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MICROBIAL CONTAMINATION OF STORED HYDROCARBON FUELS AND ITS CONTROL

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MINI-REVIEW

ABSTRACT

The major microbial problem in the petroleum refining industry is contamination of stored products, which can lead to loss of product quality, formation of sludge and deterioration of pipework and storage tanks, both in the refinery and at the end-user. Three major classes of fuel are discussed in this article - gasoline, aviation kerosene and diesel, corresponding to increasingly heavy petroleum fractions. The fuel that presents the most serious microbiological problems is diesel. The many microorganisms that have been isolated from hydrocarbon fuel systems are listed. The conditions required for microbial growth and the methods used to monitor and to control this activity are discussed. The effects of various fuel additives, including biocides, are considered.

Key words: Biocides; biofilms; hydrocarbon fuels; microbial corrosion; storage tanks

INTRODUCTION

The petroleum refining industry is one of the largest manufacturing industries in the world. After Western Europe and the USA, Latin America, including Mexico, has the highest refining capacity of the rest of the world. Huge amounts are spent each year on capital equipment, modernization and maintenance, including prevention and treatment of microbial contamination. The major microbial problem in the industry is contamination of stored products, which can lead to loss of product quality, formation of sludge and deterioration of pipework and storage tanks, both in the refinery and at the end-user. Reports of such contamination have increased substantially in recent years (25, 26), probably due to increasing demand for diesel fuel and high quality gasolines and jet fuel (4).

Crude oil is a mixture of many different hydrocarbons, straight, branched and cyclic aliphatics, aromatic and heterocyclic compounds. The composition varies with the origin of the oil; heavy crudes generally have high carbon, metal and asphaltene content and are less stable chemically than lighter crudes. The refining process can be divided into four phases: 1) separation, 2) cracking, 3) chemical reactions such as polymerization or alkylation, 4) blending. Refinery products are mixtures of compounds. Gasoline, for example, contains straight chain and branched-chain

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hydrocarbons, alkenes, naphthalenes, aromatics and other compounds. Stabilizers, octane enhancers (principally tetraethyl lead), anti-corrosion agents and other substances may also be added. Treatments such as desalting, dehydrating and desulfurization are also sometimes required. The processes employed in fuel production affect the composition of the final product, which, in turn, affects its susceptibility to microbial contamination.

CHARACTERISTICS OF HYDROCARBON FUELS

Three major classes of fuel are discussed in this article - gasoline, aviation kerosene and diesel, corresponding to increasingly heavy petroleum fractions (Table 1).

Table 1 - Fuel fractions obtained from crude oil

Fraction	C atoms	M. Wt.
Gas	1-4	16-58
Gasoline	5-12	72-170
Kerosene	10-16	156-226
Diesel	15-22	212-294

Gasoline The properties of the various grades of gasoline are controlled by specifications designating boiling point range, volatility, octane number, stability and various minor constituents. Sulfur compounds, consisting mainly of disulfides, sulfides and thiophenes, are undesirable and the level is rarely above 0.25% wt. Various additives are allowed:-

- anti-oxidants (e.g., substituted aromatic amines and phenols). These remove free radicals, which cause the production of polymeric gums
- metal deactivators (chelating agents such as disalicylal-1,2-propane diimine), which inhibit the formation of free radicals
- tetraethyl or tetramethyl lead (anti-knock compounds)
- anti-icing agents (alcohols or surfactants)
- corrosion inhibitors (surfactants).

Dyes may also be added for identification and advertising purposes. In Brazil, gasoline may contain up to 17% alcohol. This would be expected to concentrate in the water phase, inhibiting microbial growth. The range of carbon chain lengths present in gasoline (Table 1) also limits growth of microorganisms, the lower molecular weight compounds may be toxic due to the solvent effect on cell membranes (31). However, it is apparent that many of the allowed additives (e.g., surfactants) could act as nutrient sources for microorganisms, whilst others, like the anti-knock compounds and sulfur containing compounds, may be slightly inhibitory.

Jet Fuel. Specifications for jet fuel (aviation kerosene) are the most demanding. The fuel must ignite readily under all conditions and must burn steadily with no blowout or flashback. It must produce minimal levels of particulates and must have low vapor pressure and freezing point (9). These requirements are met by paraffinic kerosene or a mixture of kerosene and gasoline fractions with an aromatic content below 25%. Anti-icing agents (diethylene or triethylene glycol monomethylether; 2-methoxyethanol 2-ME) may be added to the fuel and these have a biostatic activity (32), although *Pseudomonas putida* has been shown to utilize 2-ME (15). The hydrocarbons in this fuel are readily degraded by some microorganisms.

Diesel. Diesel fuel is a hydrocarbon product boiling between approximately 150°C and 400°C, with carbon chain lengths of C15-C22 (Table 1). Various classes are available and classification differs from country to country. Some classes contain selected cracked distillates such as light cycle oils (10). In Brazil, a relatively new introduction onto the market is "urban (or metropolitan)" diesel, which has a lower permitted level of sulfur (0.5% maximum, somewhat higher than in many other countries). Reduced sulfur content can allow enhanced microbial activity. Marine diesel fuel differs in its specifications from others. In Brazil, it must reach its flash point in 60, rather than 30, minutes. A variety of additives may be used to improve the stability of the fuel; these include compounds such as aliphatic amines, chelating agents, detergents and corrosion inhibitors (4), some of which can act as a nutrient source for microorganisms. Diesel is the fuel which suffers from the most varied microbial contamination problems.

CONTAMINATION PROBLEMS IN FUEL STORAGE TANKS

Even in the best-kept tanks, microbial contamination is an occasional problem. Microorganisms are usually present in the fuel, but good housekeeping (removal of water and use of biocides) minimizes their growth. Nevertheless, reports of microbial growth in fuel tanks have increased in the last few years (26) and the holding of strategic reserves for long periods has always been problematical (12). The most important requirement

for microbial growth in fuels is water. This is almost always present, for the following reasons:

- water dissolved in the fuel can condense on tank walls
- moisture in the air can enter through floating tank lids or other vents
- poorly designed tanks do not drain efficiently
- water may be added as ballast (on ships) or to purge the delivery system.

In Brazil, the specification for diesel oil allows a maximum of 0.05% water. This is 0.5ml/litre - quite sufficient for the initial growth of microorganisms. Although Hill and Hill (26) state that 1% water is needed for substantial microbial growth, a fine film of water on the tank surfaces, or a few microliters in the fuel, is enough to allow microorganisms to begin growing, and cell metabolism, once begun, results in the production of more water. Thus the cycle continues.

Oxygen is normally present in sufficient quantities in distillate fuels (25), and is continually replenished when tanks are refilled. However, even if the fuel becomes anaerobic, it is not protected from microbial attack, since facultative organisms, such as *Bacillus*, and anaerobes, such as sulfur-reducing bacteria (SRB), continue to thrive. The limiting factor to growth is probably availability of minerals, particularly phosphorus, which is generally present at <1ppm in the fuel (25). Nitrogen and iron may also be important limiting nutrients (48). Many laboratory-based studies have shown that fungi grow much more readily in a fuel system containing mineral salts solution as the aqueous phase than with water, or even tank drainage water (6). However, apart from minerals entering in water or aerial contaminants, many of the additives now used in the fuel industry contain these vital mineral elements, removing one of the factors that limited growth in earlier times.

Problems associated with microbial growth are listed in Table 2. In diesel fuel, microbial contamination may contribute to aging instability (28), but in general the most important consequences are microbially induced corrosion of the storage tanks and pipework, and formation of microbial mats, with the ability to block filters and pipelines, and to increase wear in pumps. According to Irish and Richardson (27), as little as 1mg particulates/100ml fuel can cause filtration problems. In addition to microorganisms, these may include dirt, dust, sand, components of other filters such as paper or cotton, pump wear particles, corrosion debris and material removed from tank or pipe linings, such as fiberglass

Table 2 - Consequences of microbial growth in fuel systems

Problem	Principal types of microorganisms
Blockage of pipes, valves, filters and incorrect readings from fuel probes	Fungi; polymer-producing bacteria
Increased water content	All
Sludge formation	All
Surfactant production, causing oil/water emulsification, entry of cells into the oil phase and coalescer malfunction	Fungi and aerobic bacteria
Corrosion of storage tanks and lines	Fungi and anaerobic bacteria
Production of suspended solids in the fuel	All
Breakdown of hydrocarbons	Fungi and aerobic bacteria
Shortened filter life	All
Fouling of injectors	Aerobic bacteria and fungi
Increased sulfur content of fuel	SRB
Shortened life of engine parts	Undetermined
Penetration of protective tank linings	Fungi
Health problems	Endotoxin-producing bacteria, opportunistic pathogens, SRB.

Sources: 5, 14, 25, 26, 36, 40, 42, 55.

(54). Corrosion from within tanks and pipelines can be intense when microbial contamination is present.

In aircraft fuel tanks, made of aluminum alloys, *Hormoconis resinae* can be a major problem causing corrosion and/or penetration of tank linings. Airlines are aware of this and regular testing is undertaken. Current tests however require a minimum of 24 hours and there are still financial implications involving downtime and clean up operations when contamination is found. The major microorganisms involved in storage tank corrosion are the anaerobic SRB. Aerobic bacteria may also participate in the process (for descriptions of microbially influenced corrosion, see refs. 16, 51). Fuel storage tanks in-ground are especially prone to contamination problems, because of the difficulties of drainage. Since they are hidden from view, it may not be noticed that they are corroded and leaking and thus important environmental pollution can result. In Brazil, many fuel distribution stations are replacing in-ground tanks by aerial ones, with resulting decrease in microbial problems. There are, of course, other hazards associated with aerial tanks, such as increased fire risk. A new regulation in the USA requires in-ground tanks to be protected against corrosion by a variety of mechanisms (coatings and cathodic protection), which will mean considerable expenditure by the firms involved.

Microorganisms may enter the fuel from the soil, via the air, from polluted wash water, contaminated pipelines, or from the biofilm present on the tank walls, if the latter have not been sufficiently well cleaned. Table 3 shows the organisms which have been isolated from various stored hydrocarbon fuels. Not all of these organisms are capable of metabolizing hydrocarbons. Those that have been reported to grow, rather than simply survive, in fuel are marked with an asterisk. Apart from the hydrocarbons, organisms may gain nutrients from fuel additives, from dirt entering the system, or from the growth of primary colonizing organisms. The hydrocarbon chains most readily utilized are C10-C18. Shorter chain hydrocarbons may actually inhibit growth of some organisms. Different strains of *H. resinae* however have been reported as having different optimal chain lengths for growth. Teh and Lee (47), using isolates obtained from soil in Australia by the 'Creosoted matchstick technique' (34), found highest yields on n-alkanes C13-C18. Cafone *et al.* (8) studied two isolates from jet fuel and found these to have optimum growth at chain length C11. This may reflect the alkane chain lengths in the substrate from which the fungi were isolated, creosote containing the longer chains from approximately C12-C24.

To assess the importance of particular microorganisms in fuel deterioration, it is essential to determine their ability to grow, rather than merely exist, in these systems. Unpublished studies at UFRGS indicate that *H. resinae* and *A. fumigatus* isolated from diesel are able to grow in Bushnell-Haas mineral medium plus diesel as sole carbon source, whilst *P. variotii* grows poorly, if at all. Hettige and Sheridan (23) showed that *H. resinae*, *P. corylophilum* and *P. variotii* all grew in this system, when inoculated separately, whilst in mixed cultures of *H. resinae* and *P. corylophilum*, only *H. resinae* remained viable after 6 weeks. *P. corylophilum* was, however, able to grow in Bushnell-Haas/fuel from which *H. resinae* had been removed by filtration, indicating that it does not compete well in terms of growth, but is not inhibited by *H. resinae* metabolites. More work is needed to define those microorganisms from the above list (Table 2) which are able to utilize the hydrocarbon chains, and those that grow on other fuel components.

Importance of fuel additives

It has been suggested that microbial growth in gasoline occurs only at the expense of additives such as vegetable oil phosphatides, and certainly large-scale microbial contamination is seen only in the presence of organic additives (11). Solana and Gaylarde (45) found that *H. resinae* grew in gasoline containing 13% alcohol for only 6 days after inoculation, but that changes in the hydrocarbon composition were, nevertheless, produced. It is also rare to find SRB as gasoline contaminants, but this may be due to the toxic effect of gasoline hydrocarbons (11). The presence of various additives in diesel oil has been held responsible for increased microbial problems, but recent work at UFRGS suggests that certain new additives have no effect on fungal growth (6). It is important to test agents intended to improve chemical and physical fuel properties for their potential as stimulators of microbial growth. Utilization of an additive by microbial cells not only results in increased contamination problems, but also leads to "neutralization" (by breakdown) of the additive itself. Chromium tables introduced to naval fuels to enhance anti corrosive properties were found to act as excellent substrates for growth of chromium resistant strains of fungi, due to the degradable binders used in the tables (Kelley, Unpublished).

Table 3 - Microorganisms isolated from fuels

Bacteria		
*Acinetobacter**	*Brevibacterium ammoniagenes*	*Pasteurella sp.*
Acinetobacter calcoaceticus	*Clostridium sporogenes*	*Pseudomonas sp.**
Acinetobacter cerificans	*Corynebacterium sp.*	*Pseudomonas aeruginosa**
Actinomycetes	*Enterobacter cloacae*	*Pseudomonas fluorescens*
Aerobacter aerogenes	*Enterobacter glomerans*	*Pseudomonas maliphora*
*Alcaligenes**	*Flavobacterium arborescens*	*Pseudomonas oleovorans**
*Bacillus sp.**	*Flavobacterium diffusum*	*Pseudomonas putida**
Bacillus acidocaldarius	*Micrococcus sp.*	*Serratia marcescens*
Bacillus megatherium	*Moraxella sp.*	*Serratia odorifera*
Bacillus subtilis	*Ochrobactrum anthropii*	SRB*
Yeasts		
Aureobasidium pullulans	*Candida guilliermondii**	*Candida zeylanoides*
*Candida sp.**	*Candida lipolytica**	*Hansenula mrakii*
*Candida famata**	*Candida rugosa*	*Rhodotorula sp.**
Candida fluviatilis	*Candida tropicalis*	*Saccharomyces sp.*
Filamentous fungi		
Acremonium sp.	*Fusarium sp.**	*Penicillium hirsutum*
*Acremonium strictum**	*Fusarium acuminatum*	*Penicillium minioluteum*
Alternaria sp.	*Fusarium moniliforme**	*Penicillium notatum#*
Alternaria alternata	*Fusarium oxysporum**	*Penicillium spinulosum*
*Aspergillus sp.**	*Fusarium sambucinum*	*Penicillium thomii*
Aspergillus clavatus	*Geomyces cretaceus*	*Penicillium waksmanii*
Aspergillus fischeri	*Geotrichum candidum*	*Pestalotiopsis aquatica*
Aspergillus flavus	*Gliomastix sp.*	*Phialophora sp.*
*Aspergillus fumigatus**	*Helminthosporium sp.*	*Phialophora richardsiae*
*Aspergillus niger**	*Hormoconis resinae**	*Phoma sp.*
Aspergillus nidulans	*Humicola grisea*	*Phomopsis sp.*
Aspergillus ochraceus	*Mucor sp*	*Pseudallescheria boydii*
Aspergillus paradoxus	*Paecilomyces sp.*	*Rhinocladiella sp.**
Aspergillus sejunctus#	*Paecilomyces lilacinus*	*Rhizopus oryzae*
Aspergillus sydowi	*Paecilomyces variotii**	*Sordaria fimicola*
Aspergillus versicolor	*Paecilomyces virvus*	*Stemphylium botryosum*
Aspergillus tamarii	*Penicillium sp.**	*Thielavia sp.*
Botrytis cinerea	*Penicillium brevicompactum*	*Trichoderma sp.*
Cephalosporium sp.	*Penicillium canescens*	*Trichoderma harzianum*
Chaetomium dolichotrichum	*Penicillium citrinum*	*Trichoderma koningii*
Chaetomium globosum	*Penicillium corylophilum**	*Trichoderma viride**
Cladosporium sp.	*Penicillium cyclopium*#*	*Trichosporon sp.**
*Cladosporium cladosporoides**	*Penicillium digitatum*	*Trichothecium roseum*
Cladosporium herbarum	*Penicillium echinulatum*	*Tritirachium oryzae*
Cladosporium sphaerospermum	*Penicillium expansum*	*Ulocladium sp.*
Curvularia lunatus	*Penicillium frequentans#*	*Ulocladium atrum*
Drechslera cynodontis	*Penicillium funiculosm*	*Ulocladium chartarum*
Epicoccum purpurascens	*Penicillium glabrum#*	

* organisms with reported ability to grow in fuel

Synonyms:-

Aspergillus sejunctus = A. rubrobrunneus, Penicillium frequentans = P. glabrum, P. cylopium = P. aurantiogriseum, P. notatum = P. chrysogenum

Sources: 1, 2; 5,; 7, 11, 13, 15, 16, 19, 21, 23, 24, 30, 33, 35, 37, 40, 41, 43, 44, 49, 50, 52, 53, 54, and IMI Genetic Resource Collection Records.

Fuel testing for microbial contamination

Tests for the presence of microorganisms in fuels include filtration accompanied by microscopy and/or culture, direct culture of fuel or drained water samples and newly developed and developing immunological methods. For details of traditional techniques, reference may be made to textbooks, manuals and various published papers (e.g., 22, 26, 33, 39). In 1979, Bailey and May (3) examined the use of commercially available kits for detection of contamination in marine diesel and found that most gave results correlating well with standard plate counts. They detected <50 to 10^3 bacteria/ml fuel and 10^2 to 10^6 bacteria/ml seawater ballast, with corresponding fungal counts of 0 to <10 and 20 to 10^2. SRB were only detected in the water phase, an observation confirmed in studies in Brazil (5). Detection of SRB is an especial cause for concern because of their role in corrosion and production of the toxic gas, hydrogen sulfide. Hill and Hill (26) state that expected numbers of microorganisms per milliliter of the water bottom from slightly (highly) contaminated fuels are 10^5 (10^6-10^8) bacteria and 10^3-10^4 (10^4-10^6) yeasts/fungi, although the validity of using viable counts as an indicator of contamination by filamentous fungi in any situation is perennially contentious. It is extremely important to test the drained water sample and not merely the fuel itself, since the majority of contaminants will be present in this aqueous phase, or at the fuel/water interface. A clean fuel will contain less than 50 organisms per liter, whilst the associated water may carry >1,000 or >40,000 organisms per milliliter (25). A "rapid" detection method much used for jet fuel in Brazil is the Boron Microbe Monitor Test, in which fuel samples are incubated over an aqueous mineral salts layer with or without biocide. A positive result is one in which a change at the interface (fungal growth) is noted in the non-biocide-containing flask. This method, although simple to perform and interpret, is slow to produce results (1-2 weeks are generally required). Commercially available rapid on site kits were assessed by Stockdale and Watkinson (46) and a recently developed rapid immunofluorescence method to specifically detect *Hormoconis resinae* is described by Lopes and Gaylarde (29). This highly sensitive technique can be performed in half a day, a considerable improvement on current methodologies.

It should be noted that it is not necessary to detect viable cells to indicate a potential problem. Microbial metabolic products (e.g., acids, polysaccharides, surfactants) are also important contaminants and can be tested for by simple methods, or mere observation.

Standards - No acceptable standards have been published for microbial contaminants in fuels (26). Standards have been established using figures based on direct visual counts of fungal fragments; this an unsatisfactory and labor intensive technique. Standards based on colony forming units (especially when filamentous fungi are involved) are not acceptable because they do not correlate directly with genuine contamination and can only give estimates of the size of any problem. With the development of new rapid methods where vital activities can be measured more closely, the concept of an acceptability standard may become achievable. A number of companies use "in-house" standards for quality control of their products, but a recognized upper limit for numbers of the various classes of microorganisms does not exist. Hartman *et al.* (20) suggested a classification of risk factors for stored jet fuel into "low", "medium" and "high", based on number of moulds, number of aerobic bacteria, number of SRB, COD, pH, Eh, sulfate level and sulfide concentration. These parameters, measured in bottom and upper layers, were supplemented with chemical measurements of water separation index, water tolerance, elemental sulfur, hydrogen sulfide, copper and silver corrosion rates and total acidity to produce a Computerized Expert System for diagnosis and control of fuel storage systems. The recommendations for any necessary action were based on:-

- overall microbial activity
- overall risk of chemical deterioration
- microbial contamination in the upper level (i.e., the fuel phase)
- type of roof on the tank (fixed or floating)
- previous history
- water accumulation potential of the tank (diesel)

Once a decision to take remedial action has been made, the type of treatment must be selected. This will almost certainly involve emptying the tank and taking it out of service for a period, with concomitant economic losses.

CONTROL OF MICROBIAL CONTAMINATION

As in most cases of microbial biodeterioration of materials, the best control treatment is prevention. Cleanliness and frequent drainage of water should ensure that problems are minimal. However, these standards are difficult to maintain in practice and it is not infrequent that storage systems have to be emptied

for thorough cleaning (removal of biofilms) and biocides used. Corrosion induced in the tanks may be avoided by internal coatings, which will be effective for some years, but this is an expensive option, albeit one which is increasingly employed. Cathodic protection (impressed current or the use of sacrificial anodes) is also used to combat corrosion, sometimes in addition to resistant coatings. Although expensive, this may be less costly than the government-imposed fines which are levied in the USA against companies polluting the environment with leaked fuels. Such provisions, however, do not reduce the other consequences of contamination. Without adequate drainage, microbial slimes and sludges are likely to form in fuel systems and hence recourse to biocides may be necessary.

