently not the envelope glycoproteins that are involved in postvaccine reactions (12).

Regarding studies on the evaluation of SUVs against AD, three types of vaccines should be considered: **crude SUVs**- where viral envelope glycoproteins are extracted from infected cell cultures with detergents and the nucleocapsids are removed by ultracentrifugation (7,10); **affinity chromatography purified SUVs** - where glycoproteins are purified by affinity chromatography after detergent extraction from infected cell cultures (6) and **purified virus SUVs** - where glycoproteins are extracted with detergents from purified virions (5,9).

The aim of the present study was to evaluate the Bartha strain of ADV with deletions in gI and gp63 as source of glycoproteins for a crude SUV, prepared with a simple protocol. This type of SUV should allow the serological differentiation between vaccinated and infected animals, due to the deletion of Ig in the Bartha strain.

MATERIALS AND METHODS

ADV strains and cell cultures

The Bartha strain of ADV cultivated in PK-15 cells was used for SUV preparation.

A virulent strain of ADV isolated in Santa Catarina- Brazil and cultivated in VERO cells was used for challenge studies and ELISA antigen preparation.

Experimental Animals

CH3-Rockfeller mice were used in the challenge and humoral response studies.

SUV preparation

ADV glycoprotein solubilization and removal of nucleocapsids were based on the method of Todd *et al* (8) with some modifications. Briefly, monolayers of PK-15 cells were infected with the Bartha strain of ADV. After total cytopathic effect (48 to 72 hs post infection), the cellular debris was collected by centrifugation (5,000 g; 30 minutes; 4°C). The sediment obtained was suspended in 0.01M TRIS-HCl, pH 7.0 + 0.001M EDTA (0.5 mL of buffer per 80 cm^2 area of cell monolayer) and sonicated for 2 cycles of 30 seconds in ice bath (50% out-put; Ultrasonic Cell Disrupter - Heat Systems -USA). Next, an equal volume of 2% Triton X-100 was added to the suspension, followed by incubation for 30 minutes at 37°C and removal of nucleocapsids by ultracentrifugation (100.000 g; 120 minutes; 4°C) in a SW-28 Beckman rotor. Finally, formaldehyde at a final concentration of 0.05% was added to destroy an eventual infectivity and stabilize viral antigens (10).

A control subunit vaccine (SUVC) was prepared with non-infected PK-15 cells processed as described above.

The protein content of SUV and SUVC was assayed by a commercial kit based on bicinconinic acid (BCA)*, since it is not influenced by 1%

Triton X-100 present in the subunit vaccine preparations.

Avridine at a final concentration of 4 mg/mL was employed as adjuvant for both SUV and SUVC.

ELISA

For antigen preparation, monolayers of VERO cells infected with the virulent strain of ADV were collected after maximum cytopathic effect and submitted to 2 freeze/thawing cycles. The viral suspension (460 mL) was clarified 15 minutes at 5,000 g /4°C, layered onto a 1.0 mL/tube cushion of 30% (w/w) sucrose in TBS pH 7.0 (0.01 M TRIS-HCl ; 0.0018 M $CaCl_2$; 0.02 M $MgCl_2$; 0.0026 M KCl; 0.136 M NaCl) and centrifuged for 60 minutes at 26,600 g/4°C in a SW 28 Beckman rotor. The viral pellet was resuspended in 1.2 mL of TBS, layered onto a 15%-30% (w/w) sucrose gradient in TBS and centrifuged 60 minutes at 26,600 g/4°C in a SW 28 Beckman rotor. The virus band was collected, aliquoted and stored at -70°C until use. For ELISA, antigen, sera and conjugate were added at 100 µl/well. Microplates were sensitized overnight at 4°C with antigen at 10 µg/mL in O.O5 M bicarbonate buffer, pH 9.6. Sera were diluted at 1:200 in PBS with 4% bovine serum albumin (PBS-D), added to the wells and incubated for 45 minutes at 37°C. Goat IgG anti mouse IgG-peroxidase conjugate was diluted 1:1000 in PBS-D before addition and also incubated for 45 minutes at 37°C. Between each reaction step, microplates were washed 3 times (3 minutes each) with PBS (0.01 M PO_4^-; 0.146 M NaCl; pH 7.2) containing 0.05% Tween 20. A solution of 10 mg of OPD and 10 µl of H_2O_2 30% diluted in 25 mL of citrate-phosphate buffer, pH 5.0, was used as chromogen. The reaction was stopped with 50 µl of HCl 1N. Absorbance was measured at 492 nm in an automatic ELISA reader (Multiskan Plus- Labsystems -USA).

ELISA data were expressed by the Sample/ Positive ratio (S/P) using the formula:

S/P = xS - xN / xP - xN , where:
xS = mean absorbance of duplicate of the sample,
xN = mean absorbance of duplicate of negative reference sera
xP = mean absorbance of duplicate of positive reference sera

Protection and humoral immune response studies

For protection studies, groups of 30 days old mice were vaccinated subcutaneously with 0,2 mL of SUV or SUVC containing different protein concentrations. Twenty one days later the animals were revaccinated as mentioned and challenged subcutaneously after 7 days with 0,2 mL of virulent ADV suspension at different titers. Mice were observed daily for 15 days after inoculation.

Prior to challenge, a representative number of mice from the SUV and SUVC groups was sacrificed for collection of serum and evaluation of humoral immune responses.

RESULTS AND DISCUSSION

Protein concentrations for the two batches of SUV and SUVC ranged from 1,900 to 2,700 mg/ mL and were comparable to the values reported by Rock and Reed (7), who also produced crude SUVs without any glycoprotein purification.

Increasing the protein content of the SUV dose from 350 mg (assay # 01) to 500 mg (assays # 2 and 3) led to a significant increase ($p \leq 0.05$) in the mean humoral response of immune mice, as measured by indirect ELISA (TABLE 1)

The experimental reproduction of AD in pigs is extremely difficult because of factors such as the sublethality of symptoms upon challenge infection and the development of natural resistance associated with age (5). Therefore, although pigs are the target animals for an AD vaccine, many protection studies using SUVs against AD have been carried in species where the disease is highly lethal, like sheep (5) or mice (6,7,9,10).

Protection studies of SUVs against lethal ADV infection may differ in the protocols adopted, which vary according to routes of administration, potency of infecting virus or ADV strains and adjuvants employed (TABLE 2). Most importantly, however, all of them use a challenging virus titer able to induce 100% mortality in the control animals

TABLE 1 shows that, following infection with the higher virus titer (assay # 2), 100% mortality was observed in mice treated with the control vaccine (SUVC) yet 83% protection was induced in mice treated with two 500 mg doses of SUV,

TABLE 1. Challenge data and humoral immune responses induced by suvs and suvcs in mice.

Assay Number	µg of protein per animal SUV/SUVC	Virus tiler for challenge $TCID^{50\%}$	% Protection		Meam ELISA S/P values					
			SUV	SUVC	SUV			SUVC		
# 01	350	$10^{7.6}$	11/12[a] (92%)	1/12 (8.3%)	0.932	(4)	[b] 18.3 % [c]	0.074	(4)	54.9%
# 02	500	$10^{9.43}$	25/30[a] (83 %)	0/30 (0%)	1.734	(8)	41.7%	0.083	(8)	49.0%
# 03	500	$10^{8.66}$	54/54[a] (100%)	9 /29 (31%)	1.707	(10)	25.3%	0.107	(10)	28.6%

a = number of protected animals/number of challenged animals
b = number of animals bled in the assay
c = coefficient of variation

TABLE 2. Studies of SUVs against AD based on protection to lethal ADV infection

Authors	Type of SUV	ADV Strain	Adjuvant	Strain used	Protein content (µg) of SUV used to induce protection	Animal Species
ROCK & REED , 1980	crude	s62 /26	IFA 2 times	s 62/26	500	mice
TURNER et all, 1981	crude	Dekking	?? 2 times	Dekking	?	mice
MOREIN et all, 1989	purified virus	Phylaxia	ISCOM 2 times	Phylaxia	≥ 3	sheep
TSUDA et all. 1991	purified virus	Yamagata S-81	ISCOM 2 times	Yamagata S-81	1	mice
PUENTES et all, 1993	affinity chromatography purified	Bartha and E-974	Marcol 52	E-974	5	mice

similarly to the result obtained by Rock and Reed (7). Greater protection rates were recorded after challenge with lower virus titers (assays # 1 and # 3), but in these cases mortality did not reach 100% in the control groups. Analyzing crude SUVs produced from a virulent ADV strain, Turner *et al* (10) reported 100% protection in mice without the use of an adjuvant, however they did not state the protein content of their SUVs.

In this study, the use of Avridine as adjuvant led to protection rates comparable to those obtained by Rock and Reed with Incomplete Freund Adjuvant (IFA) (7). Avridine permits an easy emulsification of SUVs and has the additional advantage of not causing visible post-vaccine reactions at the inoculation site, which were observed with IFA in our preliminary studies (data not shown).

Using virus-purified SUVs and ISCOM as adjuvant, Morein *et al* (5) were able to protect sheep with 2-3 mg of protein and Tsuda *et al* (9) attained significant protection in mice with 1 mg of protein. Puentes *et al* (6) protected mice with a 5 mg protein dose of affinity-chromatography purified SUV in Marcol 52 as adjuvant and also reported the efficacy of the Bartha strain of ADV as source of glycoproteins for SUV preparation. Although in these investigations protection was obtained with very low concentrations of protein, both virus-purified and affinity-chromatography purified SUVs are more difficult and expensive to produce compared with the crude SUV evaluated in the present work.

Studies carried out in swine, where protection criteria were based on clinical signs and weight loss after virus challenge, also demonstrated the efficacy of the Bartha strain for the obtention of affinity chromatography purified SUVs against AD (6,12).

To conclude, the crude SUV prepared from the Bartha strain of ADV in this study protected 83% of mice from a lethal ADV infection. Considering the simpler protocol adopted for SUV production compared with other procedures and the possibility of differentiating between vaccinated and infected

animals, efforts should be made to test this crude SUV in swine.

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RESUMO

Avaliação de uma vacina de subunidades produzida com a amostra bartha do vírus da doença de aujeszky em camundongos

Uma vacina de subunidades preparada com a amostra Bartha do Vírus da Doença de Aujeszky foi avaliada em camundongos. A vacina de subunidades foi preparada através da solubilização das glicoproteínas do envelope viral com Triton X-100 a 1%, remoção das nucleocápsides por ultracentrifugação e adição de Avridine como adjuvante. Em estudos de desafio, esta vacina de subunidades protegeu 83% dos camundongos contra uma infecção letal do vírus , após duas doses contendo 500 mg de proteína. A resposta imune humoral induzida por diferentes doses de proteína foi mensurada pelo ELISA. Como vantagem, se aplicada ao suíno, a vacina de subunidades descrita poderia permitir a diferenciação sorológica entre animais vacinados e infectados, devido à deleção da glicoproteína gI na amostra Bartha.

Palavras chave: Doença de Aujeszky, vacina de subunidades, desafio em camundongos, resposta imune humoral.

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MYCOFLORA , AFLATOXIGENIC SPECIES AND MYCOTOXINS IN FRESHLY HARVESTED CORN (*ZEA MAYS* L.): A PRELIMINARY STUDY

Maria Fernanda Penteado M. Castro[1]
Lucia M. Valente Soares[2]
Regina P.Z. Furlani[2]

ABSTRACT

Grain mycoflora, occurrence of aflatoxigenic species and presence of mycotoxins (aflatoxins B_1, B_2, G_1 and G_2,, zearalenone and ochratoxin A) were evaluated in samples of freshly harvested corn collected at different locations in the State of São Paulo, Brazil, during 1992. Invasion of grains by the potentially mycotoxigenic genera *Fusarium* and *Penicillium* was rather high. The genus *Aspergillus* was also isolated but at a lower frequency compared with the other two genera. The *Aspergillus* species detected were: *A.versicolor* , *A. flavus*, A.*niger*, *A. alutaceus*, *A. wentii*, *Eurotium chevalieri*, *Eurotium rubrum*, *Eurotium amstelodami* and *Eurotium repens*. Eight *Penicillium* species were recovered, namely: *P.variabile*, *P.funiculosum*, *P.citrinum*, *P.pinophilum*, *P.brevicompactum* , *P. canescens* , *P. raistrikii* and *P. spinulosum*. Four of the seventeen *A. flavus* isolates were found to be either B1 or B1, and B2 aflatoxin producers. Despite the presence of potentially toxigenic fungi, zearalenone (produced by *Fusarium* spp.), aflatoxin (produced by *A. glavus/A. parasiticus*) and ochratoxin A (produced by *Aspergillus spp.* and *Penicillium spp.*) were not detected in the grain samples. The occurrence of non-toxigenic strains, unfavorable field and environmental conditions, interactions between fungal species and resistant varieties of corn are some of the factors that, alone or in combination, may explain the absence of mycotoxins in corn harvested during 1992 in the State of São Paulo.

Key words: **corn, mycoflora, aflatoxigenic species, mycotoxins, occurrence**

INTRODUCTION

The invasion of grains by potentially toxigenic molds prior to storage and the production of mycotoxins before, during and after harvest have recently received greater attention (3). The main genera involved in mycotoxin production in corn are *Aspergillus, Penicillium* and *Fusarium*. Aflatoxins, zearalenone and ochratoxin A are some of the toxins that may be produced by species of these genera and have been specifically associated with contaminated corn (1,17,23). *Fusarium* is considered the most common mycotoxigenic genus found in freshly harvested corn. Of no less importance, however, are *Aspergillus* and *Penicillium spp.*. These two genera, though being regarded as storage fungi, may also affect grains at pre-storage stages since infection by *A.flavus* and contamination with aflatoxins have been shown to occur in freshly harvested corn (13, 14, 25). Fungi of the genus *Penicillium* also frequently invade preharvest corn (12, 16, 18, 19), yet despite being commonly detected in our local conditions no data is available at the species level. The aims of the present investigation were to evaluate freshly harvested corn collected at different locations in the State of São Paulo during 1992, with respect to: (1) identification of grain mycoflora, with particu-

[1] Instituto de Tecnologia de Alimentos, Caixa Postal 139, 13073-001 Campinas, S.P.
[2] Faculdade de Engenharia de Alimentos, Caixa Postal 6121, UNICAMP, 13081-970 Campinas, S.P.

lar emphasis on the storage molds *Aspergillus* and *Penicillium*, (2) detection of aflatoxigenic species, and (3) detection of aflatoxins B_1, B_2, G_1 and G_2, zearalenone and ochratoxin A.

MATERIALS AND METHODS

Samples

Samples of freshly harvested corn grains, 1992 crop, were obtained from different sites in the State of São Paulo (FIGURE 1) after drying and homogenization of the lot. All the samples were sundried except for one (from São Manuel), which was treated in a continuos flow drier. Grain samples from each locality (three on average) were mixed together forming a composite sample. Sixteen sites were sampled in this way. In Lorena, two composite samples were prepared. Grain moisture content prior to evaluation ranged from 10.32% to 16.94% as determined by the oven method (2). Samples were kept in a freezer (-15° C) until use.