Selection of biocides for use in fuel systems

The criteria governing the selection of an effective biocide formulation can be summarized as follows:

- no adverse effects on fuel specifications (ref 35 lists the parameters which should be unaffected)
- no adverse effects on engines, pumps etc.
- efficacy against a broad spectrum of microorganisms
- ability to penetrate microbial slime
- chemical and physical compatibility with the fuel and other additives (e.g., corrosion inhibitors)
- suitable partition coefficient (water-oil)
- safety and ease of use and storage
- biodegradability
- cost-effectiveness.

In aircraft fuel systems, routine maintenance, rigorous monitoring and treatment with allowed biocides when necessary (EGME [in military aircraft], organoborinane, or isothiazolone [which is allowed by some manufacturers]) keep risks of serious microbial growth to a minimum. Other fuels are generally not so well protected, but a wider variety of biocides is available. These include formulations intended to partition into the fuel phase and other, water-soluble, compounds. It is generally recommended that the biocide dissolve in the oil and then partition into the aqueous phase in sufficient quantity to protect the whole system and not merely the storage tank, although for treatment of tanks only, water-bottom biocides may be used and this will be a cheaper option, since the volume to be treated is smaller. Some of the available biocides are shown in Table 4. The required properties are frequently obtained by biocide formulation, rather than being inherent to the chemical compound itself.

Table 4 - Examples of oil- and water-soluble biocides.

Oil soluble	Water soluble
Isothiazolone formulations	Morpholines
Organoborinanes	Oxazolidines
Pyridinethione	Halides
Hexahydrotriazines	Aldehydes
Imidazolcarbamate	Phenolics

Shennan (38) lists 4 categories of biocide product for use in fuel/water systems:-

- oil-dispersible products partitioning completely into the water phase, for treatment of water bottoms
- oil-soluble products which partition into the aqueous phase for use in clean fuels where little water is expected
- oil-soluble blends, separating into 2 components, one active in the water and the other in the oil, for protecting the interface and the water bottom
- water-soluble products for addition to tank bottoms.

Bento and Gaylarde (5) tested 4 biocides for their activity in oil/water systems against bacteria and fungi isolated from diesel fuels. An isothiazolone mixture and a quaternary ammonium compound were the most effective, glutaraldehyde and a formaldehyde-releasing agent being active only at relatively high concentrations, if at all. The most resistant organisms were bacteria of the genus *Bacillus*, the most sensitive isolate of which was inhibited, but not killed within 24h, by 50ppm isothiazolone (the highest concentration tested). Guiamet and Gaylarde (17) demonstrated that the same isothiazolone mixture was active against biofilms of *H. resinae* at 50ppm, but was much less effective when SRB were also present on the metal surface. The need to consider biofilm organisms is highlighted by the example quoted in Hill and Hill (26), in which seizure of all pumps and injectors on a small cargo ship was treated by physical cleaning, but no biocide addition. Hence some microorganisms remained attached to the walls of the system, with the result that the same problem recurred 2 months later when the vessel was at sea!

Costs of biocide treatment

These have been calculated at around US$3 per ton of fuel for decontamination and US$0.4 - 0.8 for

continuous preventative treatment (26). An unknown factor is the potential for resistance development of the contaminating microorganisms, which will necessitate the use of more than one biocide type. Although there are no reports in the literature of such developed resistance, it has been noted in fuel systems (Hettige, personal communication) and this highlights the need for routine microbiological monitoring. This may increase the overall costs, but reduces the risk of expensive, ineffective, treatment.

Environmental considerations

Biocides, by their nature, are toxic and may present a hazard to the fuel handlers, to personnel working in areas where fuel volatilization may occur and to organisms in the environment receiving wastes from the system. Thus their use should be restricted and their disposal monitored. If sufficient dilution is not achieved on disposal, then a chemical inactivator may have to be used. Although excessive use of biocides should be avoided, their utilization should be recommended to reduce the possibility of fuel leakage to the environment through microbially corroded pipes and tanks and to try to eliminate the need for disposal of large volumes of contaminated, unusable products.

CASE HISTORY

Many of the points discussed above are illustrated by the following example.

In 1995, UFRGS undertook an investigation on behalf of a company manufacturing engine parts. Increased wear and breakages were occurring in vehicles of bus companies in the south, south-east and central-west of Brazil, where a new type of additive had recently been introduced into the diesel. The companies were visited and details of routine maintenance procedures and perceived problems noted. Samples of fuel were collected from representative parts of the system for microbiological analysis.

Most companies did not have well defined routines for maintenance of their diesel storage systems. In some cases, tanks were drained to remove water every 15 days, in others the foreman seemed unaware of the necessity to drain tanks. A typical biomass was found in some storage tanks, accompanied by an aqueous phase of low pH (3-5). Poorly maintained storage systems were characterized by frequent need to change filters, both in the storage system and in the vehicles, and reduced engine efficiency. Increased replacement of fuel nozzles was also noted, a problem linked by other workers to microbial contamination (19).

The most significant organisms isolated were *H. resinae* and SRB, isolated from storage tanks, filters and tanks containing filtered fuel. No SRB were found in the vehicle tanks or injectors, where no water-phase was present, but bacteria of the genus *Bacillus* were frequently encountered at all the points sampled.

It was not possible to correlate the level of microbial contamination with premature failure of engine parts, but there was a relationship between the latter and the use of the new additive. However, the additive was later shown not to promote microbial growth in laboratory studies (6).

An important fact indicated by many of the companies was the decrease in vehicle maintenance problems following the replacement of underground storage tanks by aerial systems. One firm in Rio Grande do Sul was loosing considerable quantities of fuel through highly corroded in-ground storage tanks (and hence causing unknown pollution of the surrounding area). The tanks were replaced by an aboveground system with storage tanks inclined at 30° to the horizontal to facilitate water drainage and removal, resulting in a better-maintained system and fewer problems. Currently, biocides are not used by these companies, but the Brazilian petroleum company, Petrobrás, has carried out tests on lorries and diesel storage tanks to show that considerable reductions in microbiological problems can be achieved by the use of isothiazolones (37). A program of education for garage foremen has been undertaken by the Microbiological Resources Center (MIRCEN) in Porto Alegre, in an attempt to reduce both economic losses to the firms and environmental pollution caused by hydrocarbon seepage. The First Seminar on Storage of Diesel Oil took place in December, 1997, and others are planned for the future. Without doubt, the dissemination of information is the most important activity for the avoidance of the economic and environmental problems associated with diesel oil storage.

RESUMO

Contaminação microbiana de combustíveis hidrocarbonados e o seu controle

O problema microbiano maior na indústria de refino de petróleo é a contaminação de produtos armazenados, que pode levar à perda da qualidade, à formação de borra

e à deterioração de tubulações e tanques de estocagem, na refinaria e no usuário. São abordadas, neste artigo, três classes de combustível, gasolina, querosene de aviação e óleo diesel, correspondente à ordem crescente de peso no fracionamento de petróleo. O óleo diesel apresenta os problemas microbiológicos mais sérios. São relatados os diversos microrganismos isolados de sistemas de combustíveis hidrocarbonados. São apresentadas as condições necessárias para crescimento microbiano e os métodos utilizados para o monitoramento e controle desse crescimento. Os efeitos de diversos aditivos, inclusive biocidas, são discutidos

Palavras chave: biocidas; biofilmes; combustíveis hidrocarbonados; corrosão microbiana; tanques de estocagem

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PROPERTIES OF THE *BACILLUS CEREUS* STRAIN USED IN PROBIOTIC CenBiot

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SHORT COMMUNICATION

ABSTRACT

Bacillus cereus CenBiot fulfilled the requirements to be used as probiotic. The spores showed D_{80} of 14 hs, inhibited *Escherichia coli* and *Yersinia pseudotuberculosis* after 24 hs in associative culture, were innocuous for suckling and adult mice and were not inhibited by antibiotics at low concentrations.

Key words: probiotic, *Bacillus cereus*

Probiotics are "viable mono or mixed cultures of microorganisms which, applied to animal or man, beneficially affect the host by improving the properties of the indigenous flora" (10). They must promote growth, improve feed conversion and inhibit enteropathogens, without causing any undesirable effect. In addition, they must survive the stress produced during manufacturing, storage and administration at farm conditions.

The search for new probiotic strains has increased in recent years due to the necessity to find economic and effective substitutes for antibiotics used as feed additives. Several strains of *Lactobacillus*, *Pediococcus*, *Bacteroides*, *Bifidobacterium*, *Bacillus*, *Streptococcus* and *Escherichia coli*, alone or consortiated, have been used as probiotics (8), giving controversial results. Among the various species of probiotics, those belonging to the genus *Bacillus* have the advantage that, due to their capacity to sporulate, they survive at ambient temperatures as well as during desiccation by methods that involve moderate heating, such as spray dryers, avoiding the use of lyophilization or other expensive technologies (10). This property also makes possible the administration of spores mixed with powdered vehicles instead of gels or liquids used with non sporulated bacteria. Although several strains of different species of *Bacillus* are being used for this purpose, information concerning their properties was seldom reported.

Probiotic CenBiot, prepared with a strain of *Bacillus cereus* at Centro de Biotecnologia of Universidade Federal de Pelotas, Rio Grande do Sul, Brazil, was tested in commercial farms showing beneficial effects in the control of diarrhoea and feed conversion in pigs (21). The effects of some factors that could affect the survival of *Bacillus cereus* in the intestinal tract and during manufacturing, such as interaction with enteropathogens, resistance to heat and to variation of pH, are reported in this work.

The strain was classified as *Bacillus cereus* due to its morphology, Gram staining, capacity to form

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thin wall spores in aerobic conditions, growth in glucose broth under anaerobic conditions, production of lecithinase and acetyl-methyl-carbinol and absence of urease (20). To obtain spore suspensions, cultures were heated at 80°C for 15 minutes, centrifuged, and after three successive washings with ultrapure water, resuspended in ultrapure sterile water pH 7.2.

To test the effect of heat, aliquots of 300µl of spore suspensions were heated in a water bath at 50°C for 4 days, 80°C for 60 min, 85°C for 60 min and 90°C for 40 min. Samples were collected every 24 hours, 10 min, 10 min and 2 min, respectively, and immediately chilled in iced water. Decimal dilutions in saline were plated on sheep blood agar, incubated at 37°C for 24 hours and the Colony Forming Units (CFU) counted. D values were calculated following Frank and Campbell (7). At 50°C the counts increased 3.7 times after 4 days, probably due to heat activation. D values were 14.28 hours at 80°C, 10.7 min at 85°C and 1 min at 90°C. Results are the means of at least three experiments.

Trying to foresee how the pH of the gastrointestinal tract could affect viability, spore suspensions were adjusted to pH 1.0 with HCl and to pH 7.6 with NaOH and held at 37°C for 24 hours. Another suspension was incubated at pH 1.0 for four hours and then adjusted to pH 7.6 and incubated for the following 20 hours. Samples taken at 0, 2, 4, 6 and 24 hours, were processed as above to count CFU. The concentrations of viable spores after 24 hours remained almost unchanged in the suspensions at pH 7.6, but they decreased to 35 % of the initial value in those held at pH 1.0 for 4 hours and at pH 7.6 for another 20, and to 11 % in those at pH 1.0 (Fig. 1). Results are the means of at least three experiments. This decrease in viability may explain the necessity of supplying *Bacillus* based probiotics in high concentrations and on a daily basis (6). It has been shown that results obtained *in vitro* highly correlate with those obtained *in vivo*, thus allowing the use of the formers in the selection of probiotic candidates (10).

Eight-day-old mice were inoculated intragastrically with 1 x 10^9 spores and observed for 14 days. Adult mice were fed for 30 days with granulated mice feed containing Probiotic CenBiot at a concentration of 1 x 10^9 spores per kg. Undesirable reactions were not produced in suckling or in adult mice during this experiment, neither in 248 piglets in the suckling phase and 199 in the nursery phase used in field tests (21). Even considering that many strains

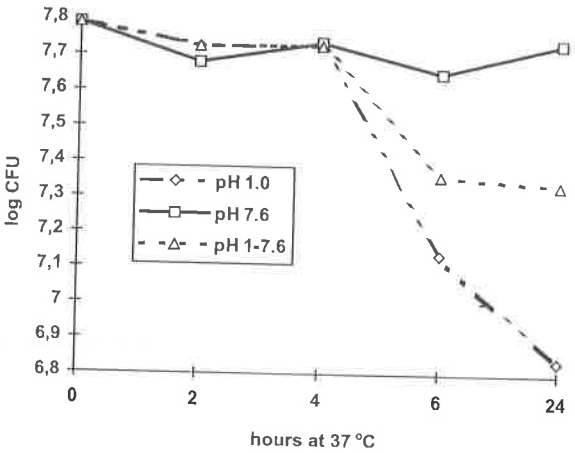

Figure 1 - Effect of pH on the viability of *B. cereus* CenBiot spores incubated at 37°C

of different species of *Bacillus* produce toxins harmful to man and animals, some are innocuous (5) or even beneficial (18), as was demonstrated for *B. cereus* CenBiot in the experiments here reported.

Yersinia pseudotuberculosis O III recovered from water buffaloes with diarrhoea, *Escherichia coli* K88ac recovered from piglets with diarrhoea, and *E. coli* O147 (O147:K89, 88ac:H19) from the Statens Serum Institut, Copenhagen, Denmark, were grown in BHI agar overnight and suspended in saline to A_{450} = 0.6. 500µl of each strain was inoculated into two 50 ml Erlenmeyer flasks containing 10 ml of BHI; to one flask 500µl of a suspension of *B. cereus* CenBiot with A_{450} = 0.6 was added. The flasks were incubated in a G24 Environmental Incubator Shaker (NBS) at 37°C and 250 rpm for 24 hours. Then, the cultures were decimally diluted in saline, plated on MacConkey agar and incubated at 37°C for 24 hours, when the CFU/ml were determined. The counts of *E. coli* K88ab and of *Y. pseudotuberculosis* dropped to 33.3 % and that of *E. coli* O147 to 30 % after growth in association with *B. cereus* CenBiot, when compared with the strains grown alone (Fig. 2). These results could be related to those of field experiments in which *Escherichia coli* K88ac was not recovered from pigs fed Probiotic CenBiot, while it was isolated from 20 % of diarrhoeic faeces collected from control animals (21). Apella *et al.* (1) used a similar procedure to show the inhibitory effect of two species of *Lactobacilli* on *Shigella sonnei*, and Hillman and Fox (11) also used associative cultures of *Lactobacillus* and enterotoxigenic *E. coli* to screen probiotic strains.

Plasmids were not obtained from suspensions of

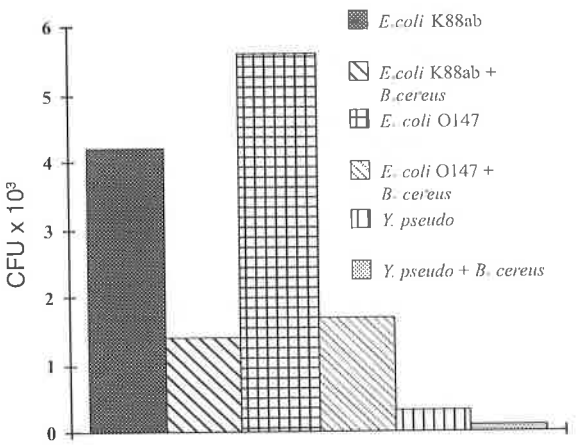

Figure 2 - Effect of *B. cereus* CenBiot on the growth of two *E. coli* strains and *Y. pseudotuberculosis*

B. cereus CenBiot by the extraction methods of Kado and Liu (3) and Birnboim and Doly (9). Plasmids encode several products related to the beneficial effects attributable to probiotics, such as the bacteriocins of *Lactobacillus lactis* (16) and lactococcal proteinases (4), suggesting that the probiotic effect showed by *B. cereus* CenBiot (21) is produced by other mechanisms not involving bacteriocins.

The effect that antibiotics commonly used in Brazil as food additives, or for therapeutic purposes, could produce on *B. cereus* CenBiot was also studied in this work. Sensitivity to Cloramphenicol (10, 40, 160 and 320μg / ml), Tetracycline (10, 40, 160 and 320μg / ml) and Kanamycin (1.25; 2.5; 5 and 10μg / ml) was tested by the minimal inhibitory concentrations method, and for Streptomycin (10μg), Erythromycin (15μg), Gentamycin (10μg), Cloramphenicol (30 μg) and Tetracycline (30μg) by the disk method (2). The strain was inhibited by all the antibiotics tested by the disk method, but not by Cloramphenicol, Tetracycline and Kanamycin at a concentration of 10μg / ml of medium. Several authors claim the utility of using probiotics in association with antibiotics, aiming to optimise the effects of both (12). Synergism between these additives, however, is not always produced. Tortuero *et al.* (19) reported that *Bacillus cereus* fed in association with Virginiamicin showed beneficial effects in piglets, while Roth and Kirchgessner (14) found that Olaquindox did not enhance the probiotic effect of *Streptococcus faecium*.

Spores were not affected by the temperatures used in feed processing. Their viability at 80°C was 94.6 % after 10 minutes and 85.7 % after one hour, showing that the spores may be desiccated in spray dryers and pelletized (10) without appreciable loss of viability. Russel (15) showed that D values increased 10 fold every 10°C fall in temperature; therefore, it may be expected that at temperatures at which the probiotic is stored, the loss in viability shall be inexpressive (17), as it was confirmed by our observations after several months of storage (data not shown). Heat activation was detected after heating at 50°C during 96 hours. However, 80°C seems to be the highest temperature to which the spores may be heated without substantial loss of viability, as can be deduced from the D_{85} of 10.7 min and D_{90} of 1 min. *B. cereus* CenBiot seems to be more resistant to heat than *Bacillus* C.I.P. 5832 used successfully as probiotic (13).

The results reported show that *B. cereus* CenBiot fulfils the requirements to be a suitable candidate for probiotic elaboration (10).

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RESUMO

Propriedades da cepa de *Bacillus cereus* utilizada no probiótico CenBiot

Bacillus cereus CenBiot possui as características necessárias para ser utilizada como probiótico. Os esporos apresentaram D_{80} de 14 hs, inibiram *Escherichia coli* e *Yersinia pseudotuberculosis* após cultivadas associativamente por 24 hs, foram inócuos para camundongos lactentes e adultos e não foram inibidos por antibióticos a baixas concentrações.

Palavras-chave: probiótico, *Bacillus cereus*.

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BOVINE S PROTEIN (VITRONECTIN) INCREASES PHAGOCYTOSIS OF *STREPTOCOCCUS DYSGALACTIAE*

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ABSTRACT

The effects of bovine S protein (vitronectin) on phagocytosis of *Streptococcus dysgalactiae* strains isolated from cattle with mastitis were investigated. Phagocytized streptococci were determined by a fluorometric microassay using glass adherent polymorphonuclear neutrophils (PMN). Preincubation of *S. dysgalactiae* with bovine S protein significantly increased their phagocytosis by PMN. Bovine S protein had no effect on phagocytic killing of non-S protein binding *S. pyogenes* cultures. Enzymatic digestion of the bovine S protein binding sites on *S. dysgalactiae* with pronase resulted in a significative reduction of the effects of S protein on phagocytosis. It could thus be concluded that in addition to its role as a promoter of cellular adhesion and complement inhibitor, bovine S protein may also influence the phagocytosis of *S. dysgalactiae* during inflammatory processes.

Key words: *Streptococcus dysgalactiae*, bovine S protein, phagocytosis, bovine mastitis

INTRODUCTION

Streptococcus dysgalactiae, *S. agalactiae* and *S. uberis* are three streptococcal species frequently reported to cause bovine mastitis (9). Although the causative organisms of bovine mastitis have been identified, the exact mechanisms of the initiation and development of infection have not yet been fully elucidated.

Selective adherence of bacteria to bovine epithelium could be a prerequisite for the organism to be a successful parasite in the mammary gland (19). Also, binding of host plasma proteins to gram-positive cocci might play an important role in pathogenicity (20,15,18). The binding of bovine complement S protein (vitronectin) to *Streptococcus dysgalactiae* isolates from cattle with mastitis and its role in adherence to bovine epithelial cells were observed (4). Vitronectin is a multifunctional protein that has important role in complement-dependent cell lysis (7), in the coagulation system (12), and in cellular adhesion (2). The bovine S protein exists in plasma with molecular weight of 76,000 and 65,000 (4) and its biological properties were indicated by the ability to spread cultured endothelial cells, as described for human S protein (11). The purpose of this study was to determine the effects of bovine S protein binding by *S. dysgalactiae* on its phagocytosis by bovine polymorphonuclear neutrophils (PMN).

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MATERIALS AND METHODS

Streptococci. Four clinical isolates of *S. dysgalactiae* recovered from cattle with mastitis were used. In addition, two cultures of *S. pyogenes* (lacking the capacity to bind bovine S protein) served as controls. Each strain was inoculated in 1-litre Erlenmeyer flasks containing 100 ml of Todd-Hewitt broth (GIBCO/BRL, GmbH, Eggstein, Federal Republic of Germany). After incubation on a rotary shaker for 18 h at 37°C and 60 rpm, the bacteria were harvested by centrifugation for 20 min at 15,000 xg.

For proteolytic treatments, two *S. dysgalactiae* strains (S.dys 8 and S.dys 12) with the highest S protein binding activities were used. One ml samples of suspensions containing 10^8 streptococcal cells/ml were incubated with increasing (2,5 to 250µg) concentrations of pronase (E. Merck AG, Darmstadt, Germany) as described (3).

Purification of bovine S protein. S protein was purified from bovine plasma as previously described (4). The purity of the protein was tested by sodium dodecyl sulfate polyacrylamide gel electrophoresis (8) and western blot analysis (16) with anti-bovine S protein antibodies. Antibodies against bovine S protein were raised in rabbits.