Analysis of Mycoflora

Isolation of fungi

From each sample, a sub-sample of approximately 30 g was taken, surface disinfected by immersion in a hypochloride solution (2%) by 3 minutes and rinsed three times with sterile distilled water. Fifty grains were selected at random and directly plated onto DRBC (10) and DG18 (9) media. Five plates and ten grains per plate were used for each medium. All the plates were incubated at 25°C for 5 days (DRBC medium) or 7 days (DG18 medium) prior to the isolation of growing fungal colonies.

Penicillium spp. isolates were transferred to CZ, *Fusarium spp.* to SNA, *Aspergillus spp.* to Cz , *Eurotium spp.* to M20, other isolates to PCA and all were stored at 5°C for further characterization.

Identification of fungi

Species of *Aspergillus* and *Penicillium* were identified according to Klich and Pitt (11) and Pitt (20, 21), respectively. Isolates of *Fusarium* were identified only at the genus level according to Brayford (4). Other frequently found isolates were also identified at the genus level according to Samson *et al.* (24).

Screening for aflatoxigenic species

Isolates of *A.flavus* were grown at 25°C for 10 days on CZ medium in test tubes. Five mL of 0.01% tween solution were poured into each tube and mixed thoroughly. One mL of suspension was collected, dropped into an Erlenmeyer containing 100 mL of 2% yeast extract plus 20% sucrose YES medium (7) and incubated for 10 days at 25 °C. The culture medium together with mycelia was extracted

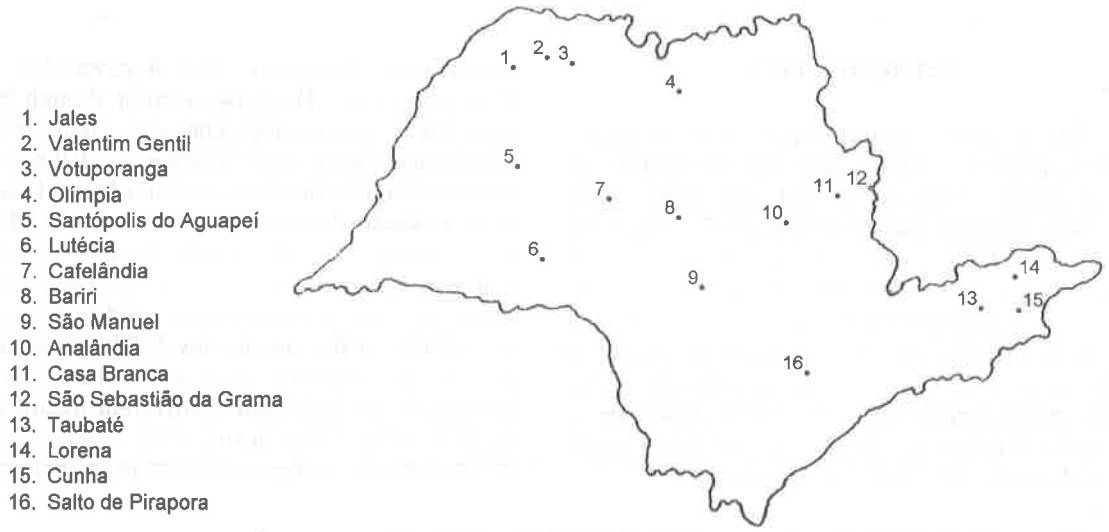

FIGURE 1. Sites in the State of São Paulo at which samples were collected for evaluation.

with 300 mL methanol/4% KCl (9+1). Each extract was analyzed for the presence of aflatoxins as described by Soares and Rodrigues- Amaya (26). The assay was conducted in triplicate.

Analysis of Mycotoxins

Detection of aflatoxins B_1, B_2, G_1, G_2 , zearalenone and ochratoxin A were carried out as described by Soares and Rodrigues-Amaya (26).

RESULTS

Analysis of Grain Mycroflora

The percentage of grains infected with fungi that were isolated on DRBC or DG18 media (TABLES 1 and 2) shows a high incidence of *Fusarium* and *Penicillium* species. *Aspergillus* species were also isolated but at lower frequencies compared with those of the other two genera.

Fungi of the genus *Fusarium* were the prevalent isolates and were detected in all the samples. With a few exceptions, higher infection rates were obtained for *Fusarium* species as compared with other fungi. As shown in TABLE 1, the percentage infection for *Fusarium* varied from 16% to 80% (DRBC agar). Zearalenone was not detected, although this mycotoxin can be produced by several *Fusarium* species.

Penicillium spp. were present in all samples but one (São Manuel). The highest percentage of infection was found in sample from Jales (92% on DRBC) followed by Cafelandia (54% on DRBC) and Lutécia (43% on DG18) samples. Eight species were detected : *Penicillium variabile* Sopp, *Penicillium funiculosum* Thom, *Penicillium*

TABLE 1. Percentage of corn kernels infected by fungi in samples collected after harvest in different localities of São Paulo State by direct planting on DRBC.

	VG	BA	SM	VO	CA	OL	CN	AN	CB	LU	SP	SSG
% Infeccion	90%	74%	66%	62%	98%	88%	78%	92%	92%	90%	100%	100%
Fusacarium spp	16%	16%	38%	39%	60%	64%	48%	80%	38%	36%	72%	42%
Aspergillus spp	-	-	-	04%	-	-	-	-	02%	02%	-	-
A. versicolor	-	-	-	-	-	-	-	-	-	-	-	-
A. flavus	-	-	-	04%	-	-	-	-	-	02%	-	-
A. alutaceus	-	-	-	-	-	-	-	-	02%	-	-	-
A. wentii	-	-	-	-	-	-	-	-	-	-	-	-
Eurotium spp	-	-	-	-	-	-	-	-	-	-	-	-
E. chevalieri	-	-	-	-	-	-	-	-	-	-	-	-
E. rubrum	-	-	-	-	-	-	-	-	-	-	-	-
E. amstelodami	-	-	-	-	-	-	-	-	-	-	-	-
E. repens	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium spp	24%	08%	-	04%	54%	10%	16%	08%	22%	35%	04%	38%
P. variabile	-	-	-	-	24%	-	04%	08%	02%	02%	04%	21%
P. funiculosum	04%	-	-	04%	16%	08%	08%	-	08%	02%	-	17%
P. citrinum	13%	-	-	-	06%	-	04%		04%	28%	-	-
P. pinophilum	07%	04%	-	-	10%	-	-	02%	02%	04%	-	02%
P. brevicompactum	-	*02%*	-	-	-	*02%*	-	-	-	-	-	-
P. spinulosum	-	-	-	-	-	-	-	-	-	-	-	-
P. raistrikii	-	02%	-	-	-	-	-	-	-	-	-	-
P. canescens	-	-	-	-	-	-	-	-	04%	-	-	-
Acremonium spp	50%	48%	10%	12%	06%	06%	15%	08%	18%	34%	13%	22%
Nigrospora spp	02%	04%	08%	04%	16%	08%	-	06%	04%	-	13%	08%
others[b]	04%	-	08%	02%	06%	10%	08%	04%	16%	04%	02%	06%

a. VG - Valentim Gentil, BA - Bariri, SM - São Manuel, VO - Votuporanga, CA - Cafelândia, OL - Olímpia, CN - Cunha, AN - Analândia, CB - Casa Branca, LU - Lutécia, SP - Salto de Pirapora, SSG - São Sebastião da Grama, SA - Santópolis do Aguapeí, JA - Jales, LO^k - Lorena (corn in kernels), LO_e - Lorena (corn in ears) and TA - Taubaté.

b. Include *Phoma sp, Diplodia sp, Stemphilium sp, Epicocum nigrum, Curvularia sp, Cladosporium sp, Alternaria sp, Rhyzopus sp* and other non-sporulating fungi.

TABLE 1. Percentage of corn grains infected with fungi in prestorage samples from different localities of the State of São Paulo. Evaluation was carried out by direct planting onto DRBC.

	VG	BA	SM	VO	CA	OL	CN	AN	CB	LU	SP	SSG
% Infeccion	78%	76%	38%	44%	62%	86%	88%	86%	94%	80%	96%	86
Fusacarium spp	02%	32%	32%	28%	18%	46%	76%	76%	48%	29%	60%	49%
Aspergillus spp	12%	04%	02%	10%	04%	-	-	-	06%	18%	-	08%
A. versicolor	-	-	02%	-	-	-	-	-	-	-	-	-
A. flavus	-	04%	-	10%	04%	-	-	-	-	08%	-	02%
A. alutaceus	-	-	-	-	-	-	-	-	-	-	-	-
A. wentii	-	-	-	-	-	-	-	-	-	*02%*	-	-
Eurotium spp	12%	-	-	-	-	-	-	-	06%	10%	-	06%
E. chevalieri	08%	-	-	-	-	-	-	-	-	-	-	-
E. rubrum	02%	-	-	-	-	-	-	-	02%	08%	-	06%
E. amstelodami	-	-	-	-	-		-	-	-	-	-	-
E. repens	-	-	-	-	-	-	-	-	04%	-	-	-
Penicillium spp	42%	14%	-	04%	40%	04%	20%	06%	14%	43%	16%	35%
P. variabile	-	04%	-	02%	28%	02%	14%	04%	10%	02%	14%	27%
P. funiculosum	-	06%	-	04%	-	-	04%	02%	-	-	-	06%
P. citrinum	42%	-	-	-	06%	02%	02%	-	04%	41%	02%	-
P. pinophilum	-	04%	-	-	04%	-	-	-	-	-	-	-
P. brevicompactum	-	-	-	-	-	-	-	-	-	-	-	02%
P. spinulosum	-	-	-	-	02%	-	-	-	-	-	-	-
P. raistrikii	-	-	-	-	-	-	-	-	-	-	-	-
P. canescens	-	-	-	-	-	-	-	-	-	-	-	-
Acremonium spp	28%	30%	04%	-	02%	08%	02%	-	20%	20%	02%	-
Nigrospora spp	-	06%	-	02%	06%	26%	04%	08%	14%	-	34%	02%
others[b]	04%	-	-	04%	08%	08%	04%	02%	02%	-	02%	10%

a. VG - Valentim Gentil, BA - Bariri, SM - São Manuel, VO - Votuporanga, CA - Cafelândia, OL - Olímpia, CN - Cunha, AN - Analândia, CB - Casa Branca, LU - Lutécia, SP - Salto de Pirapora, SSG - São Sebastião da Grama, SA - Santópolis do Aguapeí, JA - Jales, LO[k] - Lorena (corn in kernels), LO_e - Lorena (corn in ears) and TA - Taubaté.

b. Include *Phoma sp, Diplodia sp, Stemphilium sp, Epicocum nigrum, Curvularia sp, Cladosporium sp, Alternaria sp, Rhyzopus sp* and other non-sporulating fungi.

citrinum Thom, *Penicillium pinophilum* Hedgcock, *Penicillium brevicompactum* Dierckx, *Penicillium canescens* Sopp, *Penicillium raistrikii* GSmith and *Penicillium spinulosum* Thom. *P. variabile* and *P. funiculosum* were the most frequent and regular species detected but the highest rates of infection were associated with *P. citrinum* (85% on DRBC in Jales, 41% on DG18 in Lutécia and 42% on DG18 in Valentim Gentil). *P. pinophilum* was recovered from approximately half of the samples analyzed but the corresponding percentage of infected grains was low (maximum of 10% isolated in Cafelandia on DRBC). *P. brevicompactum* was detected in practically one third of the samples with a very low percentage of infected grains (2%). *P. canescens*, *P. raistrikii* and *P. spinulosum* were very seldom detected, also showing low percentage infection rates that ranged from 2%, to 7%.

Fungi of the genus *Aspergillus (* including *Eurotium* species*)* were detected in nearly 60% of the samples but at lower percentages than the other two genera (maximun of 18% in Lutécia on DG18). The *Aspergillus* isolates recovered were: *Aspergillus versicolor* (Vuill.) Tiraboschi, *Aspergillus flavus* Link, Asper*gillus niger* van Tieghem var. *niger, Aspergillus ochraceus* Wilhelm and *Aspergillus wentii* Wehmer. The *Eurotium* species identified were: *Eurotium chevalieri* Mangin, *Eurotium rubrum* Konig, Spieckermann and Bremer, *Eurotium amstelodami* Mangim and *Eurotium repens* Bary. Of these fungi, *Aspergillus flavus* and *Eurotium spp.* were the most common, being found in about one third of the samples. *A. wentii, A.versicolor, A. ochraceus* were present in just one sample each with only 2% of infected grains. Among the other genera detected

Acremonium spp. prevailed and showed a wide distribution, followed by *Nigrospora spp.*. Other fungi also isolated were *Phoma spp., Diplodia spp., Stemphilium spp., Epicoccum nigrum, Curvularia spp., Alternaria spp., Cladosporium spp.*, as well as non-sporulating fungi.

Screening of aflatoxigenic species

Four out of the seventeen *A. flavus* isolates recovered were either B_1 or B_1 and B_2 aflatoxin producers (two from Votuporanga, one from Cafelandia and one from São Sebastião da Grama)

Analysis of Mycotoxins

None of the mycotoxins assayed (aflatoxins B_1, B_2, G_1, G_2, ochratoxin A and zearalenone) were detected in the grain samples.

DISCUSSION

Invasion of commercial corn prior to storage in grain samples from the State of São Paulo (1992 crop) was rather high for the potentially mycotoxigenic genera *Fusarium* and *Penicillium spp.*. Species of *Aspergillus*, including *Eurotium spp. (A. glaucus* g), were also isolated but at lower frequencies compared to fungi from the other two genera.

Fusarium species are considered field fungi since they invade grains and seeds still in the plants. However, because they can survive in stored grain for some months (5) their presence may constitute a potential risk for producers if the grains are not properly dried. Fungi of the genus *Fusarium* were not characterized at the specie level. The *Fusarium* strains recovered might have been zearalenone producers yet no zearalenone was detected in the samples studied. *Fusarium moniliforme* is considered the most commom mold associated with corn (27). Under subsequent hermetic storage the occurrence of this species could be a potential hazard due to its apparent ability to grow in the presence of low oxygen and high CO_2 concentrations (28).