Radioiodination and binding assays. S protein was radiolabeled with ^{125}I (Carrier Free, Amersham Buchler, Braunschweig) using the chloramine-T method (6). The specific activity of each of the 3 preparations was approximately 1.4 mci/mg of protein. Radiolabelled S protein was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (8). The binding assays were performed as previously described (4), using 2 x 10^8 streptococci and 10 mg of the respective ^{125}I-labelled protein in 0.15 M phosphate-buffered saline solution (PBSS, pH 7.5) containing 0.05% Tween 20 (PBSS-Tween). After 1 h at 25°C, the streptococci were centrifuged for 3 min at 10,000 xg and washed with ice-cold PBSS-Tween. The radioactivity in the sediment was measured in a γ-spectrometer (Packard Instrument Co., Inc., Rockville, MD). The radioactivity uptake was expressed as percentage of the total activity remaining in the pellet.

Preparation of polymorphonuclear neutrophils (PMN). PMN were prepared from ethylenediaminetetraacetic acid (EDTA) treated bovine blood using ficoll-hypaque gradients (Histopaque, Sigma, Munich, Federal Republic of Germany). Remaining erythrocytes were lysed with 0.162 M ammonium chloride (pH 7.2) and the sedimented PMN washed twice in Hanks balanced salt solution (HBSS), containing 0.1% bovine serum albumin. Cell viability was higher than 95% as determined by trypan blue dye exclusion test. The PMN suspensions were adjusted to 5 x 10^6 cells/ml using a hemocytometer.

Phagocytic assays. The method of Rainard (14) for the phagocytic assays was used essentially as previously described (17). Streptococcal suspensions (10^8 streptococci/ml in HBSS) were incubated with 3 to 300 µg of bovine S protein for 30 min at 37°C. Subsequently, the streptococci were washed in HBSS, opsonized with free S protein bovine serum at a final concentration of 10% and used in phagocytic assays. Phagocytosis was observed on glass-adherent polymorphonuclear neutrophils. One hundred ml of PMN suspensions were dripped on microscopic slides and, after incubation for 1 h at 37°C under 5% CO_2, the preparations were washed with HBSS. Streptococcal suspension (100 µl) were added and after incubation for 1 h at 37°C under CO_2, the preparations were washed twice with HBSS and stained with an acridine orange (Sigma) solution (20 µg/ml in HBSS) for 1 min at room temperature. The slides were washed with HBSS, mounted with cover slips, sealed with paraffin and examined with a fluorescence microscopic. Viable streptococcal cells appeared in green and intracellular killed streptococci appeared in red (10). The phagocytized streptococci, in at least 50 randomly selected PMN, were counted. In controls either PMN or streptococci were replaced by HBSS.

RESULTS AND DISCUSSION

The purified bovine S protein migrated as a doublet, with molecular weight of 76,000 and 65,000, in the sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig.1). The purified preparation had a characteristic doublet when analysed by immunoblotting (Fig. 2), but did not cross-react with antibodies against human or bovine fibronectin (4). The functional properties of S protein were demonstrated by its ability to facilitate the spreading of cultured endothelial cells (11), to inhibit complement-dependent lysis and to neutralize the anticoagulant activity of heparin in the thrombin-antithrombin III reaction (12, 13).

The four strains of *S. dysgalactiae* bound ^{125}I-labeled bovine S protein with a mean value of 45% and those of *S. pyogenes* did not interact with this protein. Therefore *S. pyogenes* served as a negative control in the phagocytic assays.

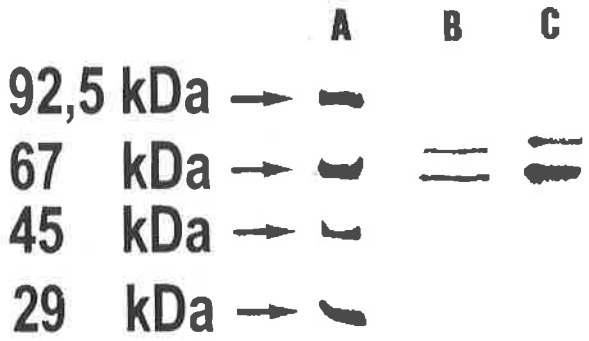

Figure 1 - SDS-Polyacrylamide gel electrophoresis of purified bovine S protein. Lanes: **A**, size markers (phosphorylase B, 92,5 kDa; bovine serum albumin, 67 kDa; ovalbumin, 45 kDa; carbonyc anhydrase, 29 kDa); **B**, human S protein; **C**, purified bovine S protein.

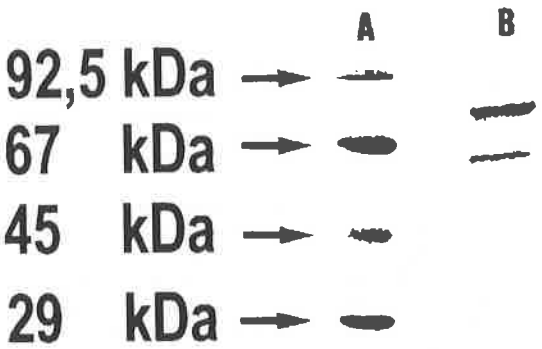

Figure 2 - Western blot of purified bovine S protein using anti-bovine S protein antibodies. Lanes: **A**, size markers (phosphorylase B, 92,5 kDa; bovine serum albumin, 67 kDa; ovalbumin, 45 kDa; carbonyc anhydrase, 29 kDa); **B**, purified bovine S protein.

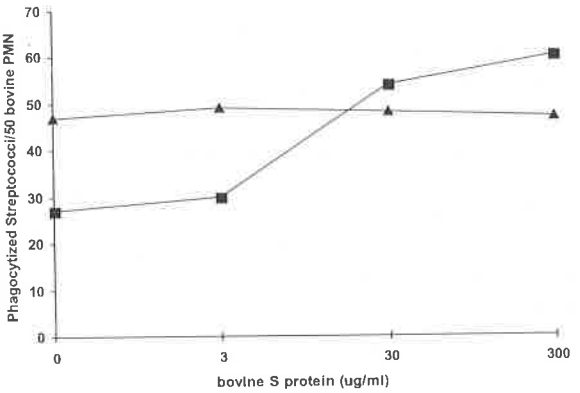

Figure 3 - Phagocytosis of *Streptococcus dysgalactiae* (■) and *Streptococcus pyogenes* (▲) by bovine polymorphonuclear neutrofils after preincubation of streptococci with increasing concentrations (3-300 µg/ml) of bovine S protein. Results represent means obtained with four cultures of *S. dysgalactiae* and two cultures of *S. pyogenes*.

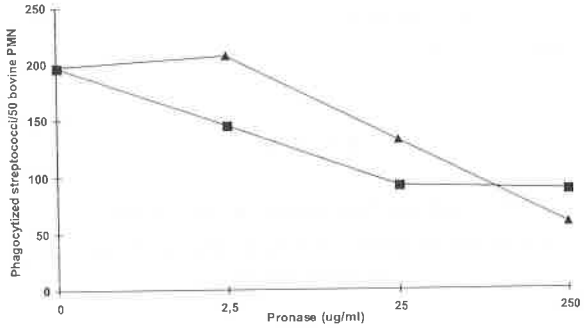

Figure 4 - Phagocytosis of *Streptococcus dysgalactiae*, strains S.dys 8 (■) and S.dys 12 (▲), by bovine polymorphonuclear neutrofils after treatment with increasing concentrations (2.5-250µg/ml) of pronase, in the presence of bovine S protein (100µg/ml). Each point represents mean of duplicates.

In the phagocytic assays, prior incubation of *S. dysgalactiae* strains with bovine S protein significantly increased their phagocytosis by bovine polymorphonuclear neutrophils. A phagocytic increase of almost 120% was observed when 300 µg/ 10^8 streptococcal cells were applied (Fig. 3). Preincubation of *S. pyogenes* strains with the same concentration of bovine S protein did not affect their phagocytosis. To further elucidate the involvement of bovine S protein binding sites in the phagocytic killing of *S. dysgalactiae*, streptococci were treated with proteolytic enzymes prior to phagocytosis assays. After enzymatic digestion of the streptococcal binding sites for S protein with pronase, the effects of bovine S protein on *S. dysgalactiae* phagocytosis were reduced (Fig. 4).

Intramammary infections of dairy cows caused by *S. dysgalactiae* have been characterized by persistence of the infection and by a poor response to conventional control measures. Significant differences in intramammary infectivity and binding capacities among certain strains of *S. dysgalactiae* were reported (5, 4). Previous reports (4, 1) demonstrated that *S. dysgalactiae* strains could adhere and invade bovine epithelial cells, induced cellular damage, and were capable of persisting inside bovine mammary epithelial cells.

Phagocytosis by polymorphonuclear neutrofils is one of the first defense lines against invading streptococci and the specific interaction of streptococci with fibrinogen plays an important role as an antiphagocytic factor (20, 17). In adherence

experiments, bovine S protein enhanced streptococcal adherence to bovine epithelial cells (4). However, no studies about the influence of S protein in the phagocytosis of *S. dysgalactiae* were described. This study presents evidence that binding of bovine S protein by *S. dysgalactiae* increased phagocytosis by PMN. The involvement of the specific S protein binding structures of S. *dysgalactiae* in the phagocytosis could be confirmed by assays using proteolyzed streptococci, a procedure known to destroy S protein binding activities of *S. dysgalactiae* (4). The results of the present study indicate that in addition to its role as a promoter of cellular adhesion and complement inhibitor (7, 2), S protein may also influence the phagocytosis during inflammatory processes. These data will be helpful in the elucidation and understanding of the pathogenesis of *S. dysgalactiae* bovine mastitis and the exact relationship between bovine S protein binding, adherence and infectivity .

RESUMO

Aumento na fagocitose de *Streptococcus dysgalactiae* pela ação da proteína S bovina (vitronectina)

Foram investigados os efeitos da proteína S bovina (vitronectina) na fagocitose de amostras de *Streptococcus dysgalactiae* isoladas de bovinos com mastite. A determinação do número de estreptococos fagocitados foi realizada pelo método fluorométrico utilizando neutrófilos polimorfonucleares (NPM) aderidos em lâminas de vidro. A pré-incubação do *S. dysgalactiae* com a proteína S bovina aumentou significativamente a sua fagocitose por NPM. A proteína S bovina não causou efeito na fagocitose de culturas de *S. pyogenes*, já que não apresentam sítios de ligação para esta proteína. A digestão enzimática com pronase dos sítios de ligação *S. dysgalactiae* para a proteína S bovina resultou numa significativa redução do efeito da proteína S na fagocitose. Pode-se concluir que além do papel como promotor da adesão celular e inibidor do complemento, a proteína S bovina pode também influir na fagocitose do *S. dysgalactiae* durante os processos inflamatórios.

Palavras-chave: *Streptococcus dysgalactiae*, proteína S bovina, fagocitose, mastite bovina.

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INCIDENCE OF *CAMPYLOBACTER* IN PIGS WITH AND WITHOUT DIARRHEA

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SHORT COMMUNICATION

ABSTRACT

Two hundred pigs (1- 21 weeks old), from five piggeries in São Paulo State, Brazil, were divided in two groups of 100 animals each, G1 with diarrhea and G2 without diarrhea. *Campylobacter* was recovered from 43% of G1 and 34% of G2 specimens, and was more frequently recovered from 0-4 week old piglets. *C. coli* was the most common species (44.2% in G1 and 32.4% in G2), followed by *C. jejuni/coli* (16.3% in G1 and 23.5% in G2). *Campylobacter* counts were significantly higher in G1 ($\leq 10^8$ UFC/g) than in G2 ($\leq 10^4$ UFC/g) ($p < 0.01$), which suggests that the bacterium may play a role at least in the aggravation of the diarrheic process.

Key words: *Campylobacter*, diarrhea, pigs

The primary habitat of some species of *Campylobacter* is the intestinal tract of warm-blooded animals. Colitis attributed to *C. jejuni* has been reported in men, dogs, cats, ferrets and hamsters, though colonization by the organism usually causes mild diarrhea without severe colitis (3,5,12). In pigs, the association of diarrhea with *Campylobacter* was first reported by Doyle (6,7), who reproduced the so-called "pig dysentery" in healthy animals through experimental inoculation. Dysentery was also observed when *Campylobacter coli* was inoculated in gnotobiotic piglets by oral route. Sala *et al.* (16) observed diarrhea and bacterial dissemination in the lungs, kidneys and liver of pigs after experimental inoculation with thermophilic *Campylobacter*. There are many other reports on pig campylobacteriosis, such as Sala *et al.* (17), Boosinger and Powe (4), Smith *et al.*(22) and Sticht-Groh (23).

Since raw and undercooked animal products may be contaminated with microorganisms derived from the stools, representing a potential health hazard, and there is limited information as to whether the presence of *Campylobacter* in substantial numbers in feces can be linked with diarrhea in pig, this study was undertaken to investigate the incidence and number of the microorganism in feces of pigs with and without diarrhea.

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Two hundred pigs (1-21 weeks old), from five piggeries in São Paulo State, Brazil, were divided into two groups, 100 animals each, G1 with diarrhea and G2 without diarrhea. Fecal samples from animals of G1 and G2 groups were collected in sterile flasks, shipped under refrigeration and stored at ±20°C after the initial laboratory handling. For isolation of microorganisms, samples were directly smeared onto thioglycolate agar plates with 20% defibrinated bovine blood and Butzler selective supplement (SR 85-Oxoid) (TSA). Following this procedure, 1 g of feces was homogenized in 9 ml of sterile saline (0.85 %). The homogenate was centrifuged at 2500 rpm/5 min and the supernatant was filtered through an acetate membrane (0.65 µM ∅ Millipore). Five drops of each filtrate were also streaked onto plates of TSA without Butzler selective supplement (TA). Plates of TSA and TA were incubated under microaerobic atmosphere for 72 h at 43°C and 37°C, respectively. Colonies were examined for morphology and motility using phase contrast microscopy (1000 x). The final identification was based on biochemical tests: catalase, hydrogen sulfide production with and without cysteine, oxidase, growth at 25°C, at 43°C, in 0.8% glycine and in 3.5% sodium chloride, reduction of sodium selenite, sodium hippurate hydrolysis and tolerance to 2, 3,5-triphenyl tetrazolium (TTC) (8,9,11,18,20, 21).

Enumeration of *Campylobacter* was done plating 0.1 ml of each filtrate, submitted to serial dilutions in sterile saline, ranging from 10^{-1} to 10^{-10}, onto TA plates, which were incubated under microaerobic atmosphere for 72 h at 43°C. After incubation, plates showing 30-300 colony-forming units (CFU) were selected for counting.

The X^2 test was used to compare G1 and G2 in relation to the incidence of *Campylobacter* according to age of the animals. Student's t test was used to evaluate the significance of the differences in the enumeration of *Campylobacter* in groups G1 and G2 (15).

The incidence and number of *Campylobacter* in fecal specimens of pigs with and without diarrhea are presented in Table 1. *Campylobacter* was recovered from 43% of G1 and from 34% of G2 specimens. These data are similar to those of Piazza and Lasta (14) and Smith *et al.* (22), but not to those of Kashiwazaki *et al.* (10). We found no statistically significant difference between the incidence of *Campylobacter* in G1 and G2, although the microorganism was more frequently recovered from the 0-4 week old piglets. Despite the presence of *Campylobacter* in all age groups, it is difficult to consider that this microorganisms is the causative agent of diarrhea in G1, in view the broad spectrum of biological factors which affect the diarrheic process, including the association of various enteropathogenic agents. There was a significant difference ($p<0.01$) between the density of *Campylobacter* in fecal specimens of G1 ($\leq 10^8$ UFC/g) and G2 ($\leq 10^4$ UFC/g), which suggests that *Campylobacter* may at least play a role in the aggravation of the diarrheic process, possibly due to intestinal colonization.

Table 2 shows that there was a higher incidence of *C. coli* in G1 (44.2%) than in G2 (32.4%), though this species was the predominant in both groups. However, other thermophilic species were more frequently recovered from G2 than G1. *C. jejuni* biotype 2 showed a higher incidence than *C. jejuni* biotype 1 in both G1 and G2. With regards to *Campylobacter lari* (nalidixic acid resistant *Campylobacter*), in five out of 11 strains the phenotypic profile of sodium hippurate hydrolysis was comparable to that of *C. jejuni,* and in six the phenotype of TTC tolerance was comparable to that of *C.coli.* In this context, previous studies (13,16) suggested that, based on the biochemical profile, *C. lari* may be considered a distinct species, and that *C. lari, C. coli* and *C. jejuni* present common phenotypic characteristics.

In this study, *C. fetus* subsp *fetus* was also isolated, which may have an epidemiological significance, in view the etiological correlation with enzootic abortion in sheep, sporadic abortion in bovines, diarrhea in calves and enteritis in dogs and cats (1,2,19).

Table 1. Incidence of *Campylobacter* in fecal specimens of pigs with (G1) and without diarrhea (G2)

Age (weeks)	G1 Positive Cultures (%)	G2 Positive Cultures (%)
0 ⊢ 4	23/47 (48.9)	10/24 (41.7)
4 ⊢ 8	15/42 (35.7)	14/41 (34.2)
8 ⊢ 21	5/11 (45.5)	10/35 (28.6)
Total	43/100	34/100

0 ⊢ 4	4 ⊢ 8	8 ⊢ 21	Total:
$x^2=0.338$	$x^2=0.022$	$x^2=1.085$	$x^2=1.710$
$p>0.50$	$p>0.50$	$p>0.30$	$p>0.10$
G1 = G2	G1 = G2	G1 = G2	G1 = G2

Table 2. Species of *Campylobacter* isolated from fecal specimens of pigs with (G1) and without diarrhea (G2)

Campylobacter	G1		G2	
	N°	(%)	N°	(%)
C. coli	19	(44.2)	11	(32.4)
C.jejuni biotype 1	3	(6.9)	4	(11.8)
C.jejuni biotype 2	5	(11.6)	5	(14.7)
C.jejuni /coli	7	(16.3)	8	(23.5)
C. lari	6	(13.9)	5	(14.7)
C. fetus subsp. *fetus*	1	(2.4)	0	
Atypical strains	2	(4.7)	1	(2.9)
Total	43	(100)	34	(100)

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RESUMO

Incidência de *Campylobacter* em suínos com e sem diarréia

Um total de 200 suínos (1 - 21 semanas de idade), originários de cinco criações localizadas no Estado de São Paulo, Brasil foi dividido em dois grupos de 100 animais caracterizando-se o grupo G1 de animais com diarréia e G2, sem diarréia. *Campylobacter* foi isolado em 43% das amostras provenientes de G1 e 34% de G2. O microrganismo foi mais frequentemente encontrado em leitões na faixa de 0 a 4 semanas de idade. *Campylobacter coli* foi a espécie mais comumente observada em G1 (44,2%) e em G2 (32,4%), seguido por *Campylobacter jejuni/ coli* com 16,3% em G1 e 23,5% em G2. As contagens de *Campylobacter* foram significativamente maiores ($p < 0,01$) em G1 ($\leq 10^8$ UFC/g) do que em G2 ($\leq 10^4$ UFC/g), fato que sugere que o microrganismo pode, pelo menos, atuar no agravamento do processo diarréico.

Palavras-chaves: *Campylobacter*, diarréia, suínos.

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PATHOGENESIS OF MENINGOENCEPHALITIS IN RABBITS BY BOVINE HERPESVIRUS TYPE-5 (BHV-5)

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ABSTRACT

This article describes the main aspects of bovine herpesvirus type-5 (BHV-5) neurologic infection and disease in rabbits, a candidate animal model for studying BHV-5 neuropathogenesis. Intranasal inoculation of weanling rabbits with a Brazilian BHV-5 isolate produced neurological disease and death in 78.8% (26/33) of the animals. Neurological signs started as early as 5 days post-inoculation and lasted from 10-12 hours up to several days. Most animals evolved to a moribund state or death within 24 (69.2%) to 48 hours (88.5%). Neurological disease was characterized by excitability or depression, tremors, bruxism, walking or running in circles, backward arching of the head and body, incoordination, backward and sideways falling, paddling, profound depression and death. Moderate levels of infectivity were detected in several areas of the brain, most consistently in the ventro-lateral hemisphere (in 16 out of 20 animals), anterior cerebrum (15/20), midbrain (11/20), dorso-lateral hemisphere (10/20) and pons (12/26). Infectious virus was also recovered from the olfactory bulb (9/20), medulla oblongata (10/26), cerebellum (7/20), posterior cerebrum (5/20) and trigeminal ganglia (4/20). No gross lesions were observed. Microscopic lesions were mild and consisted of non-suppurative meningitis, mononuclear perivascular cuffing and focal gliosis. These changes were observed most consistently in the ventro-lateral hemisphere and anterior cerebrum. Passive immunity partially protected rabbits from BHV-5-induced encephalitis. Rabbits born to immunized dams showed a significative delay in the onset of clinical disease and reduced morbidity and mortality rates compared to rabbits born to unvaccinated dams. These results demonstrate that BHV-5-induced neurological disease can consistently be reproduced in rabbits and point towards the use of this species as an animal model to study BHV-5 neuropathogenesis.

Key words: Bovine herpesvirus type-5, BHV-5, meningoencephalitis, rabbits, animal model

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INTRODUCTION

Previously classified as bovine herpesvirus type-1.3 (BHV-1.3), the alphaherpesviruses causing meningoencephalitis in cattle have been recently reclassified as bovine herpesvirus type-5 (BHV-5) (24) or bovine encephalitis herpesvirus (BEHV)(27). These neuropathogenic viruses are closely related to the respiratory and genital BHV-1 strains (BHV-1.1 and BHV-1.2, respectively) in many molecular and biological aspects (4, 6, 11, 18, 29). However, they can be differentiated from the classical BHV-1 strains by DNA restriction endonuclease analysis (6,18), reactivity with monoclonal antibodies (11, 18, 23), and differential expression of virus-encoded polypeptides (11,18). The major difference between BHV-5 and BHV-1, however, relates to their distinct ability to invade the central nervous system (CNS) and cause neurological disease (2, 3). Whereas BHV-5 has commonly been associated with meningoencephalitis in cattle, isolation of BHV-1 has been largely restricted to respiratory and genital disease (2, 12, 13, 25, 27).

The biological and molecular basis for the neuropathogenic phenotype of BHV-5 are poorly understood. This is due, in part, to the lack of a suitable animal model to study the neuropathogenesis of BHV-5 infections. The pathogenesis of neurological infections by two other neurotropic alphaherpesviruses, herpes simplex virus (HSV) and pseudorabies virus (PRV), has been largely studied in mice (1, 17, 26, 28) and rats (7, 8, 9). In contrast, studies on the pathogenesis of BHV-5 have been restricted to cattle (2, 3, 13). Fruitless attempts to reproduce the BHV-5-induced neurological disease in rabbits have been reported since the initial identification of BHV-5 as a causative agent of bovine meningoencephalitis (13). In contrast, rabbits have been used to study selected aspects of acute and latent infections by BHV-1 (21, 22). Only after the description of a special technique for intranasal inoculation (5), BHV-5 infection and disease have been reproduced in rabbits (10, 19).

The main purpose of this study was to investigate the susceptibility of rabbits to infection and disease with a Brazilian BHV-5 isolate. Intranasal inoculation of weanling rabbits with BHV-5 EVI-88 strain consistently resulted in viral invasion of the CNS and neurological disease. Moreover, the rabbit infection and disease resembled in many aspects the experimental and naturally occurring disease in cattle. Further studies are underway to better characterize the rabbits infection and disease by BHV-5 and to evaluate the suitability of rabbits as an animal model to study selected aspects of BHV-5 neuropathogenesis.

MATERIALS AND METHODS

Experimental Design. Three animal inoculations were performed to evaluate the susceptibility of rabbits to BHV-5 and to initially characterize the neurological disease associated with BHV-5 infection (Table 1). Weanling rabbits were inoculated intranasally with a Brazilian BHV-5 isolate and submitted to virological and clinical monitoring. Necropsies were performed in animals recently dead or euthanized in extremis. Tissue samples collected at necropsy were submitted to virus isolation and histological examination. A fourth experiment was conducted to evaluate the ability of passive immunity in preventing BHV-5-induced neurological disease. Two adult female rabbits were immunized twice with the homologous BHV-5 (days 0 and 15) and mated 15 days later. Rabbits born to immunized and non-immunized dams were inoculated at weaning and the course of clinical disease and morbidity/mortality rates were compared (Table 4).

Cell culture and virus. The BHV-5 isolate EVI-88 was obtained from calves with clinical meningoencephalitis in Mato Grosso do Sul state, Brazil, and partially characterized by Roehe *et al.* (23). The Argentinean isolate (A663), utilized in preliminary experiments, was provided by Dr. Laura Weber, INTA Castelar, Argentina. Madin-Darby bovine kidney cells (MDBK) were used for all procedures of virus multiplication, quantitation and isolation from tissues. Cells were routinely maintained in Eagle s minimal essential medium (MEM) containing penicillin (1.6mg/l), streptomycin (0.4mg/l), supplemented with fetal calf serum (Cultilab). The virus titers used for each animal inoculation are presented in Table 1. Adult female rabbits were immunized by two inoculations (15 days apart) with $10^{7.31}TCID_{50}$ of virus. In the experiment of passive immunity (#4), rabbits were challenged with $10^{8.22}TCID_{50}$ of virus.

Rabbits and animal inoculation. Four to five-weeks-old, recently weaned New Zealand or Chinchilla rabbits (weighing approximately 0.3 to 0.4 kg) were used throughout the experiments. Rabbits were maintained in collective cages and given food and water ad libitum. Experiment # 4 utilized three

adult female rabbits (two of which were immunized with BHV-5) and their offspring. In experiment # 1, six rabbits (three virus-inoculated and three uninfected controls) were treated with dexamethasone (Azium, Schering-Plough, 0.4mg/animal/day) during 5 days before virus inoculation. Prior to virus inoculation, rabbits were anesthetized by intramuscular administration of 0.2ml (2mg) of Zoletil (Virbac). Rabbits were inoculated into the paranasal sinuses through nephrine openings according to a protocol adapted from Brown and Field (5). Each weanling rabbit was inoculated with 0.5ml of viral suspension in each nostril. After inoculation, the heads were maintained upwards for a few seconds to allow an uniform distribution of the inoculum in the nasal cavity. The adult female rabbits were immunized by intramuscular and intranasal inoculation of virus suspension, as described above.

Monitoring and sample collection. Rabbits were monitored clinically three times a day until the onset of clinical signs. Thereafter, monitoring was performed several times a day, in an almost continuous basis. Nasal swabs for viral isolation were collected every two days in the two first experiments. Nasal swab specimens (0.2ml) were inoculated onto MDBK cells grown in 24 well plates and monitored for cytopathic effect (CPE) during 5 days. Negative samples were further inoculated onto fresh MDBK cell monolayers and monitored for additional 5 days. Blood for serology was collected from all inoculated rabbits before virus inoculation. Rabbits were tested for BHV-5 neutralizing antibodies prior to virus inoculation and females were tested at day 0, day of mating (day 30) and at the day of weaning (day 90). Serum samples were submitted to a standard microtiter virus-neutralizing (VN) assay (4), using two-fold dilutions of serum against a fixed dose of virus (100-200$TCID_{50}$/well).

Tissue samples for viral isolation and histologic examination were collected at necropsy performed in rabbits euthanized in extremis or recently dead. Different sections of CNS (Tables 3 and 4), plus lungs, liver, spleen and kidney were aseptically collected and submitted to virus isolation. Tissue samples for virus isolation were processed by preparing a 10% (wt/vol) homogenized suspension and inoculated onto MDBK cell monolayers grown in 24/well plates. Monitoring of CPE was performed as described above. Samples positive for CPE were subsequently quantitated by inoculating 10-fold dilutions onto MDBK cells cultivated in 96/well plates. Virus titers were calculated according to Reed and Muench (20) and expressed as $Log_{10}TCID_{50}$/g of tissue. Tissues for histopathologic examination were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin (HE), using routine methods.

RESULTS

Morbidity and mortality. Inoculation of weanling rabbits with the Brazilian isolate EVI-88 produced neurological disease in 25 (75.8%) and death in 26 (78.8%) of the inoculated animals (Table 1). All the animals showing neurological signs evolved to a moribund state or death. In contrast, six rabbits inoculated with an Argentinean isolate (A663) remained healthy in spite of an efficient viral replication detected in the nasal mucosa for up to 8 days (data not shown). No differences in morbidity, mortality or clinical evolution were observed between

Table 1. Morbidity and mortality in weanling rabbits inoculated intranasally with bovine herpesvirus type-5 (BHV-5) strain EVI-88

Experiment #	Virus titer ($TCID_{50}$)	Breeding	Treatment	Neurological Disease	Mortality
1	$10^{7.59}$	New Zealand	DX[a]+virus	3/3	3/3
	Controls[b]		virus	3/3	3/3
			DX+MEM	0/3	0/3
			MEM	0/3	0/3
2	$10^{8.89}$	New Zealand	virus	6/7	7/7
	$10^{8.89}$	Chinchilla	virus	6/10	6/10
	Controls		MEM	0/2	0/2
3	$10^{8.22}$	New Zealand	virus	7/10	7/10
	Controls		MEM	0/2	0/2
Total (virus inoculated)				25/33 (75.8%)	26/33 (78.8%)

[a] Animals were treated with dexamethasone (0.4mg/animal/day) during five days prior to virus inoculation.
[b] Control animals were inoculated intranasally with minimal essencial medium (MEM).

the groups treated and untreated with dexamethasone (experiment # 1). Some differences in morbidity and mortality rates were observed among the inoculations. In experiment # 2, one rabbit was found dead without previous neurological signs. None of the control MEM-inoculated rabbits showed any clinical manifestation (Table 1).

Neurological disease. The clinical features of the neurological disease induced by BHV-5 were very similar all across the inoculations, with minor differences in the onset of clinical signs and in duration of clinical disease (Table 2). Neurological signs started between days 5 (9/25 animals, 36%), day 6 (13/25 animals, 52%) and 7 pi (3/25 animals, 12%) and lasted from 10-24 h (18/25 animals, 72%), 48 h (5/25 or 19.2%) to several days (2 animals, 8%). Most rabbits (88.4%) were euthanized in extremis or found dead within the first 24 to 48h of clinical manifestations (Table 2). Clinical disease was characterized by periods of depression or excitability, crisis of tremors, bruxism, running in circles, backward arching of the head and body, flexion of the neck, incoordination, sideways and backways falling, profound depression and paddling. The evolution of clinical disease was characterized by a gradual yet progressive increase in the intensity and frequency of such crises. A few rabbits showed a prolonged clinical course, presenting mild and intermittent clinical signs. All animals showing neurological disease died or were euthanized in extremis; no recovery from disease was observed. Three animals (one in experiment # 2 and two born to vaccinated dams, experiment # 4) were found dead without previous neurological signs.

Infectivity in nasal secretions and tissues. Infectivity was detected in nasal secretions collected up to days 6 and 8 pi, in experiments # 1 and 2, respectively. After day 8, monitoring of viral shedding

Table 2. Clinical, virological and histological findings in rabbits which developed neurological disease following inoculation with bovine herpesvirus type 5 (BHV-5) strain EVI-88

Exp/ animal	Neurological disease (dpi) Onset/Death[b] euthanasia[c]		Virus in the brain[a]	Histopathology
#1.1	6	7[c]	cc, p, cb	Perivascular mononuclear cuffing (+[d])
2	6	7[c]	cc, p, cb	-
3	6	7[c]	cc, p, cb	-
4	6	7[c]	cc, p, cb	Perivascular mononuclear cuffing (+)
5	6	7[c]	cc, p, cb	-
6	6	7[c]	cc, p, cb	ne[e]
#2.1	7	12[c]	ac	-
2	6	6[b]	ac, vlh, dlh, ol	Mononuclear cell infiltrate (+) (vlh), focal gliosis (++) (mo, vlh)
3	5	6[c]	ac, vlh, p, ol	Multifocal gliosis(+)(ac)
4	6	6[c]	vlh, mb, p, ol	Multifocal hemmorrhage, congestion (+)(vlh)
5	-	13[b]	vlh, dlh, p, cb	ne
6	7	8[c]	ac, vlh, mo, mb	Multifocal gliosis(+)(vlh)
7	7	8 [b]	ac, vlh, dlh, ol, mb, p	Gliosis(+) (vlh)
21	5	16[b]	ac, vlh, dlh, cb, mo, mb	Multifocal mononuclear infiltrate(+)(dlh, vlh, mb), multifocal gliosis(+)(p)
23	5	6[c]	ac, vlh, dlh, mb, p	ne
26	6	6[c]	ac, ol, cb	ne
27	6	6[c]	ac, vlh, dlh, mb	ne
28	5	6[b]	cb	ne
29	6	6[c]	ac, vlh, cb	-
#3.1	6	8[c]	ac, vlh, dlh, mb, p, pc, tg, ol	-
3.5	5	7[c]	ac, vlh, dlh, mb, p, pc, tg, ol, cb	Focal hemmorrhage (meninge)(dlh, vlh)
3.6	5	7[b]	ac, vlh, dlh, mb, p, pc, ol	Mononuclear inflamatory infiltrate(meninge)(+)(hvl)
3.7	5	6[c]	ac, vlh, mb, p, ol	-
3.8	5	7[c]	cb, pc	Focal mononuclear inflamatory infiltrate(meninge)(+) and perivascular cuffing(vlh)
3.9	6	7[b]	ac, vlh, dlh, mb, p, tg, ol, cb	Focal mononuclear infiltrate (meninge)(+)(dlh), focal/extense infiltrate (vlh)
4.0	5	7[b]	idem 3.5	-

[a] ac, anterior cerebrum; pc, posterior cerebrum; vlh, ventro-lateral hemisphere; dlh, dorso-lateral hemisphere; mb, midbrain; cb, cerebellum; p, pons; ol, olfactorybulb; mo, medulla oblongata; tg, trigeminal ganglia; cc, cervical cord;cc, cerebral cortex. Time of the [b] Death or [c] Euthanasia; [d] mild histological change (+); moderate (++), absence of lesion (-); [e] not examined

was not conducted. Infectious virus was recovered from brains of all animals showing neurological signs (Tables 2 and 3). Infectivity was detected in several regions of the CNS, most consistently in the ventro-lateral hemisphere and anterior cerebrum (Table 3). Other areas such as dorso-lateral hemisphere, midbrain, pons, cerebellum, olfactory lobe and medulla oblongata also frequently showed infectivity upon inoculation onto cell cultures. Posterior cerebrum, cervical cord and trigeminal ganglia only occasionally were positive for virus. Low to moderate levels of virus were found all across the brain sections examined (Table 3). The highest levels of infectivity were demonstrated in the ventro-lateral hemisphere, followed by anterior cerebrum and dorso-lateral hemisphere. Infectivity was also demonstrated in the brain of one animal found dead without previous illness (experiment # 2). Attempts to isolate virus from brains of the animals found dead in experiment # 4 were not conducted. Infectious virus was rarely isolated from non-neural tissues such as lungs (1/13) and liver (2/15) and was not recovered from spleen (0/13) or kidney (0/13).

Table 3. Infectivity in the central nervous system (CNS) of rabbits showing neurological signs following inoculation with bovine herpesvirus type-5 (BHV-5) strain EVI-88.

Section	Infectivity	
	Frequency (%)	Level[a]
Anterior cerebrum	75 (15/20)	+++[b]
Posterior cerebrum	25 (5/20)	++
Ventro-lateral hemisphere	80 (16/20)	++++
Dorso-lateral hemisphere	50 (10/20)	+++
Midbrain	55 (11/20)	++
Cerebellum	35 (7/20)	++
Pons	46(12/26)	++
Olfactory bulb	45 (9/20)	++
Medulla oblongata	38 (10/26)	+
Trigeminal ganglia	20 (4/20)	+
Cervical cord	7.6 (1/13)	+

[a] Average of virus titer determined for each tissue section obtained from several animals; [b] Infectivity levels: + virus titer below 10^2 $TCID_{50}$/g of tissue; ++ between 10^2 and 10^3 $TCID_{50}$/g; +++ between 10^3 and 10^4; ++++ above 10^4 $TCID_{50}$/g.

Histological changes. Microscopic lesions were mild and characterized by rare foci of non-suppurative meningitis, occasional mononuclear cell infiltrates (perivascular cuffing), and focal gliosis. These changes were observed mostly in the ventro-lateral hemisphere and anterior cerebrum of some rabbits (Table 2).

Passive immunity and protection from neurological disease. Immunization of two adult female rabbits by two inoculations of the homologous BHV-5 induced moderate levels of virus-neutralizing (VN) antibodies (Table 4). None of the inoculated females developed respiratory or neurological signs. Most bunnies born to these dams also had VN antibodies at the time of the challenge, with titres ranging from 1:4 to 1:16 (Table 4). Rabbits born to immunized dams showed reduced morbidity (5/12 or 41.6% showed neurological signs) and mortality rates (7/12 or 58%) when compared with rabbits born to BHV-5 seronegative dams (7/10 or 70%). These rates were also lower when compared with the overall mortality observed in all four inoculations (78.8%). The onset and duration of clinical disease were also affected by passive immunity. Most animals born to immunized dams showed a significative delay in the onset of clinical disease (average 7.4 days pi) compared to animals born to non-immunized dams (5.28 dpi) ($p<0.00038$). These animals also showed an increased clinical period compared to the non-immune controls (Table 4). Death or euthanasia in these animals were concentrated mostly after days 9 and 10 (average: day 11.4), comparing with death/euthanasia in the control group (average: day 7pi) ($p<0.00066$). Additionally, two rabbits born to vaccinated dams were found dead on days 14 and 15, without previous illness.

DISCUSSION

The results presented herein demonstrate that BHV-5 neurological infection and disease can be consistently reproduced in weanling rabbits by inoculation with a Brazilian BHV-5 isolate. Intranasal inoculation of the isolate EVI-88 resulted in CNS infection and neurological signs in 75.8 % of the inoculated animals (78.8% mortality). Infectious virus was recovered from several areas of the brain of animals showing neurological signs. Mild histological changes, characterized by occasional focal meningitis, mononuclear inflammatory infiltrate (perivascular cuffing) and focal gliosis were observed in CNS sections of some animals. In addition, the BHV-5-induced neurological disease in rabbits proved to be useful for evaluating the protection conferred by passive immunity. Although several aspects of BHV-5 infection and neurological disease in rabbits remain to be elucidated, these findings point towards the use of this species as an animal model for studying

Table 4- Effects of passive immunity on neurological disease and mortality in rabbits inoculated with bovine herpesvirus type 5 (BHV-5) strain EVI-88.

Dam	VN titer	Neurological disease	Mortality	Rabbit	VN titer	Clinical Disease (dpi[a])[b]	
						Onset	Death/ euthanasia
Immunized #1	1:32	3/7 (42.8%)	4/7 (57.1%)	v11	1:8	6	11
				v12	1:8	7	10
				v13	1:8	-c	-d
				v14	<1:2	-	15
				v15	1:4	-	-
				v16	1:8	-	-
				v17	1:8	8	9
Immunized #2	1:64	2/5 (40%)	3/5 (60%)	v21	1:16	-	-
				v22	1:16	8	10
				v23	1:8	8	11
				v24	nd[e]	-	14
				v25	1:8	-	-
Non-immunized	<1:2	7/10 (70%)	7/10 (70%)	c1	<1:2	-	-
				c2	<1:2	-	-
				c3	<1:2	-	-
				c4	<1:2	6	7
				c5	<1:2	5	7
				c6	<1:2	5	7
				c7	<1:2	5	6
				c8	<1:2	5	8
				c9	<1:2	5	7
				c10	<1:2	6	7
Mock-infected/ controls			0/2	cc7	<1:2	-	-
				cc8	<1:2	-	-

[a] Day post-inoculation
[b] The means of the onset of clinical disease and death in the immunized and control groups were compared by the "F" test, at a significance of 1%.
[c] Absence of clinical signs or [d]death
[e] Not determined

selected aspects of BHV-5 neuropathogenesis.

Extensive investigations utilizing animal models have provided important insights into the pathogenesis of neurotropic alphaherpesviruses (e.g. HSV and PRV) and contributed to the overall understanding of the alphaherpesviruses neuropathogenesis (1, 7, 8, 9, 17, 26, 28). The BHV-1/BHV-5 system provides a unique opportunity to identify and to map the alphaherpesvirus gene function (s) associated with neuroinvasiveness and neurovirulence. Nevertheless, studies concerning BHV-5 neuropathogenesis have been historically hampered due to the lack of a suitable animal. Attempts to study BHV-5 neuropathogenesis in rabbits have been reported since the initial evidences of its association with meningoencephalitis in cattle (12,13). The initial experimental infections, conducted by intradermal, intravenous and subcutaneous routes in adult rabbits, failed to reproduce clinical disease (13). Almost two decades later, rabbits were shown to be suitable models and have thereafter been used to study the pathogenesis and molecular aspects of BHV-1 acute and latent infections (5,21,22). Experimental infections of rabbits with respiratory viruses have been classically conducted by inoculation into the conjunctival sac, due to the small size, particular anatomy and sensitivity of the nares and nasal cavity (21). The suitability of this route of inoculation to study neuropathogenesis is controversial, since it may not result in the same pathways of CNS invasion occurring in natural infections(19). The description of a technique for inoculation directly into the paranasal sinuses through trephine openings has allowed the reproduction of intranasal inoculation and infection by BHV-1 and BHV-5 in rabbits (5).

Using this technique, we initially evaluated the susceptibility of weanling rabbits to BHV-5 infection and disease by two isolates. In a first attempt, only

the Brazilian isolate EVI-88 produced neurological disease (100% mortality, Table 1). The Argentinean prototype strain A663 did not produce neurological signs in six inoculated rabbits (data not shown). This isolate has been shown to reproduce meningoencephalitis in calves upon experimental inoculation and recently has been used to cause neurological disease also in rabbits (4,19,25). The administration of dexamethasone prior to virus inoculation, as recommended by Chowdury *et al.* (10), had no effect in increasing morbidity or mortality rates or severity of disease with any of the viruses tested herein. The isolate EVI 88 was then used throughout the subsequent inoculations, without previous dexamethasone treatment. A total of four inoculations were performed, with the development of neurological disease in 25/33 (75.8%) and mortality in 26/33 (78.8%) of the animals. The clinical signs were typical of neurological disease and consisted of episodes of excitation/depression, tremors, bruxism, incoordination, backward arching of the head and body and inevitably death. Most animals started showing clinical signs between days 5 and 6 pi and died or were euthanized within 24 to 48 hours. The onset of clinical disease occurred earlier and the clinical course was shorter than in cattle (2, 3, 25), reflecting what usually happens in the mouse and rat models for PRV and HSV neuropathogenesis, respectively (1, 7, 8, 26, 28). The short incubation period observed in these models has been attributed, at least in part, to the the shortest path traveled by the virus to reach the CNS of these species upon inoculation into peripheric sites (19).