The high percentage of infected grains observed for Penicillia mainly in samples from Lutécia and Jales could be related to the high moisture content of corn harvested at these locations, which demanded a long period of drying. No Penicillia were found in the sample from São Manuel, where the grains were immediately placed in a drier after harvest. Of the Penicillia detected in our study, five species had already been isolated in freshly harvested corn in other countries. Mislivec and Tuite (18) recovered *P. variabile, P. citrinum and P. brevicompactum* from Indiana corn in the USA. Burguess and Hocking, cited by Pitt and Hocking (22), found *P. pinophilum* in Australia, whereas *P. funiculosum* was commonly detected in both countries as reported by these authors and by Mislivec and Tuite (18). Our results show the occurrence of *P. funiculosum, P. variabile* and *P. citrinum* in unstored corn and thus favor the inclusion of these species in the *Penicillium* field group, as proposed by Mislivec and Tuite (18). *P. variabile* and *P. citrinum* have been associated with ochratoxin A production (29) but this mycotoxin was not detected in the grain samples. Because most species do not grow competitively below 16 to 17% under commercial storage conditions (12) it is unlikely that *P. variabile* and *P. citrinum* would be able to produce ochratoxin A during subsequent storage.

The *Eurotium* species are considered typical storage molds and were isolated in six of our samples. Tuite (28) also found members of this group (A. glaucus g) in corn seed prior to harvest. If corn grains are stored with moisture content values in the range of 14.0% to 14.5%, the appearance of *Eurotium spp.* may intensify and this increase indicates that deterioration is underway (6).

The occurrence of *A. flavus* in freshly harvested corn has also been observed by other authors (13, 14, 25). According to Lillehoj (1983) cited by Hocking and Pitt (22), insect damage to cobs (8,15), high growing temperatures (above 30° C) and plant stress appear to be important factors that favor invasion. A few aflatoxin producers were isolated yet aflatoxins were not detected in the samples. This may be due to prevalent climatic conditions which may have not allowed the aflatoxigenic strains to produce the mycotoxins in the field. The grains, however, did contain inocula of aflatoxin-producing strains and therefore, since conidia can survive for years under favorable conditions (8), the toxin may be produced during storage.

The other genera found (*Acremonium spp., Nigrospora spp., Phoma spp., Diplodia spp. , Stemphilium spp., Epicoccum spp., Curvularia spp., Alternaria spp. and Cladosporium spp.)* normally colonize grains as the plant is growing in the field and also before harvest, being considered field fungi. These fungi are not important during storage since, according to Lutey and Christensen cited by Christensen and Kaufman (6), they die rapidly in grains held at moisture contents in equilibrium with relative humidities of 70% to 75%. Further surveys should be carried out on corn pre-storage mycoflora, focusing mainly on the occurrence of potentially mycotoxigenic species and their mycotoxins. Data on this subject are scarce. Although production of mycotoxins can occur during storage, it should be noted that one source of fungi may be the field and that production of storage toxins may result from the combined action of field and storage fungi. The species identification of *Aspergillus* and *Penicillium* strains is important because it gives a better idea of the occurrence of potentially mycotoxigenic species in freshly harvested corn, but it is a difficult and also very time consuming task. Due to these facts, our study could not encompass a larger number of samples. Additional studies including samples of crop from other years and locations are therefore advisable in order to attain a more comprehensive picture of the corn grown in the State of São Paulo

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RESUMO

Micoflora, espécies aflatoxigênicas e micotoxinas em milho recém-colhido (Zea Mays L) - Um estudo preliminar.

A micoflora, a ocorrência de espécies aflatoxigênicas e das aflatoxinas B1, B2, G1 e G2, zearalenona e ocratoxina A foram verificadas em amostras de milho recém-colhido coletadas em diferentes localidades do Estado de São Paulo durante o ano de 1992. A invasão dos grãos por fungos pertencentes aos generos potencialmente micotoxigenicos *Fusarium spp.* e *Penicillium spp.* foi elevada. *Aspergillus* também foi isolado mas a sua incidencia foi menor comparada à dos outros dois generos mencionados. As espécies de *Aspergillus* detectadas foram: *A.versicolor* , *A. flavus*, A.*niger*, *A. alutaceus*, *A. wentii*, *Eurotium chevalieri*, *Eurotium rubrum*, *Eurotium amstelodami* and *Eurotium repens*. Oito espécies de *Penicillium* foram isoladas, a saber: *P.variabile*, *P. funiculosum*, *P.citrinum*, *P.pinophilum*, *P.brevicompactum* , *P. canescens* , *P. raistrikii* and *P. spinulosum*. Embora fungos potencialmente toxigenicos tenham sido isolados, zearalenona (produzida por *Fusarium* spp.) e ocratoxina A (produzida por *Aspergillus spp.* e *Penicillium spp.*) não foram encontrados nos grãos. Das dezessete cepas de *A. flavus* isoladas quatro eram produtoras das aflatoxinas B_1 ou B_1 e B_2. A presença de cepas não-toxigenicas, condições climáticas desfavoráveis, interação entre espécies fúngicas e variedades resistentes de milho são alguns dos fatores que isoladamente ou interagindo com outros podem explicar a ausencia das micotoxinas estudadas em milho colhido em 1992 no Estado de São Paulo .

Palavras-chave: milho, micoflora, espécies aflatoxigenicas, micotoxinas, ocorrencia

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PHOSPHINE FUMIGATION OF STORED IN-SHELL PEANUTS FOR THE CONTROL OF *A. FLAVUS.* LINK/*A. PARASITICUS* SPEARE GROWTH AND AFLATOXIN PRODUCTION

M. Fernanda Penteado M. Castro[1*]
Ivania A. Pacheco[1]
Lúcia M. Valente Soares[2]
Regina P. Z. Furlani[2]

ABSTRACT

Freshly harvested in-shell peanuts with moisture content ranging from 12.8 to 16.9%, were threshed and sacked in interlaced polystyrene bags. In a warehouse, the sacks were piled into two stacks of 40 sacks each. One stack was fumigated with phosphine during seven days. Inicially a dose of 3.0 g of 07 Pm_3/m^3 was applied and an equal dose was applied 24 hours Later. No *Aspergillus flavus / Aspergillus parasiticus* were detected in the treated stack immediately after the treatment. Aflatoxins were found at the top (20 mg/kg B_1, 16 mg/kg G_1) and base (24 mg/kg B_1, 5 mg/kg B_2) of the untreated stack. One month after fumigation, the percentage of grains infected with *A. flavus / A. parasiticus* in the bottom and the middle layers of the untreated stack was approximately 8 and 3 times higher than the values for the corresponding layers of the treated stack. No aflatoxins were found in the bottom and middle layers of the fumigated stack but were detected in the corresponding layers of the untreated stack (270 - 600 mg/kg B_1, 18 - 92 mg/kg B_2). On the other hand, contamination with aflatoxins was observed on the top layers of both treated and untreated stacks (340 - 1200 mg/kg B_1, 51 - 220 mg/kg B_2). The results obtained indicate that phosphine fumigation may affect the growth of *A. flavus / A. parasiticus* and aflatoxin production in peanuts stored in warehouses with moisture contents above the recommended level. Further studies should be conducted to clarify some doubtful points.

Key words: Aflatoxins, peanuts, *A. flavus/A. parasiticus*, phosphine

INTRODUCTION

Phosphine has been widely used as a fumigant for insect control and more recently to control mold development (4,9,10) and aflatoxin production (5,10) in grains. The action of phosphine on fungal development and aflatoxin production was first noted in the late 1960s. Phosphine was found to have little effect on dormant fungi (14,15). On the other hand, a reduction in fungal growth was observed in wheat with water activities of 0.80 and 0.85 and inoculated with *Eurotium chevalieri* and *Aspergillus flavus / Aspergillus parasiticus* after exposure to 0.1 mg/L of phosphine for two weeks at 28°C (9). Promising results were also obtained with rice of 0.92 water activity and inoculated with *A. parasiticus* following treatment with 0.1 g/m^3 phosphine for 14 and 28

[1] Instituto de Tecnologia de Alimentos, Caixa Postal 139, 13073-001 Campinas, S.P.
[2] Faculdade de Engenharia de Alimentos, Caixa Postal 6121, UNICAMP, 13081-970 Campinas, S.P.
* To whom correspondence should be sent

days (10), when a reduction in microbial growth and 50% fall in aflatoxin production were observed with respect to untreated rice. Phosphine at a concentration of 0.3 g/m^3 caused a 10 to 100 fold reduction in aflatoxins B_1, B_2, G_1 and G_2 production by *A. flavus* and *A. parasiticus* cultured in liquid medium (12). Recently, a complete arrest in aflatoxin production on shelled peanuts was observed in our laboratory in the presence of phosphine doses of 0.5, 1.0, and 1.5 g/m^3 for a period of up to 14 days (5). All these reports point towards the need of large scale experiments, in order to verify to what extent phosphine is effective in controlling aflatoxin production under warehouse conditions. Phosphine is a good alternative due to its low price, ease of application, wide availability and the fact that it does not leave undesirable residues in the treated grain.

It is common practice among farmers of the State of São Paulo, Brazil, to dry peanuts pods in the field. This raises a serious problem especially during the raining season, when the high humidity prevents drying of the harvested pods. The use of dryers outreaches the financial possibilities of most of the farmers. In this situation, the use of simple and low-cost techniques able to prevent fungal growth and mycotoxin production would be welcome.

The aim of the present work was to evaluate the effectiveness of phosphine in controlling *A. flavus / A parasiticus* growth and aflatoxin production on unshelled peanuts with moisture content above the recommended level that were stored in open mesh bags in a warehouse.

MATERIALS AND METHODS

Experimental set up - In-shell peanuts freshly harvested during January 1993 in Herculândia, State of São Paulo, Brazil, were threshed, cleaned, and sacked inside interlaced polystyrene bags. The bags, containing about 25 kg each, were arranged into two stacks of forty sacks each (eight sacks at the base and five sacks in height) in a warehouse of the Instituto de Tecnologia de Alimentos, Campinas, State of São Paulo, Brazil. One of the stacks was fumigated for seven days with tablets of 3.0 g of aluminum phosphide/m^3 the same dose was applied again 24 hours Later. Before the application of phosphine, the stack to be fumigated was set on a plastic sheet and covered with a second sheet. The joining sides of both sheets were folded and held together with 30 x 4 cm wooden fasteners.

Control of phosphine concentration - Samples of the treated stack air were taken daily during the fumigation period through 2 mm diameter PVC pipes installed at the top, middle and base of the stack. Gas syringes were used to draw the samples. Phosphine concentrations were determined by reaction with mercuric chloride (8).

Temperature and relative humidity measurements - The stack's temperature was recorded daily during fumigation using Type T thermocouples (copper-constantan) connected to a Comark potentiometer and set next to the gas collection pipes. The warehouse temperature and relative humidity were registered daily using a thermohygrographer.

Sampling and moisture content determination - For sampling (11) purposes each stack was considered to be composed of three layers (top, middle and bottom). The top and bottom layers were comprised sixteen sacks and the middle layer eight sacks. Samples from eight sacks of each layer were taken randomly by means of a trier. About 1 kg was obtained in each case. Sampling was conducted on both stacks before and right after fumigation as well as 30 and 60 days after treatment. The peanuts were shelled by hand and separate amounts of each sample were analyzed for moisture content, presence of aflatoxins and degree of infection with *A. flavus / A. parasiticus.* The moisture content of the samples was determined in triplicate in a fan oven for 3 hours at 130 ± 3 °C (1) using 15 g of shelled peanuts.

Degree of infection with *A. flavus / A. parasiticus* - The occurrence of infected seeds was evaluated by the direct plate technique. Surface disinfection of the seeds was performed by immersion for three minutes in a 2% hypochloride solution followed by three successive rinses with sterile distilled water. The grains were placed directly on AFPA medium (13) and the plates were incubated for 48 hours at 30°C. *A. flavus* and *A. parasiticus* were recognized by their bright orange-yellow reverse colors.

Determination of Aflatoxins - Fifty-grams peanuts samples were extracted with methanol / 4 KCl (9+1), clarified with 10% cupric sulfate solution, and partitioned with chloroform. Identification and quantification was conducted by thin layer chromatography by comparison with standards (16). Confirmation was accomplished by means of a chemical reaction with trifluoracetic acid (2).

RESULTS AND DISCUSSION

Differences in phosphine concentrations within the stack were minimal, as shown by measurements from the top, middle, and bottom layers (FIGURE 1). Phosphine is reported to take 24 hours to reach a maximum fumigant concentration when evolving from aluminium phosphide tablets (17). A level of 0.8 g PH_3 /m^3 was attained 23 hours after addition of the tablet to the stack. The target concentration had been set at 1.0 - 1.5 g PH_3/m^3. Higher absorption by the pods, undetected leakage or possibly both factors may have been responsible for the lower phosphine concentration initially recorded, which became noticeably higher after a second application. Sixty four hours after the first application, the base, middle and top stack levels of phosphine were 1.69, 1.92, and 1.74 g PH_3/m^3, respectively. A drop in concentration to 0.6 g PH_3/m^3 was observed after 168 hours of fumigation. A final concentration of 0.2 g PH_3/m^3 after 120 hours of application has been suggested as indication of fumigation's efficiency (7).

The bags at the base and top of the stack followed the warehouse temperatures, while the middle layer bags kept an almost constant temperature due to the low heat conductivity of the seeds (FIGURE 2). Warehouse relative humidities ranged from 58% to 75% and temperatures varied from 22° C to 28° C during the experimental period.

The initial moisture content of the peanuts ranged from 16.9% to 12.9%, indicating lack of homogeneity for this parameter (TABLE 1). The

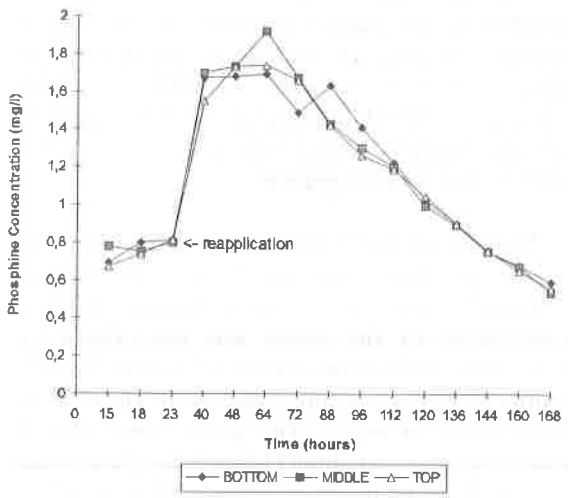

FIGURE 1. Phosphine concentrations (g/m^3) in different levels of the treated stack during fumigation.

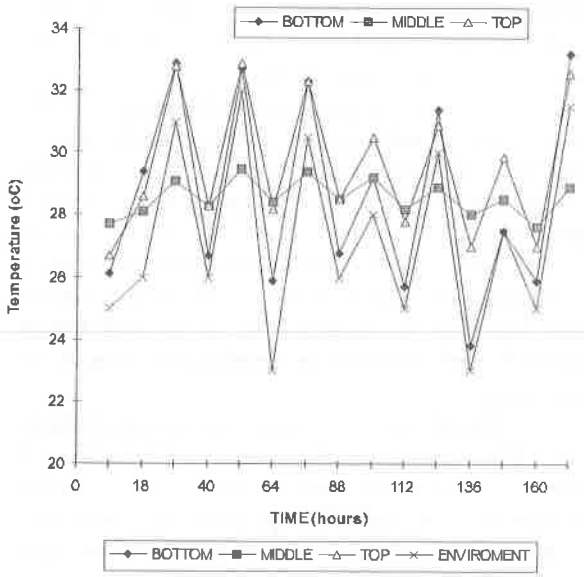

FIGURE 2. Temperatures (°C) in different levels of the fumigated stack and temperature of the warehouse during fumigation.