The neuropathogenesis of HSV and PRV has been extensively studied in animal models as well as in their natural host for PRV (1, 7, 8, 9, 14 ,15, 16, 17, 26, 28). Invasion of the CNS from peripheric sites is believed to occur primarily by transport of virus through circuits of synaptically linked neurons (1, 7, 15). After intranasal inoculation, the main pathway to the CNS appears to be the sensory neurons of the trigeminal nerve, yet the olfactory route may also be operative (14,16). This appears also to be the pathways used by BHV-1 and BHV-5 to reach the CNS of cattle (2, 3, 10). After replication in the nasal mucosa, virions invade nerve endings and are transported by retrograde axonal transport to sensory neuron cell bodies located in the trigeminal ganglia (14, 15, 16). Viral replication in ganglionic cell bodies is followed by anterograde transport of virions to second order neurons located in the brain stem (pons and medulla oblongata), from where virions are transported to third order neurons located in the thalamus and cerebellum. Replication in these sites is followed by viral spread within the cerebral hemispheres (15, 16).

In the present study, the pathway utilized by the virus to reach the CNS of rabbits could not be determined. Infectious virus was recovered from several brain sections examined in the four inoculations (Table 3). In particular, the ventro-lateral hemisphere and anterior cerebrum were most consistently infected and usually harboured the highest titers of virus. Dorso-lateral hemisphere, midbrain, pons and cerebellum were also frequently infected. Frequent infection of these areas has been also reported in natural and experimental BHV-5 infections and are believed to result from transport of virus through the trigeminal pathway (2, 3, 10). Additionally, the frequent infection of the main olfactory bulb (MOB) (45% or 9/20) and anterior cerebrum (75% or 15/20) suggests that the olfactory pathways are also utilized for BHV-5 to invade the CNS of rabbits following intranasal inoculation. The olfactory pathway is the shortest route to the CNS from the nasal cavity (1, 14) and has been shown to represent an alternative route for HSV and PRV in mice and pigs (1, 14). Likewise, natural and experimental infections of cattle have also demonstrated infections of the MOB (2, 12, 13). Recently, Meyer *et al.* (19) failed in detecting infectivity in the MOBs of rabbits inoculated with BHV-5 (strain N569) whereas Chowdury *et al.* (10) demonstrated infectivity in 8 out of 12 rabbits inoculated with a North-American BHV-5 isolate. The reasons for this discrepancy are not clear, but it may apparently result from differences in neurotropism between these viruses. The frequent MOB infection and the consistent presence of infectious virus in the anterior cerebrum observed in ours and Chowdury s studies strongly suggest that the olfactory pathway is an important route utilized by BHV-5 to reach the CNS of rabbits. following intranasal inoculation Despite of the severity of clinical disease and the widespread distribution of virus within the CNS of some animals, only mild to moderate histological changes were observed. The ventro-lateral hemisphere was the section that most consistently showed histological changes. These findings agreed with the consistent detection of infectivity in this section. Even though, these lesions were not constant in all brain sections examined and were observed only in some animals (Table 3). These findings contrast

with findings by Meyer *et al.* (19) and specially by Chowdury *et al.*(10), in which a widespread non-suppurative meningo-encephalitis characterized by perivascular cuffs, mononuclear infiltrates and neuronal degeneration was consistently observed in most animals. A possible explanation for this discrepancy may be the different time courses of the infection and disease among the experiments. In the present study, most animals (76.94%) died or were euthanized up to day 7pi, after a very fast (10 to 24h) clinical evolution. In Chowdury s study, 75% (9 out of 12) rabbits died or were euthanized between days 9 and 14 pi (average: 11.2 dpi). It is conceivable that, in most animals in the present study, a very early and massive viral invasion and replication in the CNS ensued, leading to death before a significant inflammatory response could be mounted.

A disease animal model would also be of great benefit to study the cellular and humoral mechanisms involved the immune response to alphaherpesviruses. As a preliminary study, we tested the rabbit BHV-5-induced neurological disease to measure protection conferred by maternally acquired immunity. Adult rabbits are fairly resistant to BHV-5-induced neurological disease, so the dams could be immunized by intranasal and intramuscular routes as to enhance the immune response. Likewise, the virus titers used for immunization were lower than the titers used for subsequent challenge of the bunnies. Bunnies born to these vaccinated dams showed significantly lower morbidity and mortality rates and a delay in the onset and duration of clinical disease. Although cell-mediated immunity is believed to play a major role in protection against alphaherpesviruses, serum antibodies have also been shown to confer some protection (17). In our study, the delay in the clinical disease and the reduction in morbidity and mortality rates suggest that humoral immunity may also play a role in the protective immune response against BHV-5. In any case, the BHV-5-induced neurological disease in rabbits may be useful to further approach these and other immunological questions, including vaccine development and testing.

The biological and molecular basis for the neuropathogenic phenotype of BHV-5 remains one of the most intriguing and interesting issues in animal virology. Among the questions awaiting investigation are:

1. How can two viruses (BHV-1 and BHV-5), almost identical at biological and molecular levels, be so distinct in one biological property that is fundamental for full expression of their phenotypes?

2. Are the differences in neuropathogenicity derived from differences in neuroinvasiveness or neurogrowth, or both?

3. At which level of the peripheral or central nervous system is the replication/invasion of BHV-1 restricted?

4. What are the gene functions differentially expressed in BHV-5 that enable it to invade, replicate and spread within the CNS? Is it possible to map and isolate these functions?

5. Is it possible to complement the gene functions lacking in BHV-1 with the homologous BHV-5 gene functions as to confer neuropathogenicity to the former?

As it has been demonstrated for PRV and HSV, the establishment of a suitable animal model will represent an hallmark towards the understanding of these and other issues concerning BHV-5 neuropathogenesis.

The results presented herein demonstrate that BHV-5-induced neurological disease can be consistently reproduced in rabbits upon inoculation with isolate EVI-88. These findings point out for a potential use of rabbits to study selected aspects of the pathogenesis of BHV-5-induced neurological disease. Nevertheless, a further characterization of rabbit infection and disease, particularly regarding to the pathway of CNS invasion, is required to better evaluate the suitability of this species as an animal model for studying BHV-5 neuropathogenesis.

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RESUMO

Patogenia da meningo-encefalite pelo herpesvírus bovino tipo 5 (HBV-5) em coelhos

Este artigo descreve os principais aspectos da infecção e enfermidade neurológica pelo herpesvírus bovino tipo 5 (HVB-5) em coelhos. Inoculação intranasal de coelhos recém-desmamados com uma amostra brasileira do HVB-5 produziu enfermidade neurológica e mortalidade em 78,8% (26/33) dos animais inoculados. Os sinais neurológicos iniciaram a partir do 5º dia pós-inoculação e persistiram por 10-12 horas até vários dias. A maioria dos animais evoluiu clinicamente até um estado moribundo ou morte em 24h (69,2%) a 48h (88,5%). A enfermidade neurológica caracterizou-se por crises de excitabilidade/depressão, tremores, bruxismo, andar/correr em círculos, queda para o lado e para trás, arqueamento do pescoço e corpo para trás, incoordenação, movimentos de pedalagem, depressão profunda e morte. Níveis moderados de infectividade foram detectados em várias áreas do encéfalo, principalmente no hemisfério ventro-lateral (em 16 de 20 animais), cérebro anterior (15/20), pedúnculo cerebral (11/20), hemisfério dorso-lateral (10/20) e ponte (12/26). O vírus foi também detectado no bulbo olfatório(9/20), bulbo(10/26), cerebelo (7/20), cérebro posterior (5/20) e gânglio trigêmeo (4/20). Alterações macroscópicas não foram observadas. As lesões microscópicas foram discretas e consistiram de meningite multifocal, infiltrado inflamatório mononuclear perivascular e gliose focal. Essas alterações foram observadas principalmente no córtex ventro-lateral e cérebro anterior. Imunidade passiva protegeu parcialmente os animais da enfermidade neurológica. Coelhos filhos de mães imunizadas com o HVB-5 apresentaram um retardamento no início dos sinais clínicos e taxas reduzidas de morbidade e mortalidade quando comparados com coelhos filhos de mães não imunizadas. Esses resultados demonstram que a enfermidade neurológica causada pelo HVB-5 pode ser consistentemente reproduzida em coelhos e apontam para uma possível utilização dessa espécie como modelo experimental para estudar a neuropatogênese do HVB-5.

Palavras-chave: Herpesvírus bovino tipo 5, HVB-5, meningo-encefalite, modelo experimental, coelhos

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PLASMIDS IN *MYCOPLASMA* SPECIES ISOLATED FROM GOATS AND SHEEP AND THEIR PRELIMINARY TYPING [a]

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ABSTRACT

One-hundred-five (105) clinical isolates of mycoplasma from caprine origin and one isolate from ovine were surveyed for plasmids, which were present in thirty-three (31%) of them. These mycoplasmas originated from 13 herds. Ten of them were symptomatic for mycoplasmal disease (mastitis, polyarthritis, septicemia) and three herds were asymptomatic, i.e., clinically normal. Twenty-eight isolates were *Mycoplasma mycoides* subspecies *mycoides* LC (large colony or caprine biotype), four were *Mycoplasma capricolum* subsp. *capricolum* and one was *Mycoplasma cottewii*. The isolated plasmids were linearized by *Eco*RI, *Eco*RV, *Eco*RI and *Eco*RV or *Bam*HI and *Eco*RV, and were of five sizes (1.1, 1.6, 1.7, 1.8, and 1.9 Kbp). Based on restriction enzyme digestion and size of the linearized supercoiled extrachromosomal DNA, five plasmid types were recovered (p1II, p2III, p2V, p3I, and p4IV). The small size of these DNA elements probably exclude replicative forms of DNA virus, which are equal or larger than 8.0 Kbp.

Key words: *Mycoplasma*, plasmid, goat, endonuclease, electrophoresis

INTRODUCTION

Plasmids in *Mycoplasma* species (class Mollicutes) are unusual or rare, and only a few have been documented. Most extrachromosomal DNA isolated from mollicutes have been from viruses that are specific for mycoplasma, and the majority of the extrachromosomal DNA studies among these mycoplasma have been conducted on members of the genus *Spiroplasma* because of the availability of these nucleic acid elements in these organisms (15). Since the isolation of satellite DNA from *Mycoplasma arthritidis* (12) and *M. hominis* (19) few studies relating to the isolation of plasmids from members of the genus *Mycoplasma* have been reported. Since then, plasmids have been isolated from an unspeciated mycoplasma recovered from a baboon (13), and from a caprine strain of *M. mycoides* subsp. *mycoides* (1,2,8). Thereafter, an unspeciated mycoplasma isolated from a goat was found to contain extrachromosomal DNA of probable plasmid origin (6).

Despite the presence of some reports concerning plasmid isolations from mycoplasma, the number of isolated plasmid types is small and there is little

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information on the function of these DNA elements as compared to bacteria. Additionally, information on transcriptional differences, between bacteria plasmid and the mycoplasma system (14) has incited more research in this area.

In the present study, we report on the occurrence of plasmids in 106 caprine or ovine mycoplasmal isolates (105 caprine, 1 ovine) recovered from 10 diseased and three asymptomatic herds, i. e., clinically normal goats.

MATERIALS AND METHODS

Mycoplasma strains used: One-hundred-five caprine and one ovine (isolate GM630A, Table 1) mycoplasma isolates were examined. The isolates belonged do Dr. Al J. DaMassa Collection, Department of Population Health and Reproduction, University of California, Davis, USA, and only those with retrievable informations were used. They originated from separate farms and included mycoplasma from three asymptomatic and 10 symptomatic herds showing mastitis, polyarthritis, or septicemia either singly or in combination (Table 1). *M. cottewii*, type strain VIS, (7), was obtained from G. S. Cottew, CSIRO, Division of Animal Health, Parkville, Victoria, Australia. With the exception of *M. cottewii,* and isolates from herds "C", GM261B and GM267C, recovered from the external ear canal, and GM1015A (Table 1), all other mycoplasma under study originated from caprine (nine herds) or ovine (one herd) located in the Central Valley of California, USA, which had been involved in outbreaks of mycoplasmosis as reported previously (4,5,6,9). Additionally, *M. mycoides* subsp. *mycoides* strain GM12 (4) was used as a positive control because it contains a plasmid of about 1.85 Kbp (8). Prior to identification each mycoplasma isolate was filter-cloned a minimum of two times through 300 nm filters according to a procedure described elsewhere (18). For purposes of this study, the term *M. mycoides* subsp. *mycoides* will refer only to "large colony or caprine biotypes" and not to bovine or "small colony" forms of the organism.

Mycoplasma strains and identification: Mycoplasmas were identified by a growth-inhibition procedure (3) modified by the use of agar wells rather than discs.

Growth media, culturing, and processing: All isolates were grown for 24 to 48 hours in 50 ml of modified Hayflick liquid medium "B" described elsewhere (10). The cultures were centrifuged for 15 minutes at 20,000 x G. The cells pellets were washed twice in PBS, pH 7.4, resuspended in 5 ml of PBS and stored in 1 ml aliquots at –20°C for subsequent use.

DNA extraction and digestion: The DNA was extracted from mycoplasma cells by an alkaline lysis mini-preparation procedure (17). Aliquots of the sedimented DNA from each extraction were electrophoresed in agarose gel, stained with ethidium bromide, visualized under ultraviolet light, and photographed as previously described (16). Aliquots from samples containing supercoiled extrachromosomal DNA were digested with *Bam*HI, *Eco*RI, and *Eco*RV according to a standard protocol (17) for linearization and size determination (16), for preliminary typing. Following digestion, samples from each reaction were electrophoresed, visualized, and photographed as described above.

Virus assay: The supernatant of five plasmid-positive mycoplasma strains (GM30A, GM261B, GM1013, GM1043, GM630A) were assayed for virus by a standard method (19), and also by a plaque assay method described elsewhere (11).

RESULTS

Mycoplasma strains: The mycoplasma examined in this study were identified either as *M. capricolum* subsp. *capricolum*, *M. mycoides* subsp. *mycoides* LC (large colony or caprine biotype), or as *M. cottewii.*

Data pertaining to the identity of the *Mycoplasma*, the animal species from which it was isolated, the plasmid types that were recovered, and the clinical signs caused by the mycoplasma in the herd in question are presented in Table 1. Of the 106 mycoplasma isolates examined, 31.1% (33/106) contained plasmids. The plasmid-positive isolates were recovered from 13 different herds; 12 from California, USA, and one from Australia. Of the 13 plasmid-positive herds, 10 were symptomatic for mycoplasmal disease (mastitis, polyarthritis, septicemia, singly or in combination), and 3 were asymptomatic; one of the symptomatic herd was of sheep origin (Table 1). Of the 33 plasmid-positive isolates, 85.0% (28/33) were derived from *M. mycoides* subsp. *mycoides*, 12.0% (4/33) from *M. capricolum* subsp. *capricolum*, and 3.0% (1/33) from *M. cottewii.*

Table 1. Characteristics of the mycoplasma isolates and their recovered plasmid types

Herd	Mycoplasma Species	Disease signs	Isolate-total/ herd	Plasmid size (Kbp)	Digestion assay	Plasmid types
A	Mmm	P	1	1.8	*Eco*RI	pIII
B	Mmm	M, P, S	18	1.8	*Eco*RI	pIII
C	Mmm	None[a]	1	1.8		pIII
	Mcc	None	2	1.1	*Eco*RV	*Eco*RI p2V
D	Mmm	P	1	1.8	*Eco*RI	pIII
E	Mmm	P	1	1.8	*Eco*RI	pIII
F	Mmm	P	1	1.7	*Eco*RV	p2III
G	Mmm	P	1	1.9	*Eco*RI/V	p3I
H	Mmm	P	2	1.8	*Eco*RI	pIII
I	Mmm	P	1	1.8	*Eco*RI	pIII
J	Mmm	M	1	1.7	*Eco*RV	p2III
K	Mcc[b]	P	1	1.1	*Eco*RV	p2V
L	Mcc	None	1	1.8	*Eco*RI	pIII
M	Mci	None[a]	1	1.6	*Bam*/*Eco*	p4IV

Mmm = *Mycoplasma mycoides* subsp. *mycoides*, *M* = mastitis, P = polyarthritis, S = septicemia, a = mycoplasma recovered from the external ear canal, Mcc = *Mycoplasma capricolum* subsp. *capricolum*, b = isolate GM630A from sheep, *Eco*RI/V = *Eco*RI and *Eco*RV, Mci = *Mycoplasma cottewii*, and *Bam*/*Eco* = *Bam*HI and *Eco*RV.

Mycoplasma virus and plasmids: Virus was not recovered from culture supernatant or by the plaque assay procedure from the five mycolasma isolates that were studied. The results and/or characteristics pertaining to the recovered plasmids are shown in Table 1, Fig. 1 and Fig. 2. Based on restriction enzyme digestion, the plasmids were arbitrarily assigned to groups 1 to 4 depending on whether they were linearized, respectively, by *Eco*RI, by *Eco*RV, by *Eco*RI and *Eco*RV, or by *Bam*HI and *Eco*RV, (Fig. 1). They were further assigned to subgroups I to V according to their approximate size (Kbp) of 1.9 (one digested by *Eco*RI and *Eco*RV), 1.8 (all digested by *Eco*RI), 1.7 (two digested by *Eco*RV), 1.6 (one digested by *Bam*HI and *Eco*RV) and, 1.1 (three digested by *Eco*RV) (Table 1, and Fig.1), respectively. By combining the enzyme digestion reaction and the size of the linearized form, five different types of plasmid were found, namely pIII in 25 isolates of *M. mycoides* subsp. *mycoides* and in one isolate of *M. capricolum* subsp. *capricolum*; p2III found in two isolates of *M. mycoides* subsp. *mycoides*; p2V found in three isolates of *M. capricolum* subsp. *capricolum*; p3I found in one isolate of *M. mycoides* subsp. *mycoides* and p4IV found in *M. cottewii* strain GM612 (Table 1). Only one plasmid type was detected in any single isolate, but a particular mycoplasma species was found to carry more than a single plasmid type. For example, different isolates of *M. mycoides* subsp. *mycoides* carried plasmid types pIII, p2III and p3I (Table 1). All the 18 *M. mycoides* subsp. *mycoides* strains from herd B, which were recovered during one outbreak of mastitis and polyarthritis, contained the same type of plasmid (Table 1, and Fig. 2).

DISCUSSION

Although all the plasmid-positive mycoplasma isolates were not routinely assayed for the presence of virus, the sizes of the recovered extrachromosomal DNAs were were small enough (1.1 to 1.9 Kbp) to exclude replicative forms (RF) of viral DNA. The size of the reported RF DNAs from mollicutes is equal to or over than 8.0 Kbp (15).

The rate of occurrence of plasmids among the field strains of caprine mycoplasmas in this study was relatively high, in contrast with a previously reported plasmid survey on mycoplasmas from various animal hosts (13). In the United States, severe large-scale outbreaks of caprine mycoplasmosis are on record (4,5,6,9), particularly from California, which was the origin of all except one of the isolates used in this study. The extensive use of antimicrobial drugs in

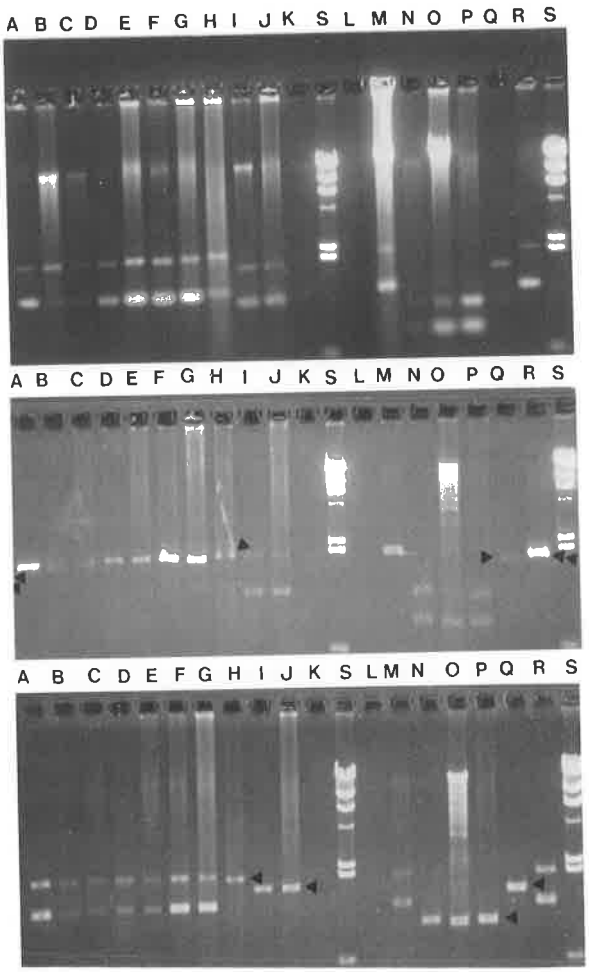

Figure 1. Agarose gel electrophoresis of plasmids from mycoplasmas recovered from 16 mycoplasma isolates under study. Top figure represents supercoiled (uncut) plasmids. Middle, represents *Eco*RI digested plasmids. Bottom, represents *Eco*RV digested plasmids. A and R = plasmid from GM12 (control), B = GM1031B, C = GM1031G, D = GM728A, E = GM975, F = GM262E, G = GM3, H = GM1019, I = GM1013, J = GM1053, K = GM1043, L = blank, M = GM1015A, N = GM630A, O = GM267C, P = GM261B, and Q = GM612. Lanes S represent molecular-weight standard (*Hin*dIII-cleaved lambda phage DNA). Single-arrows indicate the size in base pair of the linearized plasmids. Double-arrows indicate the size (1.85 Kbp) of the linearized pKMK1 plasmid recovered from *M. mycoides* subsp. *mycoides* strain GM12 (Dybvig and Khaled, 1990).