TABLE 1. Temperatures (°C) in different levels of the fumigated stack and temperature of the warehouse during fumigation.

Stack Level	Time elapsed (days) and stack sampled							
	0		7		30		60	
	T	NT	T	NT	T	NT	T	NT
Top	13.5	16.9	14.2	12.8	10.5	10.4	8.5	8.1
Middle	14.4	12.9	12.8	10.9	10.9	10.9	8.4	8.3
Bottom	13.5	13.9	11.9	13.8	10.2	10.2	8.3	8.3

a - Results refer to penuts after being shelled
T - Treated stack
NT - Untreated stack

fumigated stack did not show any marked change in water content when sampled immediately after fumigation. Understandably, the untreated stack (mainly top and middle layers) suffered a pronounced loss of moisture during the same period because it remained exposed to the warehouse air. After 30 days, the moisture content of the peanuts in both stacks was rather similar and dropped steadily until the end of the experiment.

Infection by *A. flavus / A. parasiticus* was not detected in both stacks at the beginning of the experiment (FIGURE 4). Right after fumigation, 8% of the seeds sampled from the middle and base of the untreated stack were infected. After 30 days, samples from the treated stack were noticeably less infected than those from the untreated stack. The percentage of seeds infected with *A. flavus / A. parasiticus* in the bottom and middle layers was about 8 and 3 times higher, respectively, in the untreated stack compared with the treated one. The top layers of both stacks could not be compared because of their initial large difference in moisture content (TABLE 1). After 60 days of treatment, the degree of infection in both top layers was practically the same, probably due to reinfection of the treated stack. The middle and bottom layers of the fumigated stack maintained extremely low degrees of infection with *A. flavus / A. parasiticus* even after 60 days of fumigation. The outer layer of peanuts may have acted as a natural barrier against grain recolonization with fungi.

Initial determinations showed that aflatoxin was found only in the middle layer of the treated stack (TABLE 2). These results were not confirmed by subsequent samplings (7, 30 and 60 days) of the middle layer. In fact, isolated contamination restricted to few grains in a stack is a characteristic and common occurrence with mycotoxins (3,6). The treated stack performed better than the untreated one in terms of aflatoxin contamination for up to 30 days. After 60 days, the percentage of infection with *A.flavus/ A.parasiticus* was minimal on the bottom layer of the treated stack yet a high level of aflatoxins (mainly aflatonin B2) was detected, which may be

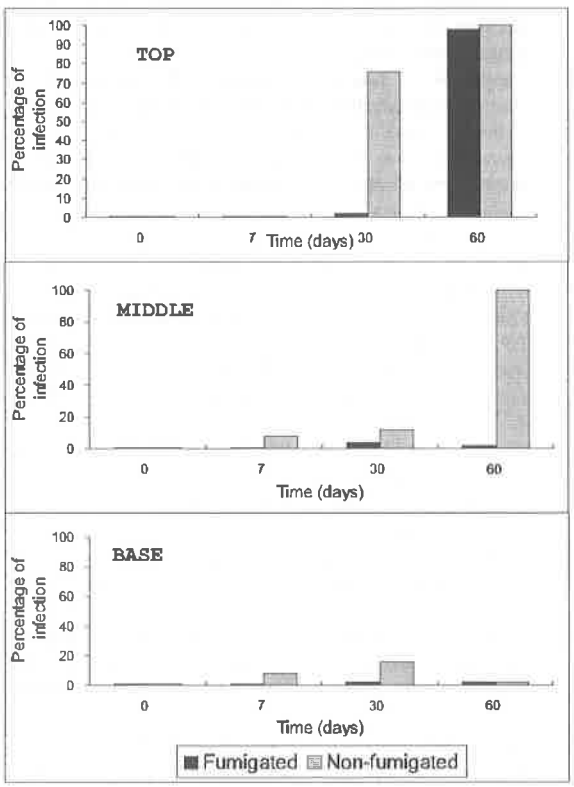

FIGURE 3. Percentage of grains infected by *Aspergillus flavus / Aspergillus parasiticus* in samples collected in the top, middle and base of the stacks during storage.

TABLE 2. Concentrations[a] (μg/kg) of aflatoxins B_1, B_2, G_1 and G_2 in samples taken from different levels of the stacks during storage in warehouse.

	Time elapsed (days) and stack sampled							
	0		7		30		60	
Aflatoxin (μg/kg)		T	NT	T	NT	T	NT	TNT
B_1	-	-	-	20	340	1200	200	400
B_2	-	-	-	tr	51	220	tr	58
G_1	-	-	-	t16	tr	170	87	26
G_2	-	-	-	-	tr	62	43	44
B_1	73	-	-	-	-	600	-	18
B_2	14	-	-	-	-	92	-	tr
G_1	-	-	-	-	-	tr	-	5
G_2	-	-	-	-	-	-	-	3
B_1	-	-	-	24	-	270	1200	-
B_2	-	-	-	-	-	18	29	-
G_1	-	-	-	5	-	-	-	-
G_2	-	-	-	-	-	-	-	-

a - Results refer to penuts after being shelled
T - Treated stack
NT - Untreated stack

explained by recolonization of the seeds with strong aflatoxin producers.

The results indicate that phosphine may affect the growth of *A. flavus / A. parasiticus* and consequently aflatoxin production in peanuts stored for short periods with moisture content above the recommended level and inside open mesh bags stacked in warehouses. Means of avoiding reinfection of the seed with fungi or a system of scheduled and repeated fumigations would be essential for long term storage. Further studies should be conduct on a warehouse scale for the establishment of an adequate control method against *A.flavus/ A.parasiticus* infection and aflatoxin contamination in stored peanuts and to clarify some doubtful points.

RESUMO

Fumigação com fosfina de amendoim em casca para o controle de *A. flavus* Link/*A. parasiticus* Speare e aflatoxinas

Amendoim em casca recém-colhido com conteúdos de umidade na faixa de 12,8 a 16,9%, foi trilhado e ensacado em sacarias de poliestireno trançado. Em um armazém, duas pilhas, de 40 sacos cada uma, foram formadas sendo que uma delas foi fumigada com fosfina durante sete dias aplicando-se 3,0 g/m³ inicialmente e após 24 horas. *Aspergillus flavus/A.parasiticus* não foram dectados na pilha tratada, imediatamente após o tratamento. Aflatoxinas foram encontradas no topo (20 mg/kg B_1, 16mg/kg B_2) e na base (24 mg/kg B_1, 5 mg/kg B_2) da pilha não fumigada. Um mes após a fumigação, a percentagem de grãos infectados com *A.flavus/A.parasiticus* foi aproximadamente de 8 a 3 vezes superior na base e no meio da pilha não fumigada em relação as camadas correspondentes da pilha tratada. Nenhuma aflatoxina foi detectada na base e no meio da pilha fumigada, mas foram encontradas nas camadas correspondentes da pilha não tratada (270 mg- 600 mg/kg B_1, 18 - 92 mg/kg B_2). Por outro lado contaminação com aflatoxinas foi evidenciado na camada superior da pilha tratada(340 mg/kg B_1 e 51mg/kg B_2) e da não tratada (1200 mg/kg B_1, 220 mg/kg B_2). Os resultados indicam que a fosfina pode afetar o desenvolvimento de *A. flavus/ A.parasiticus* e produção de aflatoxinas em amendoim armazenado em sacarias com teores de umidade acima dos limites recomendáveis em armazém. Estudos adicionais devem ser realizados para esclarecer alguns pontos que ficaram em dúvida.

Palavras-chave: Aglatoxinas, amendoim, *A. flavus/ A. parasiticus*, Fosfina.

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GROWTH AND PRODUCTION PHASES OF *PYCNOPORUS SANGUINEUS*

Artur Smânia Júnior[1]*
Elza de Fátima A.Smânia[1]
Fernando Steele da Cruz[2]
Leslie C. Benchetrit[2]

ABSTRACT

The synthesis of antimicrobial compounds was evaluated in cultures of *Pycnoporus sanguineus* MIP 89007,occurring mainly between days 18 and 23 of incubation. It was also observed that the secreted compounds are rapidly degraded when remaining in the culture broth after production has stopped. This was confirmed by a biological stability test which showed that extracts obtained from the fungus retain their activity only when stored under vacuum.

Key words: *Pycnoporus sanguineus*, production phase, antimicrobial activity, biological activity, secondary metabolite.

INTRODUCTION

Antimicrobial compounds and other secondary metabolites are usually produced after most of the cellular growth has already occurred (5, 11). In addition, Bu'Lock *et al.* (1) suggest that synthesis occurs only when the specific growth rate decreases below a certain level. On this basis, in fermentation broth, it is possible to distinguish two physiological phases. The first corresponds to a growth period and is called "trophophase"; the second corresponds to a secretory period and is termed "idiophase"(1, 5). The distinction between these two phases is most clearly observed when bacteria rather than filamentous organisms such as actinomycetes and fungi are cultured for study (6). In the latter case, cell growth continues to increase significantly during the idiophase, although usually at a lower rate than that observed at the start of the trophophase.

The present work was undertaken to highlight problems that occur during antibiosis studies with cultures of *Pycnoporus sanguineus*. We observed that the antibiotic activity of a culture varies according to the incubation period and also that extracts maintained aerated and at 4ºC loose their antimicrobial activity. Thus, this work aimed mainly at determining the greater period of synthesis of an active antimicrobial compound by *Pycnoporus sanguineus*. The biological stability of the produced compounds was also studied.

MATERIALS AND METHODS

1. FUNGAL STRAIN

The organism used in this study, *Pycnoporus sanguineus*(Class Basidiomycetes, Family Polyporaceae), is a slow growing saprophytic

1 Depto. de Microbiologia e Parasitologia, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina. Campus Universitário, C. Postal 476, 88040-900, Florianópolis, SC, Brazil.
2 Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Ilha do Fundão, C. Postal 68040, 21941-590, Rio de Janeiro, RJ, Brazil.
* Tel. 048-2319353, Fax: 048-2319258

fungus. The producer strain *P. sanguineus* MIP 89007 was obtained from a carpophore of fungus collected from a decaying tree in the State of Santa Catarina, Southern Brazil, and identified according to Gilbertson and Ryvarden (3) and Ryvarden and Johansen (10). This isolate was stored on potato dextrose agar at 4ºC until use.

2. General Culture Methods

Pycnoporus sanguineus was subcultured for 10 days at 25ºC on potato dextrose agar. The mycelium (about 1cm2) was then inoculated in 400 mL potato broth and incubated for 15 days at 25ºC. Aliquots (15 mL) of culture broth were transferred to 250 mL Erlenmeyer flasks containing 150 mL of the same medium and growth was allowed to proceed without shaking for different times (8 to 48 days) at 25ºC. On day 8 of incubation, and subsequently at 5 days intervals, mycelial and broth antimicrobial activity were assayed. Each culture was filtered (Whatman no 1 filter paper, W and R Balston Ltd) and the weight of the dried mycelium was recorded. The mycelium was added to 100 mL of sterile distilled water, macerated for 1 minute in a blender and the suspension was then filtered through gauze. The resulting liquid and the broth culture were evaporated separately in a rotary evaporator. The antimicrobial activity of both materials was tested against *Staphylococcus aureus* ATCC 25923 (American Type Culture Collection, Rockville, Md.). The potato dextrose broth for fungal growth consisted of an infusion of peeled potatoes (200 gr/L) with 2% glucose, pH 6.0. Potato dextrose agar for propagation and stock cultures of the fungus contained the broth and 1.5% agar.

3. Antimicrobial Assay

The indicator bacterium was incubated at 36ºC for 18 hours. The culture was then diluted in broth to 105CFU/mL. Thereafter, cotton swabs charged with bacterial suspension were inoculated onto plates and the bacteria were spread evenly over the surface of the agar medium. Wells of 7 mm in diameter were aseptically cut and filled with 0.1 mL of the above materials. The plates were incubated for 18 hours at 36ºC and examined for growth inhibition of the indicator strain. A positive result was defined as an inhibition zone of 9 mm or more in diameter of the bacterial strain. Muller-Hinton agar and broth (Difco Laboratories, Detroit, MI) were used for bacterial growth. All the fungal and bacterial cultures were incubated under aerobic conditions.

4. Biological Stability

Lyophilized Acetone Extract. Five distinct lyophilized broth cultures of *Pycnoporus sanguineus* were placed in a Soxhlet apparatus and exhaustively extracted with n-hexane and then with acetone. Acetone extracts were evaporated and the residues obtained were resuspended in 0.1 N NH4OH and divided into aliquots. These samples were lyophilized and at the end of the process the flasks were sealed under vacuum. On each extraction, however, the samples of some flasks were exposed to air. Immediately after lyophilization and every 30 days the antimicrobial activity of 2 samples from each lot (one sealed under vacuum and one exposed to air) were tested. The assay was carried out by the diffusion method described previously. The parameter analyzed was the monthly variation (%) of the lowest amount (mg) of material which inhibited growth of the indicator bacterium (*Staphylococcus aureus* ATCC 25923). The lyophilized extracts were stored under refrigeration (4ºC).

Acetone Extract in Aqueous Medium. The lyophilized material prepared as described was resuspended in a small volume of 0.1 N NH4OH and diluted in Mueller-Hinton broth at final concentrations of 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 mg/mL. These suspensions were distributed in 100 mL aliquots and the flasks were separated into 3 lots. One lot was maintained at -18ºC, another at 4ºC and the third one at room temperature. Antimicrobial activity was assayed by the broth dilution method (4) and the tests were carried out daily. At least a 20% loss of potency was observed (2).

RESULTS

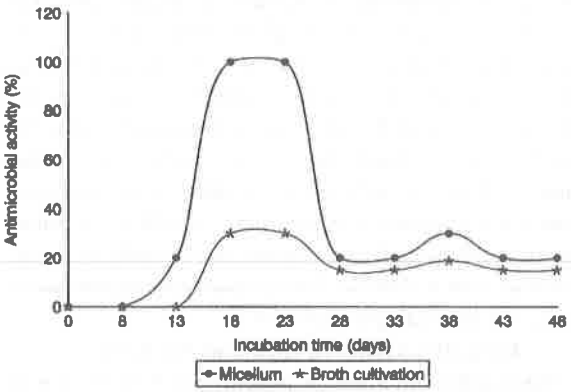
1. Growth and Production Phases of Active Substances

As shown in FIGURE 1, maximum antimicrobial activity in broth and mycelium was obtained between days 18 and 23 of culture. During

this period, the surface of the medium was covered with a thin mycelial layer that was orange in color, with some opaque white areas. The submerged mycelium was pale yellowish. It was also observed that an increase in biomass occurred during and after the production phase of antimicrobial active compounds (FIGURES 1 and 2).

2. Biological Stability of Acetone Extract

As shown in FIGURE 3, the acetone extract retained its antimicrobial activity after lyophilization only when the materials were maintained under vacuum. The aerated extracts lost their activity in the first month of storage. Growth was not inhibited when amounts 20 times greater than those of the extracts maintained under vacuum were used.



1. Each well was filled with different amounts (2.5 to 100mg) of the materials;
2. The inhibition of growth obtained with the smaller amount (7.5mg) of filtered mycelial, was considered maximum of antimicrobial activity.

FIGURE 1. Antimicrobial activity of mycelial phase and cultivation broth from *Pycnoporus sanguineus*, on *Staphylococcus aureus*, by time of growth.

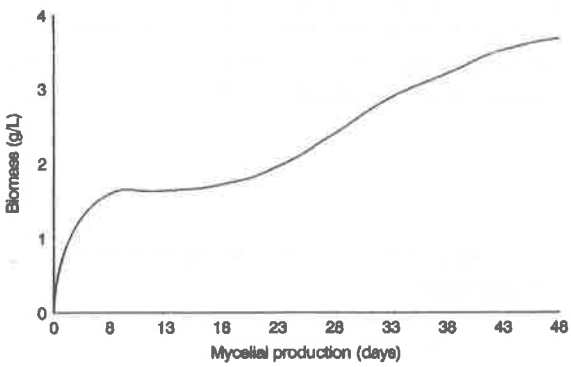
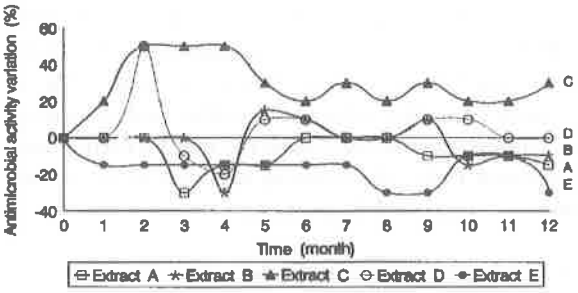


FIGURE 2. Biomass production (g/L) of culture of *Pycnoporus sanguineus*.



1. 0% variation was considered inhibitory amount at time zero;
2. The extracts exposed to the air lost 100% of activity in first month of storage.

FIGURE 3. Biological stability of extraction product from *Pycnoporus sanguineus*, under vacuum, using *Staphylococcus aureus* ATCC 25923, as indicator.

The acetone extract was biologically unstable in aqueous medium. It retained the same initial MIC only for the first 24 hours if kept at 4ºC, at room temperature or at -18ºC for 48 hours. Similar results were obtained when the parameter evaluated was the MBC. In this case, independently of storage conditions, the potency was maintained for only 24 hours (TABLE 1).

DISCUSSION

Many are the criteria that can be used to distinguish the "trophophase" from the

TABLE 1. Reduced antimicrobial activity of acetone extracts from *Pycnoporus sanguineus* in aqueous medium.

Antimicrobial Activity	Storage	conditions of the extracts	
	Freezer (-18oC)	Refrigerator Room (4oC)	temperature (25oC)
	0 h[1]	0.25[2]	0.25	0.25
	24 h	0.25	0.25	0.25
M.I.C.	48 h	0.25	0.50	0.50
	72 h	0.50	0.50 0.50	
	0 h	1.00	1.00	1.00
	24 h	1.00	1.00	1.00
M.B.C.	48 h	>1.50	>1.50	>1.50
	72 h	>1.50	>1.50	>1.50

[1] Storage time;
[2] Inhibitory concentration of the extract (mg/mL).

"idiophase" in batch culture, as for example the determination of dry weight, respiratory activity, increase in deoxyribonucleic acid, metabolization of a specific nutrient or even a combination of methods (1, 6, 7). However, for non-uniform cell development, no single method is completely adequate. In our experiments with *Pycnoporus sanguineus*, focusing only on secondary metabolites with antimicrobial activity, we established as idiophase the period of synthesis of these compounds, which corresponded approximately to 10 days. This may be considered a long period for a production phase. The duration of idiophase differs among genetically distinct producing strains and under different environmental conditions. In some antibiotic producing cultures, the phase of active synthesis can be rather short, lasting 4 to 20 hours. On the other hand, a period of synthesis of at least 10 days has been reported for industrial production using fedbatch systems (8, 9, 11). Furthermore, we noted that cell growth continues to increase during and after the idiophase (FIGURE 2) and also that the active substances are rapidly degraded when remaining in the broth after production has stopped. This instability was confirmed by a biological assay (FIGURE 3, TABLE 1). It was observed that the active compound rapidly lost its activity in aqueous medium. This was probably caused by the exposure to air. In the light of the results obtained, an incubation period of 20 days was established for the cultivation of the fungus. We also concluded that the extracts obtained from *Pycnoporus sanguineus* must be stored under vacuum. Further studies are presently being carried out to optimize the cultivation of the fungus and also to purify and identify the active substances.

ACKNOWLEDGMENTS

We wish to thank Dr. Clarice L. Leite (Depto of Botany, Federal University of Santa Catarina) for the identification of the fungus. This study was supported by the Federal University of Santa Catarina, CEPG (Federal University of Rio de Janeiro), FINEP. During the studies Dr. Artur Smânia Jr. was awarded a grant from CAPES (Brasília). Drs. Leslie C. Benchetrit and Fernando S. Cruz are Senior Investigators of CNPq.

RESUMO

Fases de crescimento e de produção em culturas de *Pycnoporus sanguineus*

Foi observado que na cultura de *Pycnoporus sanguineus* MIP 89007, a síntese de substâncias com atividade antimicrobiana ocorreu principalmente entre o décimo oitavo e o vigésimo terceiro dia de incubação. Além disso, foi também constatado que a substância produzida foi rapidamente degradada quando permaneceu no caldo de cultura após ter cessado a síntese e que os extratos obtidos a partir do fungo somente retiveram a atividade quando mantidos à vácuo.

Palavras-chave: *Pycnoporus sanguineus*, fase de produção, atividade antimicrobiana, metabolitos secundários

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CULTURE FILTRATES OF ACTINOMYCETES ISOLATED FROM TROPICAL SOILS INHIBIT *TRYPANOSOMA CRUZI* REPLICATION *IN VITRO*

Rosalie R.R. Coelho*[1]
Andrea Lopes[1,2]
Luzia T. de Azevedo Soares Semêdo[1,2]
Fernando Steele da Cruz[1]

ABSTRACT

Eighty four strains of actinomycetes isolated from Brazilian soils and 4 strains obtained from ATCC were tested for their ability to inhibit *T. cruzi* growth *in vitro*. Nine strains (ca. 10%) cultivated in liquid mineral media containing glycerol and peptone or nitrate, and occasionally supplemented with yeast extract and/or tyrosine, were able to secrete compounds present in the culture filtrates that completely inhibited parasite replication in axenic medium. Filtrates from cultures of 22 strains (ca. 25%) induced only partial inhibition of *T. cruzi* growth. For some strains, the composition of the medium and the incubation period markedly influenced the inhibitory capacity of culture filtrates. Out of 16 soil-borne actinomycete strains shown to partially restrain parasite growth in the first study, only 4 had their inhibitory effect confirmed in a second experiment using low-cost media containing soluble starch, rice flour or corn flour. Three of the 4 ATCC strains were active against *T. cruzi*, two of them already known to be producers of antibiotics effective against bacteria though not against protozoa. The effect of some of the culture filtrates on *T. cruzi* viability was followed by phase contrast microscopy, which allowed the detection of altered and no longer motile epimastigote forms of the parasite.

Key words: *Trypanosoma cruzi*, actinomycetes, inhibition of growth

INTRODUCTION

The hemoflagellate *Trypanosoma cruzi* is the etiological agent of Chagas' disease, which constitutes an important medical problem in the tropics. Studies on chemotherapeutic agents to control this disease have long been under way, but no fully effective drug is available to date. A large number of different compounds have been studied, but only two nitroheterocyclic drugs are in current use. Both, however, are highly toxic and of very restricted applicability during the chronic stage of Chagas' disease (4).

Actinomycetes are Gram-positive bacteria that form branching hyphae at some stage of their development. They are very abundant in soil and are described in the literature as the soil's main producers of bioactive compounds. More than 4000 antibiotics secreted by actinomycetes have already been described (1), in addition to low molecular weight enzyme inhibitors and modifiers (20) and a great number of degradative enzymes (16).

[1] Departamento de Microbiologia Geral, Instituto de Microbiologia , UFRJ, Centro de Ciências da Saúde, Bloco I, Cidade Universitária, Ilha do Fundão, 21941-590, Rio de Janeiro, RJ, Brasil
[2] RHAE-CNPq fellowship
* To whom correspondence should be sent

Nonetheless, most of these compounds have been obtained from isolates of temperate soils. Studies on actinomycetes derived from tropical soils are rare, yet the peculiar environmental characteristics in which they grow should lead to new species capable of producing novel bioactive molecules (12).

Brazilian tropical soils, particularly cerrado soils (savanna), are very rich in actinomycetes (2). Thus, in the present paper, our aim was to carry out a screening of actinomycetes from Brazilian soils and to evaluate their ability to produce bioactive compounds against *T. cruzi*.

MATERIALS AND METHODS

Isolation Procedures - The actinomycete strains were obtained from three different latosols: two cerrado soils, one red-yellow and one dark-red (Experimental Station, EMBRAPA, Brasilia, D.F.), and a red-yellow forest soil (Tijuca Forest, Rio de Janeiro, R.J.). Soil samples were taken from a 1-15 cm litter-free top horizon and then screened through a 1 mm mesh sieve. Following the conventional dilution plate technique, 10 g soil samples were shaken for 30 min. in 100 mL of sterile water and serially diluted in the same diluent. Five 0.1 mL replicate samples of each dilution were evenly applied to the surface of agar media on Petri dishes. Using another procedure, this dilution plate technique was applied after a dispersion and differential centrifugation procedure (6). The use of a detergent, an ionic resin and the ultrasonic treatment of soil suspensions guaranteed a more effective detachment of microorganisms from soil particles.

Starch-casein-agar (8) and glycerol-asparagine-agar (7) were employed for the forest soil, and starch-agar, peptone-glycerol-agar and nitrate-glycerol-agar for the cerrado soils, as previously described (3). The media were adjusted to pH 7,0 and supplemented with amphotericin (100 mg/mL), an antifungal antibiotic.

After 2-3 weeks of incubation at 28°C, typical colonies were selected on the basis of morphological observations, transferred onto yeast extract-malt extract-agar (17) and checked for purity by conventional techniques. Pure cultures were maintained by freeze-dried cells and/or frozen glycerol 20% spore or mycelium suspensions from cultures kept in the appropriate media (22), and stored at -20°C.

Antibiotic Activity of Actinomycetes Against *T.cruzi* - Actinomycete isolates were grown for 2 to 5 weeks at 28°C in liquid medium without shaking, followed by addition of glycerol-peptone medium (15). Glycerol-nitrate-yeast extract and glycerol-nitrate-yeast extract-tyrosine (3) were occasionally added. Cells were harvested by centrifugation and the supernatant sterilized through a Millipore filter (GSWP-0,22 mm). The cell-free extracts were kept at -20°C until use.

Four strains from ATCC were subjected to the same procedure. These were: *Streptomyces viridochromogenes* (ATCC 1490), *Streptomyces alboniger* (ATCC 19722), *Streptomyces halstedii* (ATCC 19770) and *Streptomyces phaechromogenes* (ATCC 3338).

Epimastigote forms of *T. cruzi*, strain Y, were grown in BHI liquid medium with 10% hemoglobinized fetal bovine serum (21). The same medium was employed for inoculum preparations and for test cultures. Screw-cap tubes containing 5 mL of liquid medium were inoculated with 0.5 mL of a suspension of the parasite containing 100% epimastigotes (approximately 105 parasites/mL). Different volumes (100, 250 and 500 ml) of each bacterial extract were added to two replicate tubes and then incubated at 28°C for 7 days, along with the controls (tubes that received no extracts). Parasite replication was evaluated by visual observation of growth according to the following criteria: (+++) total growth (no inhibitory action), (++) moderate growth, (+) poor growth (partial inhibitory action), and (-) absence of growth (total inhibitory action).

Additional testing of isolates from cerrado soils employing different low-cost media were also tried for those actinomycetes which exhibited promising results in the first tests. These low-cost liquid media contained soluble starch (1%), corn flour (1%), or rice flour (1%) as ingredients, in addition to the following salts: 0.1% NaNO3; 0.03% K2HPO4; 0,05% NaCl; 0.1% MgSO4.7H2O. Actinomycetes were grown in Roux bottles containing 200 mL of each medium and incubated at 28o C for 4 weeks. The bottles were shaken manually three times a day. After the incubation

period, cells were harvested and the filter-sterilized extracts were tested with or without previous concentration at 50°C in a rotating vacuum evaporator.

Microscopic Observations - Morphological modifications and parasite motility were evaluated by phase contrast microscopy (400X magnification) in all tubes where total inhibition of *T. cruzi* growth was detected.

RESULTS AND DISCUSSION

Eighty four actinomycetes isolated from different Brazilian soils and four ATCC control strains were screened for bioactive compounds against *T. cruzi*. In the first study, 9 strains (7 from soil and 2 from ATCC) corresponding to approximately 10% of the total were selected due to their ability to completely inhibit parasite growth under the test conditions used. Twenty two strains corresponding to approximately 25% of the total (21 from soil and one from ATCC) had only a partial inhibitory effect (Table 1).

Some strains were grown in more than one culture medium and/or with different incubation time, and a total of 122 different extracts was obtained. Of these, 91 were completely or almost completely ineffective against *T. cruzi* at any of the concentrations assayed. The remaining 31 extracts had a partial or total growth inhibition effect, as shown, respectively, by the poor replication or absence of replication of *T. cruzi* at one or more concentrations (Fig. 1).

Differences between the culture media used in the first screening were mainly related to the source of nitrogen, since the carbon source was always glycerol. Table 2 shows that the nature of

TABLE 1. Actinomycetes isolated from Brazilian soils.