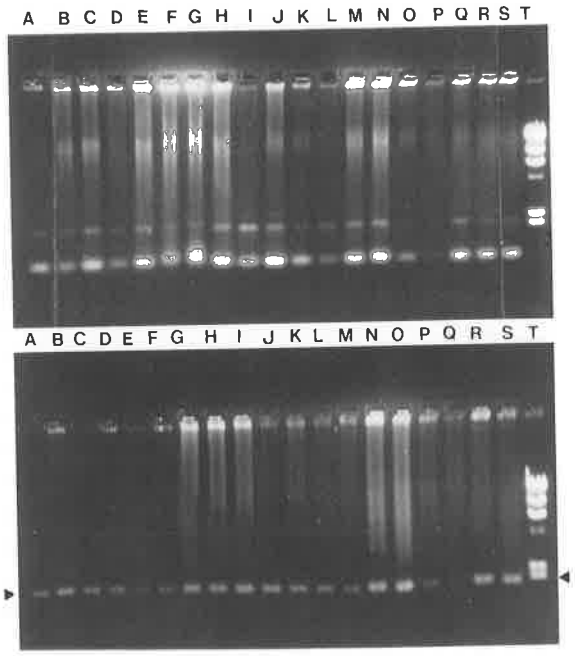

Figure 2. Agarose gel electrophoresis of plasmids from mycoplasmas recovered from all mycoplasma isolates of herd B. Top figure represents supercoiled (uncut) plasmids. Bottom, represents *Eco*RI digested plasmids. A = plasmid from GM12 (1.85 Kbp, control), B = GM32G, C = GM32D, D = GM28F, E = GM32B, F = GM32H, G = GM32A, H = GM28H, I = GM32C, J = GM32E, K = GM28D, L = GM30A, M = GM32J, N = GM28G, O = GM30B, P = GM32K, Q = GM30G, R = GM30L, and S = GM28C. Lane T represents molecular-weight standard (*Hin*dIII-cleaved lambda phage DNA). The arrows indicate the migration size in Kbp of the linearized plasmids.

these herds may have contributed to the high occurrence of plasmid in the mycoplasma strains isolated in this study. Most of the plasmids were recovered from *M. mycoides* subsp. *mycoides* isolates, but *M. capricolum* subsp. *capricolum* and *M. cottewii* were also found to contain plasmids. Excluding one plasmid from a single symptomatic sheep herd studied, most of them (26/33) originated from mycoplasma isolates from disease outbreaks in caprines. Plasmids of different types were found in different isolates of both *M. mycoides* subsp. *mycoides* and *M. capricolum* subsp. *capricolum*.

Based on size and herd of origin the p1II plasmid is probably of the same type as the pKMK1 described previously (8). Plasmids of about 1.7 Kbp have been reported previously (1), but there seems to exist no previous report of a mycoplasma plasmid as small as 1.1 Kbp. The finding of new types of plasmids, more importantly the smaller ones, because they can easily be sequenced and engineered, may add to gene expression studies in mycoplasmas as bacterial plasmids are not fully compatible with the transcription system of the former organism as evidenced previously (14). It is hoped that some of the findings reported in this study will add further to the understanding of the role of plasmids within mycoplasma cells, as well as in mycoplasma infection.

RESUMO

Plasmídios em espécies de *Mycoplasma* isoladas de caprinos e de ovinos e sua tipagem preliminar

Um total de 105 amostras clínicas de micoplasma, originárias de caprinos, e uma de ovino foram investigadas quanto a presença de plasmídios, que foram observados em trinta e três (31%) delas. Esses micoplasmas provieram de 13 rebanhos. Dez desses rebanhos eram sintomáticos para micoplasmose (mastite, poliartrite, septicemia) e três eram assintomáticos, i.é., clinicamente sadios. Vinte e oito amostras eram *Mycoplasma mycoides* subespécie *mycoides* LC ("large colony" ou biotipo caprino), quatro eram *M. capricolum* subsp. *capricolum* e uma era *M. cottewii*. Os plasmídios foram linearizados com endonucleases *Eco*RI, *Eco*RV, *Eco*RI e *Eco*RV ou *Bam*HI e *Eco*RV e apresentaram cinco tamanhos (1,1; 1,6; 1,7; 1,8 e 1,9 Kbp). Com base na digestão pela enzima de restrição e o tamanho dos DNA extracromossomais, cinco tipos de plasmídio foram encontrados (p1II, p2III, p2V, p3I e p4IV). O reduzido tamanho desses DNA circulares muito provavelmente exclui formas replicativas de vírus DNA, cujo tamanho é igual ou maior que 8,0 Kbp.

Palavras-chave: *Mycoplasma*, plasmídio, caprino, endonuclease, eletroforese

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ENZOOTIC BOVINE LEUKOSIS: DEVELOPMENT OF AN INDIRECT ENZYME LINKED IMMUNOSORBENT ASSAY (I-ELISA) IN SEROEPIDEMIOLOGICAL STUDIES

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ABSTRACT

Bovine Leukemia Virus (BLV) is the etiologic agent of Enzootic Bovine Leukosis, a retrovirus exogenous to the bovine species. Once infected, there is no detectable viraemia but instead there is a strong and persistent immunological response to BLV structural proteins, essentially the gp51 envelope glycoprotein and the mayor core protein p24. We describe the test procedure of an indirect ELISA (I-ELISA) using polyclonal reagents for the detection of antibodies to BLV. For comparison, the sera were simultaneously tested by agar gel immunodiffussion (AGID) test, which is currently used as diagnostic standard for BLV infection. The antigen applied does not require a high degree of purification and the data from the analysis of the negative sera showed that the establishment of a cut-off level corresponding to 3 times the standard deviation (SD) above the mean for the negative control set of sera provided acceptable specificity, reducing the risk of false positives results. A comparison of the results obtained by AGID test and I-ELISA showed that considering a total of 465 serum samples, all of the 234 samples (50%) that were positive by AGID were positive to the I-ELISA. Of 225 serum samples which yielded negative results in the AGID test, 69 (15%) were found to be positive by the I-ELISA and 156 (33%) were negative by both techniques. Few sera (2%) that were non-specific by AGID were defined as negative or positive by I-ELISA.

Key words: Bovine Leukaemia; indirect ELISA; seroepidemiology

INTRODUCTION

Bovine Leukaemia Virus (BLV) is the etiological agent of Enzootic Bovine Leukosis (EBL), a retrovirus exogenous to the bovine species. EBL is highly contagious and cattle become infected by transfer of BLV lymphocytes. The disease can be present without obvious clinical signs as persistent lymphocytosis, a benign proliferation of lymphoid cells, or lymphocarcinoma. Once infected, there is no detectable viraemia but instead there is a strong and persistent response to BLV structural proteins, essentially the gp51 envelope glycoprotein and the mayor core protein p24.

The control and eradication of EBL is based on detection of antibodies against BLV. Our preliminary

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experience was in applying the Agar Gel Immunodiffussion (AGID) test. The simplicity, reliability and economical advantages of this test performed on individual sera have been extremely important for epidemiological studies and are the only official serological test in Argentina. Since both eradication programmes and surveys determining disease prevalence require testing of a large number of samples, there has been continued interest in methods more suitable than the AGID test for large-scale application.

Considerable effort has been spent evaluating various technical modifications and practical uses of the ELISA test (1, 2, 3, 4, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 21, 22). However, not always the results obtained were entirely satisfactory and the routine use in clinical laboratories is therefore restricted. Naif *et al.* (12) demonstrated false negative readings for some herds even with new generation kits.

In this communication we describe the test procedure of an indirect ELISA (I-ELISA) using polyclonal reagents for the detection of antibodies to BLV and determine the sensitivity, specificity and predictive value of the test. For comparison, the sera were simultaneously tested by AGID test, which is currently used in many countries around the world as a diagnostic standard for BLV infection.

MATERIALS AND METHODS

Antigen: The persistently infected cell line FLK-BLV (kindly supplied by the National Institute of Animal Health, Tsukuba, Japan) was grown in polystyrene tissue culture flasks (Corning) in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical CO. Ltd, Japan) with antibiotics (penicillin 100 IU/ml, dihydroestreptomycin 100 µg/ml) and supplemented with tryptose phosphate broth (TPB) 0.28 %, glutamine 0.03 % and 8% of foetal calf serum. After the complete monolayer was established, the cultures were washed 3 times with sterile Phosphate Buffer Saline (PBS) and medium without foetal calf serum was added. The supernatant fluid of cultures was harvested on 10 occasions; each separated by five days. The final volume was cleared by centrifugation at 12,000 x *g* for 30 min. Ammonium sulphate was used for the overnight precipitation of viral particles. After centrifugation at 6,000 x *g* at 4°C, the obtained pellet was dialysed against PBS and concentrated with polyethylene glycol (PEG 6,000) at a concentration 1,000-fold of the initial volume. Disruption of the virus was performed at 0°C with Triton X 100, 1% for 1 h. The preparation was cleared by centrifugation at 10,000 x *g* for 15 min. at 4°C. This crude antigen was available at a concentration between 33 and 60 mg/ml protein and stored in 0.1 ml aliquots at –20°C until used as the ELISA antigen.

I-ELISA: Polystyrene microtitration plates (Maxisorp F 96 Immuno Plate; Nunc) were used. Hundred microliters per well of the previous selected antigen dilution (using carbonate-bicarbonate buffer 0.05 M pH 9.6) was used. After incubation overnight at 4°C, the plates were washed three times (5 min. each) with PBS containing 0.05% Twin 20 and skimmed milk powder 0.2 % (PBS-T-SMP). To standardise the method each volume of serial two fold dilutions of reference positive and negative control sera starting from a 1:20 dilution were performed in PBS-T-SMP (0.050 ml/well) and incubated overnight at 4°C. The plates were then washed three times, 5 min. each with PBS-T, and 0.050 ml of a 1:1,000 dilution of horseradish peroxidase-labelled rabbit anti-bovine immunoglobulin G (Dakopatts) in PBS-T-SMP was added. After being incubated for 1 h at 37°C, the plates were washed, and substrate solution was added: 2.2'-Azino diethyl-benzothiazoline sulfonic acid (ABTS), Boehringer Mannheim, Germany 10^{-4} M in citrate phosphate buffer (0.1 M) pH 4.0 and 10^{-3} M H_2O_2. After incubation for 35 min. the optical density (OD) at 405 nm was measured in a microplate spectrophotometer (Multiskan, Flow Laboratories). For the ELISA of field sera, the samples were tested at 1:20 dilution and each one was run in duplicate. The test was carefully performed in order to get uniform results. On each microplate, known negative and positive sera were added.

The results were expressed as following: (Xs – Xn) / (Xp – Xn)

Were Xs: Average of two serum sample readings
Xn: Average of two negative control serum readings
Xp: Average of two positive control serum readings

The negative and positive control samples (giving a negative and positive reaction in the AGID test, respectively) were obtained from cattle from geographic regions in which BLV infection was nonendemic (patagonic region) and endemic respectively (central region).

Sera: The following groups of sera were used in this experiment:

1) Sera collected from 99 cattle (between 2 to 3 years old) belonging to three herds which had been proved to be free from BLV infection during 3 consecutive investigations applying AGID test with 3 months interval.
2) Four reference sera: A local positive bovine anti-BLV, a European reference positive serum (E4, Council Directive 64, EEC, July 1988, Annex G), a negative bovine anti-BLV and a positive bovine anti-BVDV (kindly supplied by NIAH, Tsukuba, Japan and the Virology Institute, INTA, Castelar, Argentina).
3) 465 serum samples (between 1 to 8 years old) obtained from different geographical regions of Argentina. These bovine serum samples were previously tested by the AGID test.

AGID test: This test (10) was performed on all the samples with a commercial kit produced in our laboratory (approved by SENASA, National Service for Animal Health, Argentina). Agar plates were placed in humidified trays and incubated at 37°C for 1-3 days. The AGID test detects undiluted positive reference sera and E4 diluted 1:10 in negative serum (Council Directive 64, EEC, July 1988, Annex G) as weak positive.

RESULTS

The choice of the optimal concentration of antigen used for the coating onto the wells was based on obtaining maximal absorbance readings for BLV-positive control samples and minimal background values for BLV-negative control samples (Fig. 1). The coated plates were usually used immediately but could be stored at 4°C for up to 3 months without a decrease in reactivity. As shown in Fig. 1, for all concentrations of the antigen tested, there was no increase in the background values from BLV-negative control samples. An optimal level of discrimination between positive and negative control samples was obtained with protein concentrations ranging between 77 to 192 mg/ml (equivalent to a dilution 1:500 to 1:1,000. of the original antigen preparation). An antigen working concentration of a 1.9 10^{-2} mg of total protein by well (dilution: 1:1,000) was selected and used for coating onto the wells throughout this study.

The more convenient dilution of the serum samples should provide a quantitative answer derived from the average of two readings at a determined dilution. We choose a working dilution of samples 1:20 at which both, the positive and negative control samples gave reliable results.

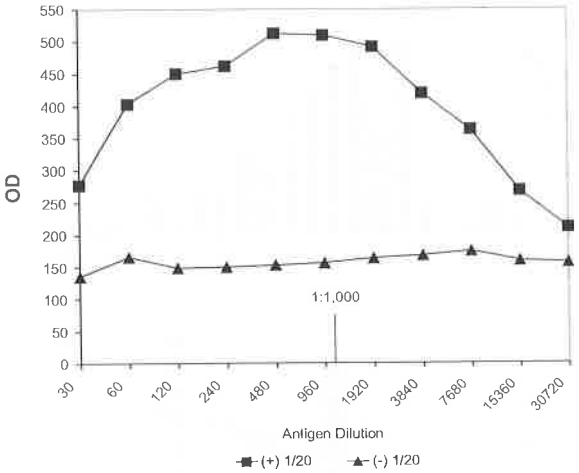

Figure 1: Titration curves of the I-ELISA antigen using AGID positive and AGID negative sera

For determination of a reliable cut-off, a total of 99 serum samples from BLV-free herds were used to determine the normal range of variability applying the I-ELISA test. The I-ELISA values of these sera showed a normal distribution as shown in Fig. 2. Therefore, assuming a 1% probability of misclassification (mean value plus 3 standard deviation, X + 3 SD), the upper limit of an ELISA value for the negative population was determinated as 0.18. On the basis of these results, cattle with ELISA values of more than 0.18 were regarded as positive reactors.

Specificity of I-ELISA: Since some sublines of FLK-BLV are known to be contaminated with BVDV, anti-BVDV reference sera were tested against our I-ELISA system. Sera having antibodies to BVDV, but not to BLV, showed no reaction in the ELISA, although sera having antibodies to BLV, but not to BVDV, showed a positive reaction. As was expected, reference European positive serum E4 (originally diluted 1:10 in negative serum) gave a positive result.

A comparison of the results obtained by the AGID test and ELISA is shown in Table 1. A total of 465 serum samples previously tested by the AGID technique were subjected to the I-ELISA. All of the 234 samples that were positive by AGID were positive by ELISA (50%). Of 225 serum samples which yielded negative results in the AGID test, 69 sera (15 %) were found to be positive by the I-ELISA. From 6 sera (2%) non-specific by AGID, 3 were negative and 3 were positive by I-ELISA. None sera negative by I-ELISA was positive by AGID.

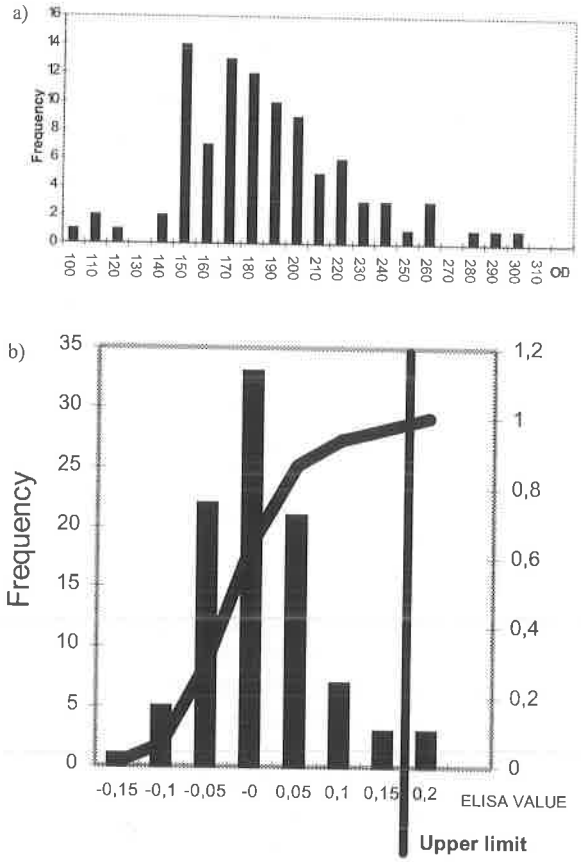

Figure 2: a) Distribution of I-ELISA values of sera collected from BLV free herds. b) Normal distribution of calculated values of negative serum samples applying I-ELISA. All sera showing I-ELISA values more than 0.18 (mean value plus 3 standard deviation, X + 3 SD) were considered positives.

Table 1: Comparison of results obtained with 456 serum samples using AGID and I-ELISA

	ELISA +	ELISA -	Total
AGID +	234 (50%)	0 (0%)	234
AGID –	69 (15%)	156 (33%)	225
AGID non-spec.	3 (1%)	3 (1%)	6
Total	306	159	465

DISCUSSION

This article reports the evaluation of an I-ELISA developed in our laboratory and its comparison with the AGID technique, which is currently used as a diagnostic standard for BLV infection. However, the interpretation of AGID results is sometimes subjective and reliability depending on a great extent on the training and experience of the laboratory personnel.

The sensitivity of both tests strongly depends on the quality of the BLV antigen used. Impurities cause relatively high background values or non-specific reactions. In general, the use of highly purified antigen significantly increases the cost of the test. In this I-ELISA test the antigen applied does not require a high degree of purification. In consequence, purification by sucrose-gradient followed by pelleting was not necessary.

Since pre-coated plates can be prepared in advance and stored at 4°C for at least 4 month, this I-ELISA reduces by less than 2 days the time required for BLV serological detection.

For any given serological test, sensitivity and specificity are determinated by the cut-off value. There are considerable benefits associated with standardised diagnostic tests, particularly in relationship to export testing, animal health regulations, and disease control (20). Arbitrary methods include the recommendation of a subjective cut-off value or use of a statistical parameter (such as 3 SD above the mean for the negative control set of sera). Our main effort was to focus on overcoming the inherent problem of significant background associated with the indirect technique. The data from the analysis of the negative sera showed that the establishment of a cut-off level corresponding to 3 times the SD above the mean for the negative control set of sera, provided high specificity, reducing the risk of false positives results. We established a reliable cut-off working with high number of negative sera -99- from animals of different ages and from three different areas. Our current cut-off value (0.11: 2 SD) or another somewhat lower value is appropriate for pre-export testing by exporters to ensure that very few false-negative diagnoses are made. This value is in accord with Ridge *et al.* (20) who pointed out that the two extreme cut-off values, 0.05 and 0.25 represent the points that provide nearly perfect sensitivity and perfect specificity, respectively. Depending on analytical variation, it is possible, within certain limits, to select a lower cut-off level, in order to increase sensitivity with only a slight reduction in specificity (4). The cut-off value could be changed to a higher value (in our case 0.18 3 SD) for almost all routine diagnostic testing and for herds for which a planned eradication program is undertaken.

As with many infections, the initial appearance of the antibodies at detectable levels and their continuing detection during long periods even using a very sensitive test, may depend upon differences in the

immune system of individual cattle, as well as upon other factors such as persistent infection with bovine viral diarrhoea virus (BVDV) which has been shown to depress antibody responses to BLV.

Comparing our results from the I-ELISA and AGID tests, they were found to agree in the vast majority of cases but ELISA sensitivity was found to be higher than the AGID test. The epidemiological significance of I-ELISA positive but AGID negative cattle is that they might be a source of BLV infection.

Both tests are used routinely in the laboratory because ELISA may complement the AGID test in a number of cases. The AGID test run in optimal conditions is the most practical, cheapest and easiest technique with high sensitivity. Besides, the crucial point is that the ELISA test system includes a number of steps and must be correctly carried out with trained operators.

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RESUMO

Leucose Enzoótica Bovina: desenvolvimento de um teste ELISA indireto (ELISA-I) e sua aplicação em estudos epidemiológicos

O Vírus da Leucose Bovina (VLB) é o agente etiológico da Leucose Enzoótica Bovina, um retrovirus exógeno da espécie bovina. Uma vez infectado, não se detecta viremia, mas uma forte e persistente resposta imunológica às proteínas estruturais, sobre tudo para gp51 da coberta e para proteína p24 do core. Neste trabalho se descreve o desenvolvimento de um ELISA indireto (ELISA-I), em que se utiliza o soro policlonal para a detecção de anticorpos para VLB. Os soros foram analisados pela prova de IDGA (imunodifusão dupla em gel de ágar) rotineiramente usada como prova padrão. O antígeno escolhido não necessita de alto grau de purificação e os resultados obtidos com os soros negativos permitiram estabelecer um valor de corte de três desvios padrão (DP) sobre a média do grupo de soros negativos, mostrando aceitável especificidade e reduzindo o risco de falsos positivos. A comparação dos resultados conseguidos por IDGA e ELISA-I demonstrou que em um total de 465 soros, 234 (50%) foram positivas por IDGA e ELISA-I. Dos 225 soros negativos por IDGA, 69 (15%) foram positivos por ELISA-I e 156 (33%) foram negativos pelas duas técnicas utilizadas. Apenas 2% dos soros foram inespecíficos por IDGA e se definiram como positivos ou negativos pelo ELISA-I

Palavras-chave: Leucose Bovina; ELISA indireto; estudos epidemiológicos.

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FILTRATION ENRICHMENT METHOD FOR ISOLATION OF AUXOTROPHIC MUTANTS OF *TRICHODERMA HARZIANUM* RIFAI

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ABSTRACT

The isolation of genetic markers, like drug resistance and auxotrophy, is a laborious but important step in genetic research. The isolation of auxotrophic mutants of *Trichoderma harzianum* using the filtration enrichment technique was more effective than using the total isolation technique. Most of 12 auxotrophic mutants exhibited similar growth rate and higher sporulation when compared with the wild type, but only two mutants (TWS-410 and TW5-523) could grow in 500µg/L of benomyl.

Key-words: Filtration enrichment technique, auxotrophic mutants, *Trichoderma harzianum*, benomyl

INTRODUCTION

Trichoderma spp. Rifai are the most promising producers of cellulolytic and chitinolytic enzymes, and also are currently investigated as biological control agents of plant pathogens. Their sexual state is unknown, but parasexual cycle has been studied with auxotrophic markers permitting selection of heterokaryons and of possible diploids (5). The classical method used, total isolation following mutagenic treatment, is laborious and yields a low frequency of auxotrophics among tested survivors (6). Several methods have been described for the selection of auxotrophic mutants of fungi. These include biotin-starvation methods that have been used for *Aspergillus nidulans* and comparable methods have been used in other fungi (6). To induce mutants of *Aspergillus niger*, Bos *et al.* (4) used low doses of the mutagen ultraviolet light (UV) in order to avoid background mutations or chromosomal rearrangements. Usually, this procedure results in high survival and low frequency of mutants among surviving prototrophics. Consequently, an efficient enrichment step can be a prerequisite. In this paper we describe the isolation of auxotrophic mutants of *Trichoderma harzianum* by a filtration enrichment technique based on the technique developed by Silveira and Azevedo (8) for *Metarhizium anisopliae*.

MATERIALS AND METHODS

Microorganism – *T. harzianum* TW5, originally isolated from a soybean field in Brazil, was obtained from the fungi collection of National Research Center for Monitoring and Assessment of Environmental Impact, EMBRAPA, Brazil. This strain has been shown to be antagonist to the plant pathogens *Sclerotinia sclerotiorum* and *Sclerotinia minor*.

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Culture medium – Mineral medium (MM) and complete medium (CM) were those of Pontecorvo *et al.* (6), modified by Azevedo and Costa (1).

Filtration technique and isolation of auxotrophic mutants – Eight layers of stretched gauze were fixed to polypropylene filters with adhesive tape. Filters, wrapped in aluminum foil, were autoclaved at 121°C for 15 minutes. Conidial suspensions were prepared in aqueous Tween 80 (0.1%), diluted and irradiated for 5 minutes with U.V. light (97.72 $\mu W/cm^2 x 10^{-2}/s$) to give 5% survival.

a) Isolation of auxotrophic mutants by the conventional technique

Treated conidia suspensions were diluted and plated on CM supplemented with sodium deoxycolate (0.1%) and incubated for 48-72h at 25°C. Colonies were tested for growth by transfer onto MM and CM supplemented with 0.1% sodium deoxycolate, in a 5x5+1 arrangement, with a total of 26 inocula per dish (2). Colonies failing to grow were considered possible auxotrophic mutants and were tested for auxotrophy on test plates with combinations of amino acids or nucleotides or vitamin mixtures.

b) Isolation of auxotrophic mutants by filtration enrichment technique

Treated conidia were transfered to 50ml liquid MM and shaken for 20h at 25°C. After filtration, the filtrate was incubated again with agitation for additional 20h. The procedure was carried out three times and after the last filtration the filtrate was centrifuged at 2,900g for 15 minutes, resuspended in 3ml distilled water, diluted and plated on CM supplemented with 0,1% sodium deoxycolate (0.1%).

Each resulting colony was inoculated on MM supplemented with 0.1% sodium deoxycolate in a 5x5+1 arrangement, with a total of 26 inocula per Petri dish (2). Colonies failing to grow were considered possible auxotrophic mutants and were tested for auxotrophy on test plates with combinations of amino acids or nucleotides or vitamin mixtures.

Characterization of auxotrophy marks in the mutants

Mutants were plated from monospore cultures and characterized for deficiency by supplementation with individual growth factors. Phenotypically similar mutants were combined in complementation tests.

Growth and sporulation of auxotrophic mutants on Potato-Dextrose-Agar (PDA) and on Oat-Agar media

Disks (5mm in diameter) of 4-day old colonies of auxotrophic mutants of *T. harzianum* TW5 were transferred to the center of Petri dishes containing PDA or Oat-Agar media. The supply of amino acids or vitamins required by each auxotrophic mutant was added in appropriate quantity (w/v) to the media after autoclavation. Colony radii were measured after 72h. Conidia were suspended in 0.1% Tween and used to determine the conidia production after 12-days. There were three replicates per treatment, and data were expressed as percentage of growth inhibition.

Growth and sporulation of auxotrophic mutants on PDA medium supplemented with benomyl

The fungicide benomyl, methyl-1(butycarbamoyl) -2-benzimidazole carbamic acid (Benlate 50% WP, Du Pont Co., Wilmington, DE) was suspended in acetone. The fungicide was tested at 1, 5, 10, 50, 100, 500 and 1000µg of active ingredient (a.i.) per milliliter of medium. The benomyl and the supply of amino acids or vitamins required by each auxotrophic mutant were added in appropriate quantities (w/v) to the PDA medium after autoclavation. Disks (5mm in diameter) of 4-day old colonies of auxotrophic mutants of *T. harzianum* TW5 were transferred to the center of Petri dishes containing the above described medium. Colony radii on solid media were measured after 6-day at 25°C. There were three replicates per treatment and the data were expressed as percentage of growth inhibition.

RESULTS AND DISCUSSION

Using the total-isolation method, only morphological mutants were isolated (Table 1). All the auxotrophic mutants isolated were obtained by the filtration enrichment method. The results in Table 2 show the variety of amino acid-deficient mutants that were isolated. Only one vitamin-deficient mutant was found using the filtration enrichment method.

These results are in agreement with those of Bos *et al.* (3), with *Aspergillus niger*. They showed that 123 auxotrophic mutants were isolated by filtration enrichment technique, but only 9 of them were vitamin-deficient mutants. The mutants obtained in this way were predominantly amino acid requiring.

The efficiency of the total-isolation technique

was considerably lower than that of the filtration enrichment technique for isolation of auxotrophic mutants of *T. harzianum* TW5. One explanation for this low efficiency is that the number of colonies tested were too small. The use of the filtration enrichment technique resulted in 11 auxotrophic mutants, in spite of having 50% less colonies than in the total-isolation technique.

Those mutants, when tested for growth speed and sporulation on PDA and Oat-Agar media, did not differ from the wild type, except by the mutants TW5-600 and TW5-53, which showed the lowest growth rates. On the other hand, these mutants exhibited higher sporulation on PDA medium (Table 3).

Table 1. Frequency of isolation of auxotrophic and morphological mutants of *T. harzianum* TW5.

Treatments	Number of colonies	Characteristics of mutants	Number of mutants	Frequency of isolation (%)
Total isolation	3920	morphologic	29	0.74
		auxotrophic	0	0.00
Filtration enrichment technique	703	morphologic	0	0.00
		auxotrophic	11	1.56
Total	4623		40	0.86

Table 2. Auxotrophic mutants of *T. harzianum* TW5, their markers, and number of tested colonies.

Mutants	Auxotrophic marker	Number of tested colonies	Mutants	Auxotrophic marker	Number of tested colonies
TW5-612	met	93	TW5-574	arg	75
TW5-537	leu	66	TW5-560	arg	93
TW5-533	leu	57	TW5-609	arg	66
TW5-523	rib, bio	75	TW5-534	arg	48
TW5-600	met, cis, arg	66	TW5-555	arg	60
TW5-410	met, cis, arg	75			

arg = arginin; **bio** = biotin; **cis** = cistein; **leu** = leucin; **met** = metionin; **rib** = riboflavin

Table 3. Mycelial growth and sporulation of auxotrophic mutants of *T. harzianum* TW5 on PDA and Oat-Agar media.

	Radial growth (cm)		Sporulation (x 10^6/ml)	
Mutants	PDA medium	Oat Agar medium	PDA medium	Oat-Agar Medium
TW5	8.50aA	8.50aA	1.637bFG	4.733aCD
TW5-534	8.50aA	8.50aA	4.153bDEFG	18.067aAB
TW5-555	8.50aA	8.50aA	12.200bCDE	26.133aAB
TW5-560	8.50aA	8.50aA	19.933bC	29.333aA
TW5-574	8.43aA	8.50aA	0.14bG3	2.233aA
TW5-612	8.50aA	7.90aAB	6.133bDEF	20.200aAB
TW5-523	8.50aA	8.50aA	13.400bCD	15.633aAB
TW5-609	7.33bAB	8.50aA	3.403bEFG	14.300aBC
TW5-410	8.50aA	8.50aA	3.643aEFG	3.340bD
TW5-533	8.13aAB	8.50aA	22.633aBC	22.300bAB
TW5-600	5.60cC	7.10bBC	45.300aA	15.233bAB
TW5-537	6.93bBC	6.27bC	38.000aAB	20.700bAB

Means followed by different letters (capital on vertical and small on horizontal) differ significantly according to Tukey' s multiple range test ($P \leq 0.05$).
Data are means of three replicates.

According to Silveira and Azevedo (8), the filtration enrichment method is very efficient for *M. anisopliae*. These authors reported a predominance of mutants able to synthesize amino acids and nucleic acids over mutants unable to synthesize vitamins. They justified the results based on the fact that mutants with vitamin requirements may have grown as a result of vitamins released by growing prototrophs or by spontaneous cell lysis, as suggested by Strauss (9). However, the diversity of mutant types according to isolation procedure is actually valuable when a wide range of types of nutritional requirements is needed (9).

Using the methods described, benomyl resistance was obtained in two of the auxotrophic mutants (TW5-410 e TW5-523). These mutants presented stable fungicide resistance and were able to grow at concentration of up to 500 and 1000µg/L, respectively (Table 4). We believe that these stable fungicide resistant isolates were produced as a direct result of the mutageneses treatment and are not naturally occurring spontaneous mutations.

Table 4. Mycelial growth reduction of *T. harzianum* TW5 mutants on PDA medium supplemented with benomyl.

Mutants	Mycelial growth reduction							
	Concentration (µg)							
	0	1	5	10	50	100	500	1000
TW-410	0	0	0	0	0	0	0	42
TW5-523	0	0	0	0	42	62	61	61
TW5-555	0	0	67	68	87	89	100	100
TW5-537	0	0	65	69	100	100	100	100
TW5-574	0	0	71	72	100	100	100	100
TW5-534	0	0	76	78	100	100	100	100
TW5-560	0	0	64	81	100	100	100	100
TW5-600	0	0	71	82	100	100	100	100
TW5-609	0	4	82	84	100	100	100	100
TW5-612	0	32	69	100	100	100	100	100
TW5-533	0	9	71	100	100	100	100	100
TW5	0	55	100	100	100	100	100	100

Data are means based on three replicates.

RESUMO

Técnica de enriquecimento por filtração para isolamento de mutantes auxotróficos de *Trichoderma harzianum* Rifai

A obtenção de marcas genéticas, quer sejam para resistência a drogas, quer para auxotrofia, é uma etapa trabalhosa mas importante em pesquisa genética. Esse trabalho visou a obtenção de mutantes auxotróficos de *Trichoderma harzianum* utilizando-se a técnica de enriquecimento por filtração. A técnica mostrou-se superior à técnica convencional de isolamento total. Doze mutantes auxotróficos obtidos foram testados quanto a estabilidade, crescimento e resistência ao fungicida benomil. Eles apresentaram taxas de crescimento e esporulação comparáveis à linhagem parental e dois mutantes foram resistentes a benomil em uma concentração de 500µg/ml.

Palavras-chave: enriquecimento por filtração, mutantes auxotróficos, *Trichoderma harzianum*, benomil

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DIFFERENTIATION OF THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM FLAVOVIRIDE* (HYPHOMYCETES)

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SHORT COMMUNICATION

ABSTRACT

The differentiation of a Brazilian isolate of *Metarhizium flavoviride* (CG 423), a promising candidate for the biocontrol of grasshoppers, was investigated. Conidia were spread onto solid medium (1% yeast extract, 2.8% agar, 96.2% distilled water), incubated at 28°C and observed during 26 h. Germination initiated as conidia size increased from 5.3 (±0.6) x 3.1 (±0.3) µm (0 h incubation) to 8.1 (±0.2) x 6.1 (±0.2) µm (8 h incubation). Germ tubes started to appear after 10 h incubation showing a high degree of multipolarity. Twenty six hours after inoculation, hyphal differentiation and anastomosis among hyphae from adjacent conidia were recorded. Appressoria were formed only from conidia incubated in liquid medium containing minimum concentration of yeast extract (0.06%; w/v). Appressoria were firmly adhered to the bottom of plastic dishes.

Key words: Hyphomycetes, entomopathogenic fungus, germination, appressorium

The fungus *Metarhizium flavoviride* is a promising candidate for the biological control agent of grasshoppers (3, 10) showing encouraging results in Africa (4) and Australia (8). In Brazil, this entomopathogen was originally isolated from *Schistocerca pallens* (Orthoptera: Acrididae) in 1992 (9). Ever since, this pathogen has been studied with the aim of efficiently controlling different grasshopper pests, including *Rhammatocerus schistocercoides* (6) and *S. pallens* (14).

Germination is one of the first steps in the infection process actively affecting pathogenicity or virulence. High virulent strains tend to germinate more quickly than strains with low virulence (7). For many fungi, the pathogenic process is also related to the formation of infection structures or appressoria after germination, which aid the fungus in the invasion of the host cuticle. *Metarhizium anisopliae*, *Beauveria bassiana*, *Aspergillus parasiticus*, *Paecilomyces farinosus*, *Coelomomyces psorophorae* and *Zoophthrora radicans* are examples of entomopathogenic fungi forming these structures (7, 11).

However, appressorium formation is not a universal prerequisite for successful infection and some fungi are able to penetrate their host cuticle without morphological differentiation. In contrast to other morphological events, appressorium

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differentiation may not always appear in the life cycle, but be a specific reaction to the environment (12). In this study we investigated the patterns of *in vitro* germination and appressorium formation of *M. flavoviride*.

Metarhizium flavoviride (CG 423) used in this study was isolated from *S. pallens* in Rio Grande do Norte state and deposited in the Collection of Entomopathogenic Fungi at Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil.

In order to avoid a possible attenuation process caused by successive transferring and storage, *M. flavoviride* was revigorated by passing it through its host *S. pallens*. After profuse sporulation, conidia were plated on selective medium (2% gross oat Quaker®, 0.1% crystal violet, 0.046% Dodine - monoacetate de N-dodecil-guanidine, 0.05% tetracycline, 2% agar). The fungus was then grown on complete medium (0.4 g $NaHPO_4$, 0.6 g $MgSO_4$, 1 g KCl, 0.7 g NH_4NO_3, 10 g glucose, 5 g yeast extract, 15 g agar, 1 liter of distilled water) during 12 days.

Germination was investigated by seeding *M. flavoviride* onto solid medium (1% yeast extract, 2.8% agar) using a conidial suspension of 300 µl (10^6 conidia/ml) and incubated at 28°C. Starting immediately after inoculation (0 h), samples were taken at regular intervals of 2 h. At each sample, a culture medium block (± 2 cm^2) was prepared for microscopic examination as follows. Samples were fixed for 15 min with formaldehyde (3.7%) and washed with PBS buffer (0.8% NaCl, 0.02% KCl, 0.11% Na_2HPO_4, 0.02% KH_2PO_4, pH 7.4). To better visualize the fungus development, a cell wall dye Tinopal (Sigma Chemical Co.) was used (1, 5). Preparations were examined and photographs taken using black & white Fuji Neopan-400 film in a fluorescence microscope (Zeiss III R5). Germination and polarity were determined for 100 conidia from each of the three replicates. Conidia was considered to have germinated when germ tubes measured at least their length and those with more than one germ tube were considered multipolars. In addition, conidial dimension (width and length) was recorded before germ tube emergence and growth ratio was determined by measuring germ tube length in a time-course experiment at 2 h intervals, from 10 to 26 h incubation.

To induce appressorium formation, conidia were suspended (10^5 conidia/ml) in liquid medium containing yeast extract at two concentrations (0.06% and 1%). The suspension was poured into plastic Petri plates (3.5 cm diameter; 2.5 ml/plate) and incubated at 30°C. A conidial suspension prepared in distilled water was used as control. After incubation during 17 and 30 h, supernatant was removed and the plates were washed with distilled water and stained with Tinopal to examine under a fluorescence microscope the germlings adhered to the bottom of the plates.

SigmaStat™ and SigmaPlot™ (Jandel Scientific, Corte Madera, CA, USA) were used to calculate statistics and plot data. The mean (± SD) is indicated in the figures.

The beginning of the germination process was visualized by the increasing volume of the conidia (Fig. 1). At inoculation, conidial dimensions ranged from 5.3 ± 0.6 µm long by 3.1 ± 0.3 µm wide (at 0 h) to 8.1 ± 0.2 µm by 6.1 ± 0.2 µm (8 h after inoculation). First germ tubes appeared at 10 h incubation and the proportion of germinated conidia increased considerably in the following two hours until the last hour of observation (26 h). By this time, 88.8 ± 4.5 % of conidia had germinated (Fig. 2).

Germ tubes presented uniform growth rate (2.6 ± 0.9 µm h^{-1} ; R = 0.99; y = -20.8 + 2.6 x) until 18 h incubation (Fig. 3). In the following hours, the growth rate increased to15.8 ± 3.8 µm h^{-1} (R = 0.98; y = -328.5 + 18.5 x). At 26 h, germ tubes reached 150 µm and it was possible to see anastomoses between adjacent hyphae. At 14 h incubation, i.e. 4 h after production of the first germ tubes, 45.3% of germlings showed multipolarity (Fig. 4). There was an increasing number of multipolar conidia until 18 h, when 88.2% of the germinated conidia had more than one germ tube, and at 26 h almost all (99.3%) were multipolar. Polarity is related to the accumulation of vesicles containing enzymes responsible for cell wall synthesis in a particular conidial pole (2). Under these conditions, no appressoria were formed at this time. However, when conidia were suspended in the liquid medium with a minimal concentration of yeast extract (0.06%), appressoria were formed against the plastic Petri plate 17 h after inoculation. In contrast, there was no appressorium formation when the yeast extract concentration was 1%. In the absence of yeast extract, germination was very low (< 10%) and no appressoria were formed. The entomophthoralean *Zoophthora radicans* produces appressoria in 1% yeast extract medium but there is no appressorium formation at higher (2%) or lower (0.2 and 0.5%) concentrations (7).

Appressoria formed from swelling hyphal tips (Fig. 5) and were delimited by a septum and firmly adhered to the bottom of the plastic plates. It was not

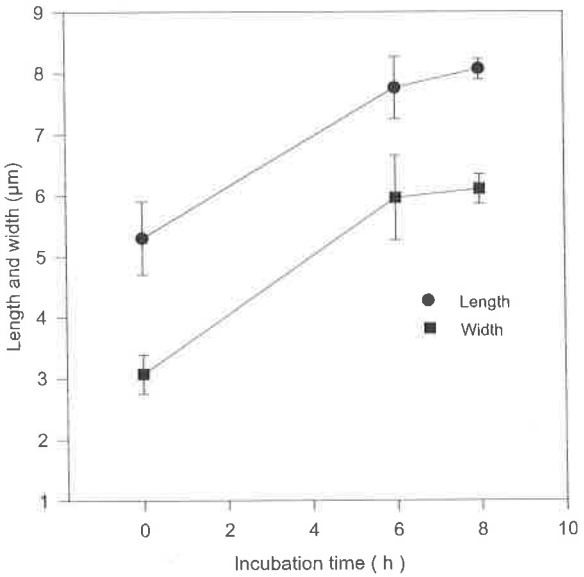

Figure 1. Conidial dimensions of *Metarhizium flavoviride* on 1% yeast extract agar at 28°C.

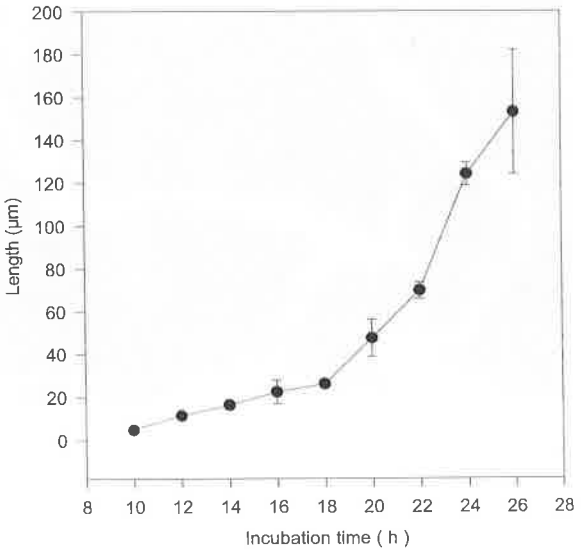

Figure 3. Lengtht of *Metarhizium flavoviride* germ tubes on 1% yeast extract agar a 28°C.

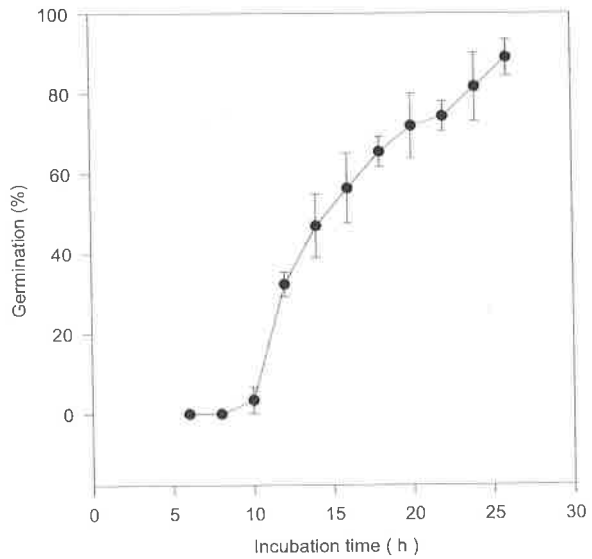

Figure 2. Germination of *Metarhizium flavoviride* on 1% yeast extract agar at 28°C.