Soil type	Isolation procedure	Isolates
cerrado soil (yellow-red latosol)	conventional technique	32,39,(43)*,44, 45, 46,49, 65, 66, 68,70, (71), 74, 75,77, 78a,78e, 78f,78h, 96,98, 99, 101, 102, 103, 104, (106), 107 (total: 28)
cerrado soil (dark-red latosol)	conventional technique	01, 06, (07), (08), 09,15,20, (51), 52, 53, (54), 55, 57, 58, (60), (62), 79, (80), 83, 85, (87), 88, 92, 94, (95), (95a), (95b) (total: 28)
Forest soil (dark-red latosol)	dispersion and differential centrifugation technique	20l, 203, 204, 205, (211)*, 215, (218), 220, (221)*, 223, (224)*, (226), (227), 228, (230), 232a, (232b)*, (234)*, (235), 236, 237, 238, (239), (241), 244, 247, 254, (255)* (total: 28)

Isolates that produced extracts with total* or partial effect on *T. cruzi* growth are given in parenthesis.

TABLE 2. Effect of nitrogen source and incubation period on the anti-*Tcruzi* growth inhibitory activity of some actinomycete culture filtrates.

Strain	Extract number	Nitrogen source	Incubation period days	Inhibitory activity
08	1084	nitrate + YE*	14	partial
	1085	peptone	28	none
51	1059	peptone	28	partial
	1078	peptone	14	none
	1079	nitrate + YE	14	none
54	1005	nitrate + YE	14	partial
	1006	peptone	14	none
	1019	peptone	35	none
80	1039	peptone	28	partial
	1041	nitrate + YE	28	partial
	1048	nitrate + YE+ tyrosine	28	none
87	1011	peptone	35	none
	1073	peptone	35	partial
106	1003	peptone	35	none
	1056	peptone	35	partial
S. alboniger	1068	peptone	14	none
	1075	nitrate + YE	14	total
	1083	peptone	28	none
S. halstedii	1069	peptone	14	none
	1076	nitrate + YE	14	partial
S. phaechromogenes	1070	peptone	14	none
	1077	nitrate + YE	14	none
	1081	peptone	28	total

* YE - yeast extract.

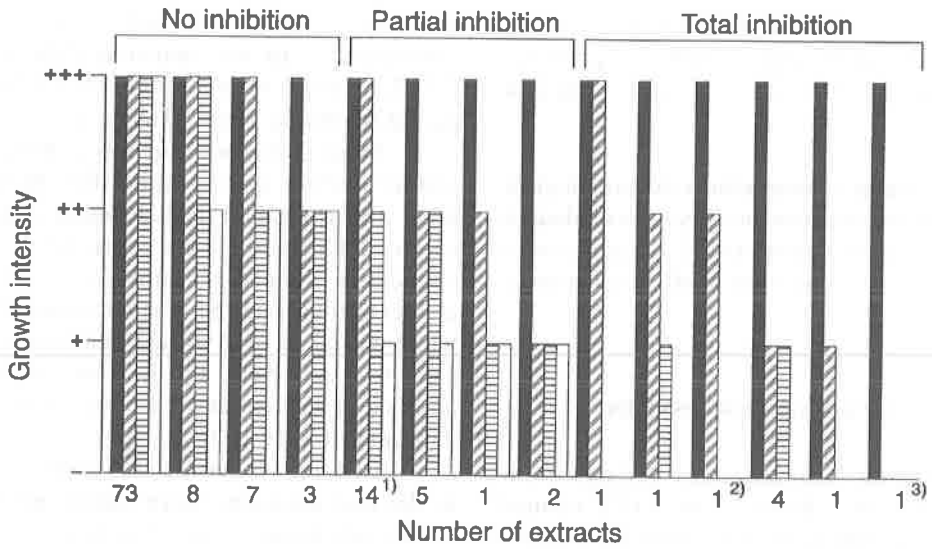

FIGURE 1. Extent of *T.cruzi* replication in the presence of different concentrations of actinomycete extracts ■ no addition of culture filtrate; addition of ▨ 100ml; ▤ 250 ml; □ 500 ml, of culture filtrate (for details see text). The results include one extract from *S. halstedii* (1), one extract from *S. alboniger* (2) and one extract from *S. phaechromogenes* (3).

the nitrogen source and incubation period had an influence on the inhibitory activity of the extract. These results were predictable, since growth conditions are known to control microbial metabolism. Thus, the sources of N and C, the time of incubation, aeration conditions (shaking or still cultures) and type of culture medium (solid or liquid media) are very important parameters to be considered (13). However, for two strains (106 and 87), the same medium and incubation period resulted in two different extracts with conflicting biological activity. In this particular situation, the inoculum size may have influenced the results, since it can control microbial morphology in submerged cultures (9) and consequently affect antibiotic production. For some actinomycete strains, only the filamentous growth, and not the fragmented mycelium, is associated with production of bioactive compounds (18).

Further studies were conducted with some strains derived from the two cerrado soils and from ATCC cultures. Fourteen of 16 strains with partial inhibitory effect and two of three strains with total inhibitory effect initially detected were additionally grown in three different low-cost liquid media that contained either soluble starch, rice flour or corn flour. In the second study, 48 new extracts were obtained after growing each of the 14 partially inhibitory strains. These extracts were tested against *T. cruzi* and 12 of the producing strains proved to be completely or almost completely ineffective in their anti-parasite effect, even at the highest concentrations assayed. Only four strains grown in low cost media still exhibited a partial inhibitory effect (Fig. 2). In fact, all these strains secreted active extracts in medium containing soluble starch but only one produced active extracts in all the three low cost media. The same results were observed with some concentrated extracts which had been vacuum evaporated at 50°C.

The four strains capable of secreting compounds active against *T.cruzi* during the first screening and in the subsequent experiments with low cost media were soil-borne. In contrast, two strains from ATCC which were originally fully active against the parasite showed no activity when grown in low cost media. Although most liquid media utilized for antibiotic production contain glycerol, glucose or starch as carbon source (13), the absence of certain micronutrients may have been responsible for the loss of anti *T.cruzi* activity by ATCC strains grown in the low cost liquid media.

Three of the four ATCC strains were able to produce extracts that completely (*S. alboniger* and *S. phaechromogenes*) or partially (*S. halstedii*)

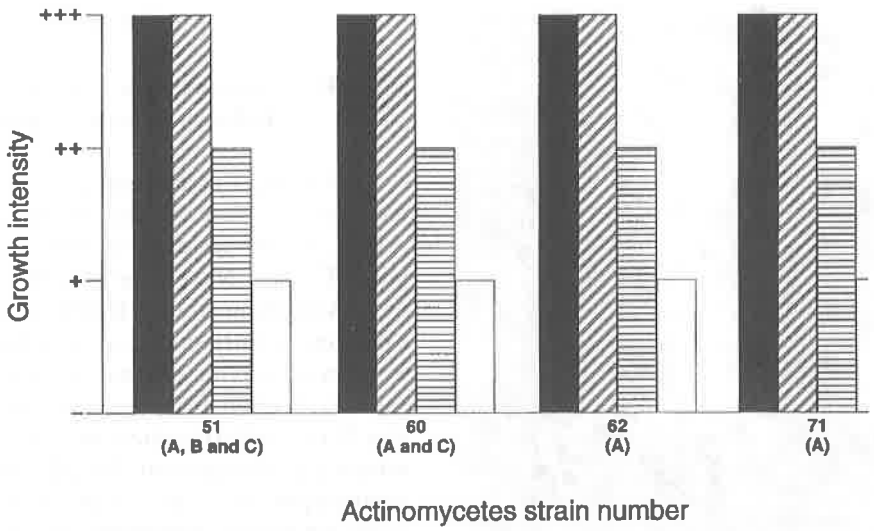

FIGURE 2. Extent of *Tcruzi* epimastigote replication in the presence of different concentrations of actinomycete extracts obtained from cultures in low cost media ■ no addition of culture filtrate; addition of ▨ 100ml; ▤ 250 ml; □ 500 ml, of culture filtrate. A = medium containing soluble starch. B = medium containing rice flour. C = medium containing corn flour (for details see text).

inhibited *T. cruzi* growth. Of these species, two are known to be producers of antibiotics. *S. alboniger* produces puromicin (19) and also paulomicin A and B (11), whereas *S. halstedii* produces N-carbamoyl-D-glucosamine, which is weakly active against some Gram-negative bacteria and fungi (14). *S. phaechromogenes* is also cited in the literature as having little or no antimicrobial activity against bacteria and fungi (10). No anti-protozoan effects have been previously described for any of these actinomycetes. The remaining ATCC strain, *S. viridochromogenes*, was completely inactive against *T. cruzi* in the present investigation. A strain distinct from those is mentioned by Hasegawa et al. (5) as having a strong inhibitory effect against *Trichomonas vaginalis*. It should be remembered, however, that antibiotic production is a strain rather than a species characteristic.

The effect of some extracts which caused total inhibition of parasite growth in the first screening was also evaluated by phase contrast microscopy. After three days of incubation in the presence of active compounds (Fig. 3), normal elongated epimastigotes along with a few abnormally rounded epimastigotes were observed, both types appearing poorly or totally unmotile. After 7 days of incubation (Fig. 4), all the epimastigotes had become rounded and completely unmotile.

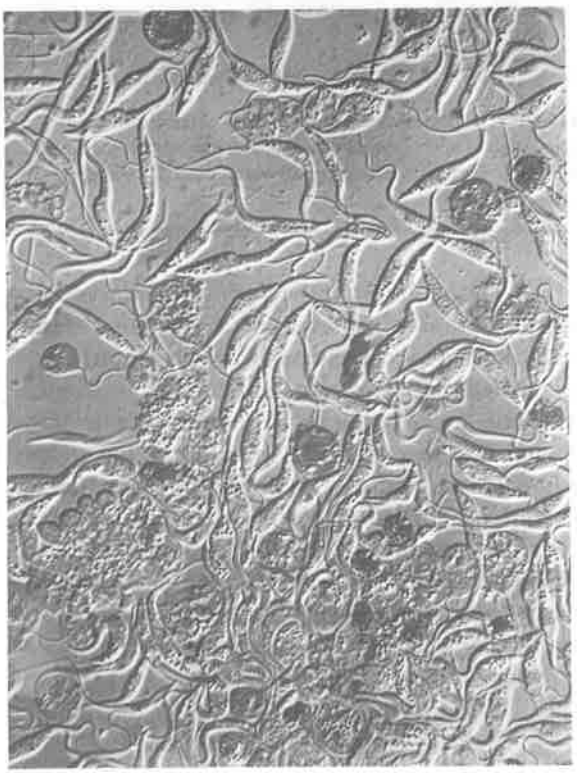

FIGURE 3. *T. cruzi* epimastigotes beginning to show morphological alterations after 3 days of incubation with actinomycete culture filtrates. Normal (A) and modified (B) epimastigotes (phase contrast microscopy; 400X).

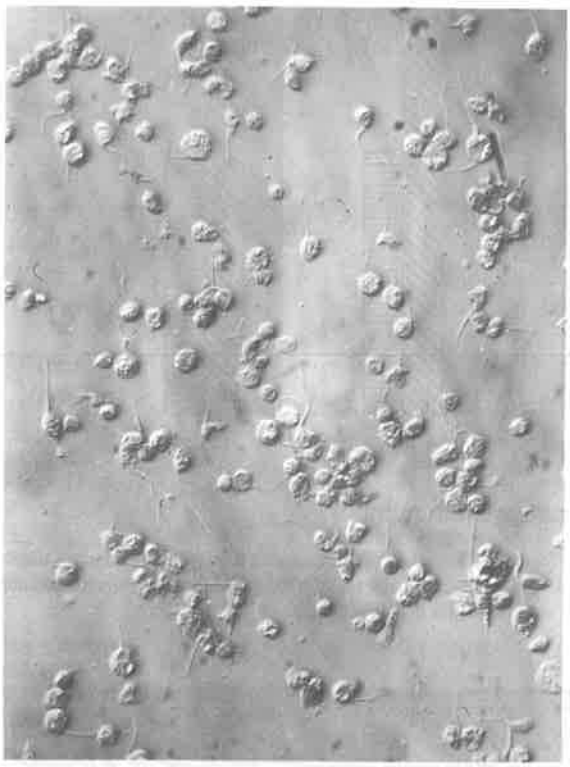

FIGURE 4. Extensive morphological alterations of *T.cruzi* epimastigotes after 7 days of incubation with actinomycete culture filtrates. Only modified epimastigotes are observed (B). (Phase contrast microscopy; 400X).

The inhibitory activity of some actinomycete culture filtrates reported in the present paper suggests that a more extensive investigation should be carried out on extracts obtained from tropical soil actinomycetes. Strains from culture collections should also be examined. In addition, the strains with promising anti-*Tcruzi* activity described herein should be further investigated. The bioactive compounds obtained need to be isolated, purified, chemically characterized and tested in vitro against tissue culture derived trypomastigote forms of the parasite, as well as *in vivo* in animals experimentally infected with *T.cruzi*.

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RESUMO

Inibição de *Trypanosoma cruzi* por actinomicetos isolados de solos tropicais

Oitenta e quatro amostras de actinomicetos isolados de solos brasileiros, mais 4 obtidas da ATCC, foram testadas quanto à capacidade de inibir *T. cruzi*. Nove delas (ca. 10%), quando cultivadas em meio mineral líquido contendo glicerol e peptona ou nitrato, com eventual adição de extrato de levedura e/ou tirosina, excretaram compostos capazes de inibir completamente os parasitos. Outras 22 (ca. 25%) produziram compostos que mostraram um efeito parcial. Para algumas amostras, a composição do meio e o período de incubação foram parâmetros importantes na capacidade inibitória dos filtrados das culturas. Em meios contendo ingredientes de baixo custo (amido solúvel, farinha de arroz, farinha de milho), a maioria das amostras testadas, que no estudo prévio havia determinado um efeito inhibitório parcial, não confirmou este efeito neste segundo experimento. Apenas 4 dentre as 16 amostras testadas foram parcialmente ativas. Três dentre as 4 amostras da ATCC foram ativas contra *T. cruzi*, duas das quais já conhecidas como produtoras de antibióticos ativos contra bactérias, mas não contra protozoários. O efeito de alguns sobrenadantes de cultura sobre ***T. cruzi*** foi acompanhado por microscopia de contraste de fase, para a detecção de células alteradas e imobilizadas.

Palavras-chave: *Trypanosoma Cruzi*, actinomicetos, efeito inibitório.

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GENETIC MANIPULATION OF *ASPERGILLUS NIGER* FOR INCREASED SYNTHESIS OF PECTINOLYTIC ENZYMES

Sonia Couri[1]
Antonio Xavier de Farias[1]

ABSTRACT

A group of 35 morphological mutants with potential for pectinolytic enzyme synthesis was obtained from conidia of *Aspergillus niger* by conventional induced mutation with EMS followed by UV irradiation. In the first stage, the mutant strain with highest pectinolytic activity (described as *A. niger* 5T25A) was used for induction of second stage mutants. The selected strains were tested for synthesis of pectinolytic enzymes under solid state conditions. After 72 h of fermentation, one mutant (*A. niger* 3T5B8) showed pectinolytic activity as measured by released reducing groups and viscosity reduction, with values that were, respectively, 56% and 282% greater than the obtained for wild *A. niger* and 31% and 147% greater than those of A. *niger* 5T25A. Mutant *A. niger* 3T5B8 showed a markedly reduced capacity for sporulation and grew in low water activity (a_w) media (basic medium, aw = 0.95 and fermentation medium, aw = 0.92), favoring the process of solid state fermentation. Another advantageous feature of this selected mutant was the absence of sectors in its colonies when grown in complete and basic medium.

Key-words: Induced mutation, Aspergillus niger, pectinolytic enzyme, solid state fermentation.

INTRODUCTION

Aspergillus niger van Tieghem has a high potential for the production of organic acids and enzymes, in addition to other metabolites of industrial interest. Previous research with this fungus has shown promising results with respect to microbial synthesis of pectinases used in the maceration of fruit pulps and extraction and clarification of juices and wines.

Ilczuk (7) obtained A. niger mutants with increased pectinolytic activity using ultra-violet (UV) irradiation. Fiedurek and Ilczuk (5) produced spontaneous revertants from auxotrophic *A. niger* strains with considerable increase in pectinolytic activity.

Bailey and Pessa (2) obtained *A. niger* mutants induced by UV irradiation and developed a process for the production of polygalacturonase (PG) enzyme using wild *A. niger* as standard strain.

The present work was conducted to select *A. niger* strains with high efficiency for pectinolytic enzyme synthesis in solid state fermentation, employing conventional techniques of induced mutation by chemical and physical agents.

MATERIALS AND METHODS

Microorganism. *Aspergillus niger* was isolated from black peppers (*Piper nigrum*) in the CTAA/ EMBRAPA laboratory.

1 Centro Nacional de Pesquisa de Tecnologia Agroindustrial de Alimentos-CTAA/EMBRAPA
Área de Engenharia de Alimentos. Av. das Américas 29 501, Guaratiba, CEP: 23020-470.
Telephone nº: 021- 410 1353. Fax nº: 021-410 1090. Rio de Janeiro, RJ. Brazil.

Media (in g/L of distilled water).

Basic (selection): citric pectin 10.0; $NaNO_3$ 3.0; KH_2PO_4 1.0; $MgSO_4.7H_2O$ 0.5; KCl 0.5; $FeSO_4.7H_2O$ 0.01, agar 20.0; pH 4.2.

Complete: in accordance with Pontecorvo et al. (9).

Culture (fermentation): 100g of commercial corn bran and 60 mL of 0.90% ammonium sulfate solution in 0.1N hydrochloric acid.

Isolation of mutants. Wild type *A. niger* strain was treated with a 2% ethylmethanesulphonate (EMS) solution according to Rosato (10) and with UV irradiation (2537 A). Survival curves were constructed by plating in complete medium at the appropriate dilutions (4).

Selection of pectinolytic mutants. Mutants obtained from treatments with mutagenic agents were morphologically selected for the presence of pigmentation, sporulation and colony sectors. Pectinolytic activity was assayed according to Hankin et al. (6), substituing the cited medium for basic medium. Another technique used was the infusion of 1 cm^2 of orange peel in a salt solution of the basic medium inoculated with conidia of the test colony. After 96 hours, the degradation of the peel was compared with that in non-inoculated medium (control). Selected mutants were assayed for enzymatic activity by solid state fermentation in trays.

Preparation of inoculum. The microorganisms were grown in basic medium at 32°C for seven days. Inocula were prepared by suspending the conidia in 0.01% Tween 80 solution to a concentration of approximately 10^8 conidia/mL.

Solid state fermentation. Selected mutants were tested for pectinolytic enzyme synthesis during solid state fermentation in stainless steel trays (dimensions: 3 x 10 x 13 cm). In each tray, 40g of the sterile culture medium were inoculated with 4 ml of spore suspension and incubated at 32°C in a chamber.

Preparation of the enzymatic solution. 100 ml of acetate buffer (pH 4.2) were added per 40 g of fermented medium, stirred for 1 hour at 230 rpm in a controlled temperature chamber, and filtered through Whatman 40 filter paper.

Pectinolytic activity. The activity in the enzymatic solution was determined using two methods. The first method measured PG activity by quantifying the number of reducing groups released as galacturonic acid after incubation of the enzymatic solution with 0.25% polygalacturonic acid (SIGMA P.3889), as described by Somogy (11). One unit was defined as the amount of enzyme that released 1 mol of galacturonic acid per minute per mL of enzymatic solution, under conditions of the assay. The second method measured the viscosity change in 10 mL of a 1 % citric pectin solution (INLAB Cod. 6240), pH 4.2, for 10 minutes using a rotational viscosimeter (MLW-Rheotest 2) in a thermostatic bath (35°C). The apparent viscosity was determined and one viscosimetric unit was defined as the volume (mL^{-1}) of enzymatic solution which reduced 50% of the viscosity of a citric pectin solution for ten minutes, under conditions of the assay (8).

The average results of the four fermentations were submitted to the Student's "t" test ($P<0.01$) (3).

RESULTS AND DISCUSSION

Using the technique of induced mutation, morphological mutants were isolated. Three cultures of wild type *A. niger* on agar slants were treated with 2 % EMS solution. From each culture, viable cells with morphological modifications in the 1 to 5 % survival range were tested for total pectinolytic activity. Cultures 1 and 2 showed no mutants with positive characteristics for selection (sporulation absent). However, after two hours of treatment, fifteen mutants from culture 3 were screened by the same methods and two were selected (*A. niger* 5T25A and *A. niger* 15T24A).

These two mutants were tested for their ability to synthesize pectinolytic enzymes during solid state fermentation and the results were compared with those obtained with wild type *A. niger*. For screening, 0.2 mL of the enzymatic solutions (filtrates obtained after 24, 48 and 72 hours of fermentation) was used to determine enzyme activity by reduction in viscosity. A slow decrease

in activity (percent reduction in viscosity) was observed for both mutants after 24 and 48 hours of fermentation, whereas wild type *A.niger* showed an increase (TABLE 1). Both mutants exhibited a stable behavior after 72 hours of fermentation, while the wild strain showed a slight decrease in activity.

TABLE 1. Activity of the pectinolytic enzyme complex from two morphological mutants of *A.niger* as compared to the wild strain. Results are expressed as percent reduction in viscosity.

A. niger	*Fermentation time (hours)*		
	24	48	72
	Viscosity reduction (%)		
15T24A	48	46	47
5T25A	58	56	55
Wild	24	48	42

Due to the greater activity and stability observed at all the tested times during preliminary screening, mutant *A.niger* 5T25A was selected for subsequent treatment with UV irradiation.

A.niger 5T25A was treated with UV irradiation for 5 minutes. After this time (5% of survival), twelve morphological mutants were selected and submitted to a series of tests, as previously described. Six *A. niger* mutants were chosen and designated T53B6, T53B7, 3T5B8, T53A9, T53A14 and 5T25A12.

The effect of induced mutation by UV irradiation on the synthesis of pectinolytic enzymes, as measured by reduction in viscosity, is presented in TABLE 2. Because the activity of mutant *A. niger* 3T5B8 was significantly higher than that of the standard strain and of the other mutants, it was selected for further tests.

The pectinolytic activity of wild type *A. niger*, *A. niger* 5T25A and *A. niger* 3T5B8, expressed in units (U), is shown in TABLE 3.

Both mutants had a higher enzymatic activity than wild type *A. niger* for all the fermentation periods. After 72h, the reduction in viscosity and release of reducing groups for mutant *A. niger* 3T5B8 were, respectively, 282% and 56% higher than the obtained for wild type *A. niger* and 147% and 31% higher than those of mutant *A. niger* 5T25A. *A. niger* 3T5B8 also showed a markedly

TABLE 2. Percent reduction in viscosity of a 1% pectin solution by the action of enzimatic solutions produced by different mutants of *A. niger*.

Mutant	Fermentation time (hours)	
	24	48
	Viscosity reduction (%)	
5T25A*	52.2	49.5
T53B6	42.9	41.0
T53B7	43.8	34.8
3T5B8	67.5	61.0
T53A9	45.7	39.8
T53A14	47.0	39.9
5T25A12	43.8	42.1

*Standard mutant

TABLE 3. Enzymatic activity measured by reduction in viscosity and release of reducing groups. Results are expressed in units (U).

A. niger strain	Fermentation time (hours)					
	24		48		72	
	Activity (U)					
	a	b	a	b	a	b
Wild	6.0	4.8	12.0	5.8	11.0	4.6
5T25A	9.0	5.0	13.0	6.5	17.0	5.5
3T5B8	14.0	6.8	28.0	7.3	42.0	7.2

a: reduction in viscosity (ml^{-1}/10min)
b: reducing group released (μmolcs/min/ml.)

reduced capacity for sporulation without affecting its capacity to produce the amount of conidia necessary for inoculation.

The results demonstrate that treatment of *A. niger* conidia with EMS followed by UV irradiation is an efficient procedure for obtaining a morphological mutant with higher potential for the synthesis of pectinolytic enzymes. Ilczuk (7), using a multi-stage induced mutation with *A. niger* strains, increased PG and pectin esterase (PE) microbial synthesis.

Several morphological mutants obtained in this work were not tested due to complete loss of sporulation capacity. With mutant *A. niger* 3T5B8, a partial loss in sporulation capacity was observed after UV treatment. However, this was the mutant with highest stability as expressed by the absence of sectors in colonies grown in complete media. This makes it highly adaptive to solid state

fermentation, since sporulation was not observed during the fermentation period studied.

Antier et al. (1) reported a new approach for selecting pectinase producing mutants of *Aspergillus niger*. They found that low levels of water activity (a_w), induced by adding 15% of ethylene glycol to agar plates (a_w= 0.96), significantly affected the obtention of strains well adapted to solid state fermentation. In the present work, mutant *A. niger* 3T5B8 grew in low water activity media (basic medium, a_w = 0.96 and fermentation medium, a_w=0.92), which also favored its adaptation to the solid state fermentation process.

Further experiments are currently underway to optimize solid state fermentation for the production of pectinolytic enzymes by *A. niger* 3T5B8, with potential application to industrial production.

RESUMO

Melhoramento genético de *Aspergillus niger* para o aumento da síntese de enzimas pectinolíticas.

Um grupo de trinta e cinco mutantes morfológicos com potencial para síntese de enzimas pectinolíticas foi obtido de conídios de *A. niger*, empregando a técnica convencional de mutação induzida por EMS seguida por irradiação com UV. A linhagem mutante selecionada na primeira etapa (descrita como *A. niger* 5T25A) foi usada para a segunda etapa de indução de mutação. Todas as linhagens selecionadas foram testadas para a síntese de enzimas pectinolíticas por fermentação em estado sólido. Após 72 h de fermentação o extrato enzimatico obtido pelo mutante *A. niger* 3T5B8 foi o que apresentou maior atividade enzimática. A atividade, medida por grupos redutores liberados e por redução de viscosidade. Os valores obtidos para estes parametros foram, respectivamente, 56% e 282% maiores que os registrados para *A. niger* selvagem e 31% e 147% maiores que s obtidos com o mutante *A. niger* 5T25A. O *A. niger* 3T5B8 mostrou uma acentuada redução na sua capacidade de esporulação e cresceu em meios com baixas atividades de água (a_w) (meio básico, a_w = 0,96 e meio de fermentação, a_w = 0,92), favorecendo o processo de fermentação em estado sólido e formação de colônias ausentes de setores em meios de cultura completo e básico.

Palavras-chave: mutação induzida, *Aspergillus niger*, enzimas pectinolíticas, fermentação em estado sólido.

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PARTIAL PURIFICATION OF A POLYGALACTURONASE PRODUCED BY SOLID-STATE CULTURES OF *ASPERGILLUS NIGER* 3T5B

Maria Alice Z. Coelho[1]*
Ricardo A. Medronho[1]
Selma G.F. Leite[1]
Sônia Couri[2]

ABSTRACT

An extracellular polygalacturonase, obtained from extracts of solid-state cultures of *Aspergillus niger* 3T5B8, was purified using salting-out fractionation, dialysis and gel-filtration chromatography. Addition of ammonium sulfate at different levels of saturation lead to a compromise between purification and recovery of enzyme activity. Gel-filtration chromatography showed the best results when sodium azide (0.02% w/v) was added to the elution buffers, decreasing losses in enzyme activity by 14%. The molecular weight determined for this polygalacturonase was 34700 daltons.

Key words: polygalacturonase, enzyme purification, *Aspergillus niger*.

INTRODUCTION

Pectic enzymes of fungal origin, especially from *Aspergillus niger*, are industrially produced for use as processing aids in the extraction and clarification of fruit juices and wines, the extraction of oils, flavors and pigments from plant materials and the preparation of cellulose fibers for linen, jute and hemp manufacture (4, 15, 16).

These enzymes can be divided according to their action on the substrate molecule. They act on pectin degradation through desterification of the substrate catalyzed by pectinesterase and sequentially through polygalacturonase hydrolysis of pectate, a heteropolymer of which the backbone consists mainly of a-D-galacturonic acid units connected by a-1.4 glicosidic bonds.

Purification and characterization of the individual enzymes lead to a better understanding of their contribution to the degradation process. The use of enzymes in food processing requires a good control of toxicity and knowledge of their properties.

In the last few years, there has been an increasing effort to associate protein purification techniques to the fermentation process, as the extraction and purification of proteins are an important part of the whole biotechnological process (16).

This work relates to the partial-purification of a polygalacturonase produced by *Aspergillus niger* 3T5B8 through the utilization of known methods. The aim was to obtain a polygalacturonase enzymatic extract with relatively high specific activity at low costs.

MATERIALS AND METHODS

Detailed information about the methods used can be found in Coelho (1).

[1] Escola de Química, Universidade Federal do Rio de Janeiro, CT Bl. E, 21941-900, Rio de Janeiro, RJ, Brazil. Fax: 55(021)5904991.
[2] CTAA/EMBRAPA, Av. das Américas, 29501, 23020-470, Rio de Janeiro, Brazil.
* To whom correspondence should be addressed.

Microorganism: *Aspergillus niger* 3T5B8, from the National Research Center of Agro-industrial and Food Technology (CTAA / EMBRAPA - Rio de Janeiro, Brazil) collection. This is a strain selected from induced mutation (3).

Enzyme preparation: Crude enzyme was provided by CTAA / EMBRAPA. It was obtained through solid-state culture containing 40 g of wheat bran with 60% of humidity (ammonium sulfate solution). The medium was seeded with 10^8 spores of *Aspergillus niger* 3T5B8 per gram of culture and incubated at 32°C for 48 hours.

The extraction was performed using 70 mL sodium acetate buffer pH 4.4 at 30oC, under agitation (230 rpm) for 30 minutes. A conventional filtration step was used to remove insoluble impurities eventually present. The crude enzyme solution was stored at -30°C for further purification.

This temperature was chosen based on the results presented by Langone et al. (10). The authors verified that it is possible to store this type of solution at -30°C for at least three months without any change in relative polygalacturonase activity. It was not necessary to use an agent, as glycerol, to maintain enzyme stability.

Assay of enzyme activity: Polygalacturonase activity was determined by measuring the reducing groups released by enzyme hydrolysis of the pectin substrate. The reaction mixture consisted of one mL of 0.25% polygalacturonic acid salt (Sigma Chemical CO., USA) in sodium acetate buffer pH 4.4 and 0.1 mL of suitably diluted enzyme. Incubation was carried out at 45°C for 10 minutes. Galacturonic acid released was measured by the method of Somogyi(18), using galacturonic acid monohydrate (Sigma Chemical CO., USA) as standard.

One polygalacturonase unit was defined as the amount of enzyme which produced 1mmol of galacturonic acid per minute under the above conditions.

Protein determination: Protein concentrations were measured as described by Lowry (11) using bovine serum albumin as standard.

Polygalacturonase Purification:

Salting-out fractionation - Different levels of ammonium sulfate saturation were used (25, 40, 55, 70, 85, 90 and 95%). The crude enzyme solution was treated with these levels of saturation overnight at 5°C and then centrifuged at 8500 rpm (20000 x g) for 30 minutes. Both precipitate and supernatant were analyzed for polygalacturonase activity and protein content.

Dialysis - Supernatant obtained after ammonium sulfate 25% saturation treatment and precipitate obtained after ammonium sulfate 70% saturation treatment were dialyzed in a cellulose bag (Spectrapor no 132700) against sodium acetate buffer pH 4.4, overnight at 5°C under agitation. An aliquot was collected and analyzed for polygalacturonase activity and protein content.

Gel-filtration chromatography - The dialyzed enzyme solution was added to a Sephadex G-100 (Pharmacia Ltd.) column (1.2 x 29 cm) previously equilibrated with sodium acetate buffer pH 4.4 and eluted with the same buffer without change in ionic strength, but containing 0.02% (w/v) sodium azide in some experiments. The flow rate was 19 mL/h and fractions of 9 mL each were collected during 11 hours and 20 minutes. Both protein content and polygalacturonase activity were determined in the same manner as above. Protein eluted was monitored by the spectrophotometric method (E280/E260) of Warburg and Christain (20).

SDS-Polyacrilamide electrophoresis - The procedure followed the and Osborn method (21).

RESULTS AND DISCUSSION

Salting-out fractionation: Fractionation steps usually imply a compromise between recuperation of enzyme activity and the desired level of purification. The concentration of salt necessary to promote precipitation is not an absolute property of the enzyme, but depends on the properties of other proteins present (co-precipitation) and on the initial protein concentration (17).

Different levels of ammonium sulfate saturation were added at constant intervals to evidence this compromise and to select an adequate salt concentration to work with.

The results presented in TABLE 1 show the best purification at 70% ammonium sulfate (3-fold), while 100% recovery of activity was obtained only at ammonium sulfate levels higher than 90%. It was observed that 52% of the total protein content

TABLE 1. Results obtained by ammonium sulfate fractionation

% Saturation	Specific Activity (U/mg)	Purification (fold)	Recovery of enxyme activity (%)	Protein Recovery (%)
0	4.42	1.0	100.0	100.0
25	8.08	1.8	9.0	4.9
40	10.46	2.4	18.4	7.8
55	11.98	2.7	37.8	14.0
70	13.15	3.0	68.3	23.0
85	11.00	2.5	87.4	35.1
90	10.50	2.4	100.0	42.0
95	9.00	2.0	100.0	49.1

remains in the supernatant even after addition of higher concentrations of ammonium sulfate, like 95% saturation. The addition of salt does not precipitates all the proteins because protein solubility is dependent on polar interactions with the aqueous solvent, ionic interactions with the salts present and electrostatic forces (17).

These results are in some respect better than those described in the literature. Hara et al. (5,6), using 90% ammonium sulfate to precipitate a polygalacturonase from *Aspergillus niger*, reached around 1.5 fold purification levels. In our study, for the same salt concentration, the purification obtained was higher (2.4-fold) than that reported by Hara et al. or by Obi and Moneke (1.6-fold) (14). Under this condition of salt saturation, the recovery of enzyme activity in the mentioned studies was around 85%, whereas in the present work it reached 100%. On the other hand, for the best purification level (3-fold) presently obtained at 70% sulfate saturation, the activity recovered did not exceed 68%.

Gel-filtration chromatography: Preserving agents are added to elution buffers in order to inhibit the action of proteases possibly present. 2-mercaptoethanol (9) and sodium azide (8, 13, 19) are examples of preserving agents.

The precipitate obtained after treatment of the crude enzyme extract with 70% ammonium sulfate was dissolved in sodium acetate buffer pH 4.4 and dialyzed. This led to an approximate 3-fold increase in specific polygalacturonase activity with an activity recuperation of 97.5%, showing that dialysis is an important step in the purification process (2).

After dialysis, the enzyme solution was applied to a gel-filtration column and protein elution was made with acetate buffer, pH 4.4, with or without 0.02% (w/v) sodium azide. TABLE 2 shows the results obtained in both cases.

As can be seen, the use of sodium azide decreases the losses in enzyme activity by 14% and apparently improves the protein elution profile (FIGURES 1 and 2), approaching it to the activity profile. This may indicate that a proteolytic degradation of the protein molecule occurs when sodium azide is absent.

Data from other investigations are worse than the 3-fold purification and 91% activity recuperation presently reported using sodium azide in the elution buffer. Our levels are higher than the

TABLE 2. Partial purification by gel-filtration chromatography

Elution Na3N (0.02%)	Specific Activity (U/mg)	Purification (fold)	Recovery of enzyme activity (%)
with	31.5	3.03	91.0
without	21.2	1.85	78.1

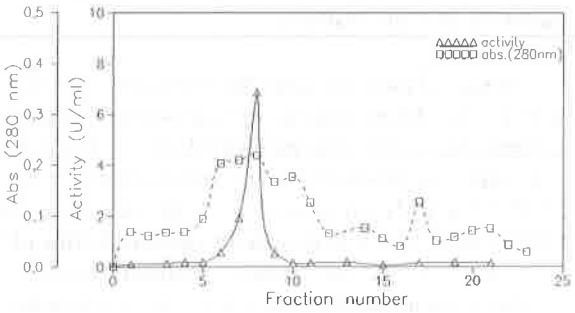

FIGURE 1: Elution chromatography profile using precipitate obtained at 70% saturation (without NaN_3 addition).

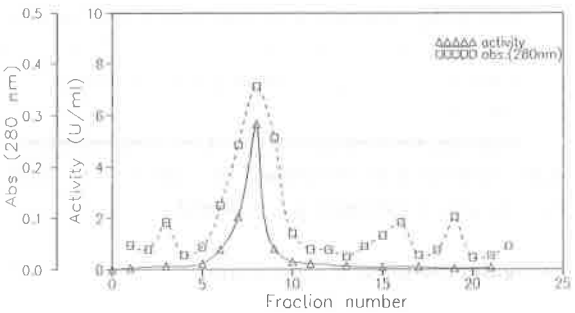

FIGURE 2: Elution chromatography profile using precipitate obtained at 70% saturation (with NaN_3 addition).

values described by Hara et al. (5,6), who obtained a 2.5-fold purification with 29% recovery in enzyme activity for an exo-polygalacturonase from *Aspergillus niger* after gel-filtration chromatography in Sephadex G-100. Manachini et al. (12) reached 89% recovery in enzyme activity but only a 1.1-fold increase in purification for an endo-polygalacturonase of *Rhizopus stolonifer*.

Purification schemes: Considering the results obtained for the different steps, two purification schemes are presented in TABLE 3. Increases of 9.2 and 9.5-fold in specific polygalacturonase activity were detected in both schemes, but with different yields.

As can be seen in both cases, gel-filtration chromatography led to dilution of the enzyme and the need of a concentration step, which is considered essential in the separation of polygalacturonase from pectinesterase (7).

The choice between the schemes presented in TABLE 3 depends on the commercial use intended for the polygalacturonase. It was possible to separate a portion of a partially purified enzyme for applications not dependent on higher levels of purification. However, separation of the polygalacturonase using less ammonium sulfate permitted a higher yield (88%) for the same degree of purification.

Molecular Weight Determination: The relation between protein mobility and molecular weight can be obtained through SDS-polyacrilamide gel electrophoresis. Using protein standards (bovine serum albumin, ovoalbumin, carbonic anhydrase and soybean trypsin inhibitors) it is possible to determine the unknown polygalacturonase molecular weight through a linear relationship (FIGURE 3).

TABLE 3. Purification schemes

	Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification (fold)	Activity Yield (%)
Crude Enzyme	5	352.5	106.0	3.3	1.0	100.0
$(NH_4)_2SO_4$ at 25% (supernatant)	5	346.5	34.0	10.2	3.1	98.3
Gel-Filtration	36	124.1	4.1	30.3	9.2	88.0
Crude Enzyme	5	352.5	106.0	3.3	1.0	100.0
$(NH_4)_2SO_4$ at 70% (precipitate)	5	116.7	11.3	10.4	3.1	66.2
Gel-Filtration	37	84.9	2.7	31.5	9.5	60.2

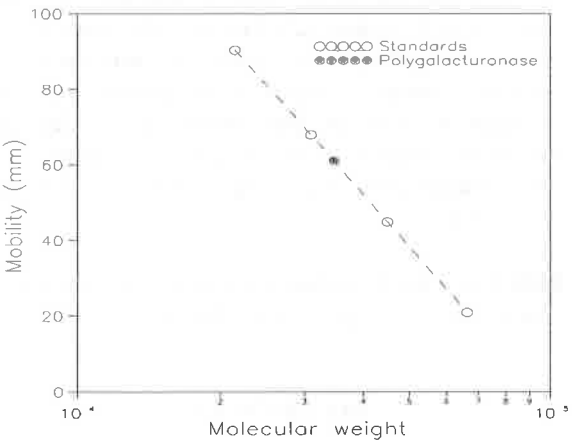

FIGURE 3 - Determination of polygalacturonase molecular weight.

The preparation of purified enzyme obtained, which was homogeneous, was used for molecular weight (MW) determination. The MW found for the polygalacturonase was 34700 daltons. This result is in agreement with MW values described for polygalacturonases of *Aspergillus niger* (20000 - 66000 daltons) by other workers. The polygalacturonase presently obtained may be an endo-enzyme since its MW is similar to that of others described in the literature (5, 8, 14). This possibility agrees with the 79% viscosity reduction obtained for a pectin solution.

Hara et al. (5) estimated a MW of 35000 daltons for an endo-polygalacturonase of *Aspergillus niger*. Similarly, Kester and Visser (8) obtained different endo-polygalacturonases (isoenzymes) from this fungal species with molecular weights ranging between 38000 and 50000 daltons. Finally, Obi and Moneke (14) describe a polygalacturonase from *Aspergillus niger* of 38000 daltons in their work.

RESUMO

Purificação parcial de poligalacturonase produzida por *Aspergillus niger* 3T5B8

A poligalacturonase produzida em cultura semi-sólida de *Aspergillus niger* 3T5B8 foi purificada usando fracionamento por adição de sal ("salting-out"), diálise e cromatografia de gel-filtração. A adição de sulfato de amônio em diferentes níveis de saturação apresentaram um compromisso entre purificação e recuperação da atividade enzimática. A cromatografia por gel-filtração forneceu bons resultados quando azida de sódio foi utilizada nos tampões de eluição, diminuindo as perdas na atividade enzimática em 14%. O peso molecular determinado para esta poligalacturonase foi de 34700 daltons.

Palavras chave: poligalacturonase, purificação, enzimas pectinolíticas, *Aspergillus niger*

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Genetic stability, conjugal transfer and expression of heterologous DNA inserted into different plasmids and the genome of *Pseudomonas fluorescens* in soil

Eric Smit*, Anneke Wolters; Jan Dick van Elsas*

*To whom correspondence should be sent.
Institute for Plant Protection
P.O. Box 9060
6700 GW Wageningen The Netherlands

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Thin-Layer chromatography of mycobactins and mycolic acids for the identification of clinical mycobacteria

Clarice Q.F. Leite; Angela M.W. Barreto; Sergio R.A. Leite

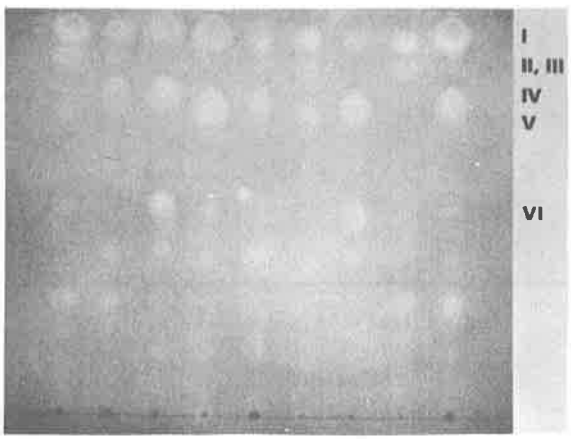

FIGURE 3. Chromatogram of a mycolic acid methyl esters. Sovent: diethyl ether-petroleum ether.
Reference strains of micobacteria, from left to right: *M.chelonae* (I.II), *M.tuberculosis* (I.(III).IV), *M.smegnatis* (I.(II).V), *M.serofulaceum* (I.IV.VI), *M.chelonae* (I.II), *M.fortuitum* (I.V).

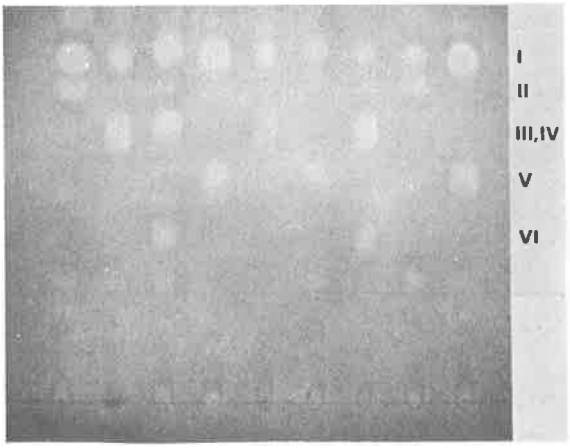

FIGURE 4. Chromatogram of mycolic acid methyl esters. Solvent: dichloromethane.
Reference strains of micobacteria, from left to right: *M.chelonae* (I.II), *M.tuberculosis* (I.III.IV), *M.avium-intracellulare* (I.IV.VI), *M.fortuitum* (I.V.), *M.bovis* (I.(III.)IV), *M.smegmatis* (I.(II).V), *M.scrofulacetum* (I.IV.VI), *M.chelonae* (I.II), *M.fortuitum* (I.V).

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

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

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

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

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Anonymous. The economy of by-products. *Álcool Alcoolquim.*, 2;33-40, 1985.

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

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