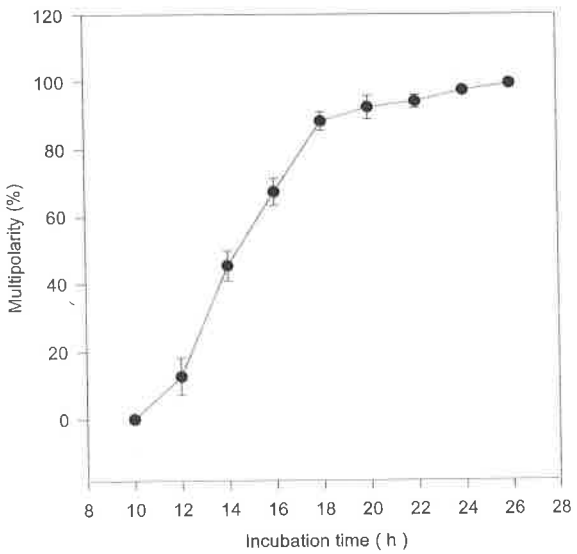

Figure 4. Polarity of *Metarhizium flavoviride* germinated conidia on 1% yeast extract agar at 28°C.

possible to remove these appressoria after several washes. The formation of appressoria after germ tubes contacting a rigid surface was also observed for *M. anisopliae* (12). In this case, the adhesive capability of *M. anisopliae* was attributed to a mucilaginous layer covering the appressorium surface. However, we did not observe such a mucilaginous layer in the development of *M. flavoviride* (CG 423).

In conclusion, germination of *M. flavoviride* starts at 10 h incubation at 28°C, in a typically multipolar fashion. Differentiation is affected by the environment and nutrition, but appressorium formation is more sensitive to the medium than is conidial germination. A nutrient gradient associated to the germ tube contact with a rigid plastic surface may be the main stimulus triggering appressorium

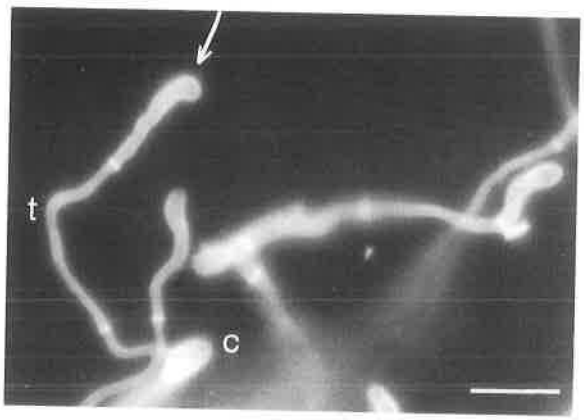

Figure 5. Fluorescence micrograph of *Metarhizium flavoviride* (CG 423) grown in yeast extract (0.06%) liquid medium stained with Tinopal (0.5%). Arrow indicates appressoria. t = germ tube, c = conidium (barr = 10μm).

formation. In addition, as observed by St. Leger *et al.* (12), this contact is similar to the natural contact of the germ tube with the rigid insect cuticle. Such a hypothesis is supported by the fact that no appressoria were detected in solid culture medium or in liquid media containing high concentrations of nutrients. A possible explanation for this would be that in the solid medium conidia are onto a soft surface whereas in liquid medium decanted conidia maintain a contact with a rigid surface. *M. flavoviride* formed appressoria on the cuticle of *R. schistocercoides*, but it was able to cause infection without forming appressoria (13). Although our results indicated that appressorium formation by this pathogen is affected by nutritional and environmental factors, further investigation on the actual role of these structures is needed.

ACKNOWLEDGEMENTS

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RESUMO

Diferenciação do fungo entomopatogênico *Metarhizium flavoviride* (Hyphomycetes)

A diferenciação de um isolado brasileiro de *Metarhizium flavoviride* (CG 423), candidato a agente de controle biológico de gafanhotos, foi investigada. Conídios semeados em meio de cultura sólido (extrato de levedura 1%, ágar 2,8%, água destilada 96,2%) e incubados a 28°C, foram observados durante 26 horas. Para induzir a formação de apressórios, conídios foram suspensos em meio líquido contendo duas concentrações de extrato de levedura (0,06 e 1%) e transferidos para placas de Petri plásticas (3,5 cm de diâmetro). A germinação teve início com o aumento do tamanho dos conídios de 5,3 ± 0,6 x 3,1 ± 0,3 μm (0 h de incubação) para 8,1 ± 0,2 x 6,1 ± 0,2 μm (8 h de incubação). Os primeiros tubos germinativos começaram a surgir após 10 h de incubação dos conídios, os quais apresentaram acentuada multipolaridade. Vinte e seis horas após a inoculação foi observado o início da diferenciação micelial e formação de anastomoses entre hifas de conídios adjacentes. Apressórios foram formados somente quando conídos foram incubados em meio líquido contendo concentração mínima de nutriente (extrato de levedura 0,0%; peso/volume). Os apressórios formados encontravam-se fortemente aderidos à superfície do fundo plástico da placa de Petri.

Palavras-chave: Hyphomycetes, fungo entomopatogênico, germinação, apressório.

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OCCURRENCE OF *PSEUDOMICRODOCHIUM SUTTONII* IN BRAZIL

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SHORT COMMUNICATION

ABSTRACT

Pseudomicrodochium suttonii was isolated from the soil of Derby Square, a leisure area in Recife city, Pernambuco, Brazil. For the isolation, suspensions were made in distilled sterile water. According to the literature, this is probably the first occurrence reported in South America.

Key words: Taxonomy, *Pseudomicrodochium suttonii*, soil

The genus *Pseudomicrodochium*, class Hyphomycetes, was first described by Sutton (7). The type species is *P. suttonii*.

The taxon was isolated from *Castanea sativa* Mill. Gard. Later, new species were described, including *P. fusarioides*, *P. lauri*, *P. candidum*, *P. cilindricum* and *P. aciculare* (8).

This work describes the isolation of *P. suttonii* from the soil of Derby Square, Recife, Pernambuco, Brazil. Surface and depth soil samples were collected and pooled, in a total of six pools.

The isolation procedure was done according to the method of Warcup (9). Suspensions were prepared in distilled sterile water, containing chloramphenicol (75mg/l) and cycloheximide (50mg/l). Ten grams of each pool were weighed, diluted 1:100 and 0.1ml was plated on the surface of Mycozel agar (5), in triplicate. Plates were maintained at room temperature (28°C ± 1°C) for seven days.

For identification, the criteria of Ellis (2), Ellis (3), Sutton (6) and de Hoog and Guarro (4) were used. The main characteristics of this species areas follows. Growth on potato dextrose agar (PDA) at room temperature (28°C ± 1°C) is slow, reaching 2.9 cm in diameter after 15 days. The colonies present a fluffy aspect, regular edge and a pale brown color. The reverse is smooth and presents a dark color. Hyphae are 1.5-2µm wide, pigmented, branched and septate. Conidiophores are absent. Conidiogeneous cells are intercallary or lateral, often without basal septum, cylindrical, whith an indistinct collarette, frequently producing conidia in a sympodial order. Conidia are straight to falcate, acicular, pale brown, smooth-walled, 16-28 x 0.8-1.1µm, 3-6 septate (Fig. 1).

This Hyphomycetes species, *P. suttonii*, is a species of rare incidence, and this is probably the first citation in Brazil. Other authors have reported the isolation of *P. suttonii* from animal phaeohyphomycosis (1).

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This strain is stored in the culture collection of the Federal University of Pernambuco, Biological Sciences Center, Department of Mycology (**Micoteca URM**), with register number 4006.

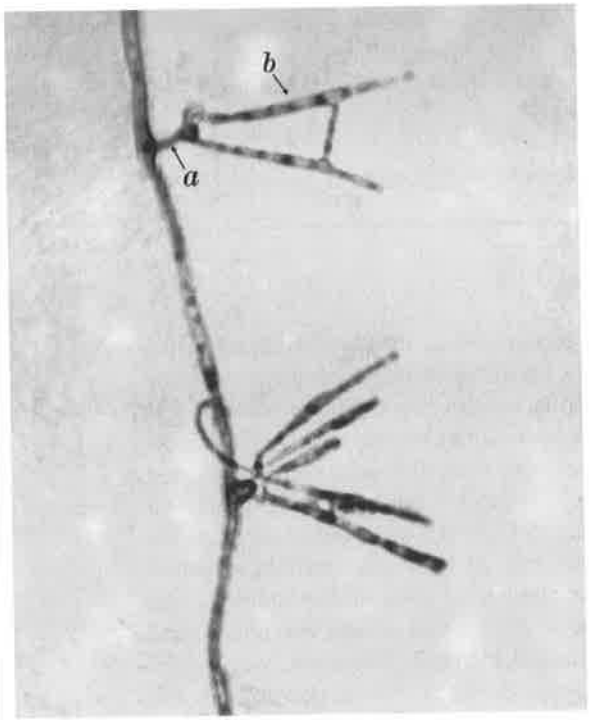

Figure 1- *Pseudomicrodochium suttonii.* Conidiogenous cells (a), conidia (b). (scale - 100X)

RESUMO

Ocorrência de *Pseudomicrodochium suttoni* no Brasil

Pseudomicrodochium suttonii foi isolado do solo da Praça do Derby, uma área de lazer da cidade do Recife, Pernambuco, Brasil. Para o isolamento, foram feitas suspensões utilizando-se água destilada e esterilizada. De acordo com literatura consultada, provavelmente esta é a primeira ocorrência notificada na América do Sul.

Palavras-chave: Taxonomia, *Pseudomicrodochium suttonii*, solo

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ROLE OF THE CONCENTRATION PROCESS IN THE RECOVERY OF *CANDIDA ALBICANS* FROM BLOOD

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ABSTRACT

The lysis-centrifugation system (Isolator ™) is recognized as the standard method for recovery of *Candida* spp. from blood. In this study, the effect of the concentration process of this system was compared with conventional methods of blood culture using liquid and biphasic media. The tests were performed *in vitro* using *Candida albicans* in counts of 100, 10, 1, and 0.5 cells/ml of blood. Cultures onto chocolate agar were performed with the sediment obtained after centrifugation of Isolator™ tube and with liquid and biphasic media after their incubation for 24 h at 35°C. Gram stain prepared from the conventional methods were also evaluated in the first 24 h. It was possible to detect *Candida albicans* in blood, regardless both the number of cells or methodology. As blastoconidia were observed in Gram stains at the same time that growth was noted, time for diagnosis was also not different for the compared methods. Therefore, we suggest that the process of concentration is not the single important factor responsible for the recovery rates of *Candida albicans* from blood by the Isolator™ system.

Key words: candidemia, blood culture, lysis-centrifugation system

INTRODUCTION

The incidence of fungemia has increased considerably in hospitalized patients (1, 21, 28). *Candida* spp. are the most commonly fungi recovered from blood, and more than 50% of the episodes of candidemia are due to *Candida albicans* (25, 31). In fact, deep fungal infection due to *Candida* spp. is responsible for an excess of hospital stay and for an overall mortality rate that may range from 38% to 75% (32, 33). Patients using inadequate antimicrobial treatment had a mortality rate higher than those receiving appropriate antifungal therapy (24, 30). Considering that patients with fungemia rarely present characteristic clinical manifestations, the diagnosis relies upon the findings of the microbiology laboratory, in particular, observation and isolation of fungi from blood. Although an increase in life-threatening invasive yeast infection has been observed, many of these infections either remain undetected or are detected too late to beneficiate patient management (4, 21, 22, 23). The number of fungal cells in blood is generally small, and this poses the major problem to obtain a positive result (27, 4).

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The conventional, visually monitored, broth-based blood culture consists of culturing blood in broth (liquid) or biphasic (liquid and solid) media. Their advantages include relative inexpensive supplies, the convenience of collecting specimens and most importantly, the benefits that can derive from the use of a technology that existed for so many years (12, 14). Therefore it is still largely used, specially in small laboratories.

The critical importance of sensivity and speed of processing blood cultures for proper diagnosis and management of patients stimulated continuous efforts to develop more sensitive blood culture methods. A great deal of work has been done to define optimal media, blood-to-medium ratios, types of anticoagulants and other routine procedures (12).

The development of a lysis-centrifugation system (Isolator; Wampole Laboratories, Cranbury, NJ) improved the recovery rates of fungi from blood. In this system blood cells are lysed to liberate microorganisms from phagocytes and blood is concentrated by centrifugation to prepare an inoculum that is plated onto agar plates. Most clinical comparisons of Isolator™ with broth-based blood culture systems showed higher sensitivity (3, 5, 11, 13, 18, 22), and earlier results using the Isolator™ system (8, 13). In a quantitative study, Kiehn *et al.* (15) reported advantage of Isolator™ versus broth culture method only if the number of fungal cells present in blood was less than 10 /ml.

Although lysis-centrifugation system has advantages, processing of specimens is labor-intensive and expensive (18), mainly due to the centrifugation process rather than the lysis. Another disadvantage of Isolator™ is the high propensity for contamination due to manipulation of samples (9, 12).

Reports of Murray *et al.* (19) and Zierdt (35), comparing the of Isolator™ system with conventional broth method plus lytic agent, indicate that the higher performance Isolator™ is due to the release of intracellular microorganisms, so that broth supplemented with lytic agent saponin has been introduced in automated systems (13, 17).

The aim of this work is to evaluate the influence of blood concentration, using the Isolator™ system, in the recovery of *Candida albicans*.

MATERIALS AND METHODS

Methods of blood culture and culture media

The concentration process was done using the Isolator™ system. Blood was concentrated by centrifugation using a fixed angle rotor, and the remaining pellet was directly plated onto solid media.

Tubes of Isolator™ contain saponin to liberate microorganisms from white blood cells and sodium polyanetholsulfonate (SPS) to prevent clotting. Although *in vitro* phagocytosis does not occur easily (16), in this study blood was mixed with the chemical components of the tube before inoculation of *Candida albicans*, in order to allow the action of SPS and saponin, avoiding "in vitro" phagocytosis (12). Therefore, the lysis interference was eliminated in all samples, assenting to determine the effect of the concentration alone.

The conventional methods were represented by biphasic bottles and by 45 ml broth bottles, containing brain heart infusion (BHI - DIFCO Laboratories, Detroit, Michigan), supplemented with 0.05% SPS.

Chocolate agar plates used to subculture *Candida albicans* were prepared with BHI agar (DIFCO Laboratories, Detroit, Michigan).

Blood

A total of 30 ml of blood was collected from healthy volunteers after appropriate desinfection of the venipuncture site. From each sample of blood a volume of 9 ml was immediately inoculated into one Isolator™ tube, one biphasic bottle and one broth bottle.

Candida albicans

Candida albicans isolated from a patient with candidemia, identified by API 20C (BIOMÉRIEUX VITEK, Missouri, USA), was maintained on agar slant at 22°C (20). The inoculation sample was prepared as described elsewhere (2, 6, 16). Briefly, the sample was inoculated onto a chocolate agar plate and incubated for 24 h at 35°C. Five to ten colonies were suspended in sterile saline and centrifuged for 10 minutes at 1,500 rpm. The pellet was washed twice and a suspension of turbidity equivalent to 1000 cells/ml was prepared. The number of cells in this suspension was confirmed by counting the cells in Neubauer's chamber (7).

Inoculation and culturing procedures

The original suspension of 1000 cells of *Candida albicans*/ml was serialy diluted to obtain an inoculum ten fold more concentrated than the final tests, i.e., 100, 10 and 5 cells/ml. One ml of these suspensions was inoculated into each of the bottles or tubes, which

had been previously inoculated with 9 ml of blood. Accordingly, to perform tests with 100 cells of *Candida*/ml of blood, 1 ml of the suspension containing 1000 cells/ml was used; to obtain tests containing 10 cells of *Candida*/ ml of blood the inocula with 100 cells/ml was used, and so on.

After the inoculation of blood and suspensions of *Candida albicans*, the samples of conventional methods were incubated at 35°C for 24 h. Subcultures were made as recommended (12): an amount was drain to prepare a Gram smear and a volume of 0.1 ml was plated onto chocolate agar plates.

The Isolator™ tubes were processed as described in the instructions of the manufacturer (29). Finally, 0.1 ml of the pellet was plated onto chocolate agar plates.

Chocolate agar plates were incubated at 35°C for 24 h. In view of the excessive number of colonies, it was impossible to perform individual colony count (Fig. 1). Therefore, magnitude of growth was estimated as degrees of confluent growth.

All tests were performed three times, using fresh suspensions of *Candida albicans* prepared immediately before each inoculation.

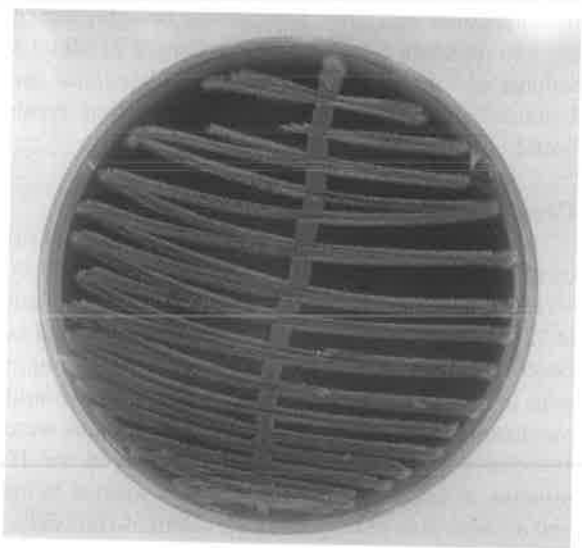

Figure 1 - Confluent growth of *Candida albicans* onto chocolate agar plates incubated for 24 h at 35°C.

RESULTS AND DISCUSSION

Reports of blood culture using clinical specimens are sometimes difficult to interpret due to problems in standardization of blood samples. Another concern is that clinical specimens usually render a small number of positive cultures for a specific organism such as *Candida albicans*, and this has statistical implications. Furthermore, in the majority of the studies, the quantification of microorganisms in the sample was not performed (3, 19, 26).

The methodology applied in this study allowed the use of equal volumes of blood in each method as well as inoculation of a previously established number of cells. The number of cells tested was related to those more frequently associated with candidemia (14, 26). Counts of 100 and 10 cells/ml of blood were used to correspond to the counts observed in patients with infections related to catheters. Suspensions of 1 and 0.5 cells/ml were used to resemble patients with candidemia related to other sources of infection (27).

Our results showed that it was possible to detect *Candida albicans* in blood, regardless both the number of cells and the methodology. In fact, the amount of colonies on agar chocolate plates in each dilution was comparable, despite the method employed (Table 1).

Table 1 - Growth on chocolate agar plates inoculated with sediment after concentration by Isolator™ and with conventional methods (broth and biphasic) previously incubated for 24 h.

Number of cells of *Candida albicans*	Method	Growth
100 cells/ml of blood	Concentration	P +++
	Conventional	P +++
10 cells/ml of blood	Concentration	P +++
	Conventional	P +++
1 cell/ml of blood	Concentration	P ++
	Conventional	P ++
0.5 cells/ml of blood	Concentration	P +
	Conventional	P +

P = positive results; +++ / ++ = confluent growth; + = uncountable colonies.

In order to determine the time required by each method to obtain positive results, we compared observations made in the first 24 h of test, i.e., presence of blastoconidia in Gram stain prepared from the conventional method with growth on solid media from the Isolator™. Blastoconidia were observed in all Gram stains and the number of yeast seen by microscopy was directly related to the original number of cells in the sample (Fig. 2).

According to our results, it appears reasonable to conclude that the concentration method alone did not

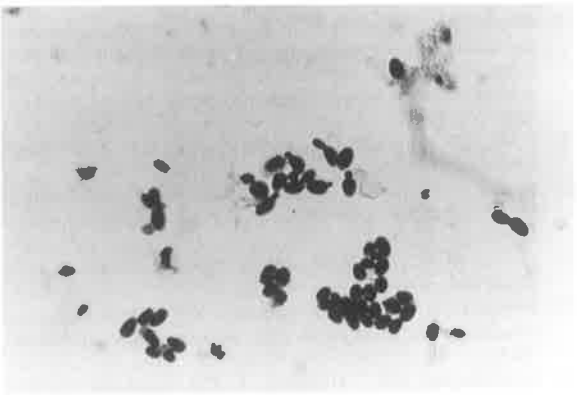

Figure 2 - Blastoconidia in Gram stain prepared from conventional methods after incubation for 24 h at 35°C. Magnification, X 1000.

reduce the time required for diagnosis, since positive results were obtained 24 h after inoculation of *Candida albicans* in both conventional and concentration methods. The mentioned advantage of Isolator™ in clinical reports may, therefore, be explained by the process of lysis which liberates microorganisms from phagocytes, increasing the number of viable yeasts (19, 35).

We suggest that the conventional method of growth in BHI for 24 h, either in liquid or biphasic media, and the concentration method by Isolator™ display comparable ability for the recovery of yeasts from blood.

Although the process of concentration appears not to improve the recovery of *Candida albicans* from blood, other advantages of the Isolator™ system deserve consideration. Quantitative results, supplied by Isolator™, although unable to determine clinical importance of the microorganism, may be important to determine the source of the infection and to follow therapy (26, 34). It has to be considered, however, that quantitative results are difficult to interpret because the method is not reproducible, and routine variation in the concentration of yeasts in blood during the course of infection is not well delimited (34). Another advantage of Isolator™ is that direct inoculation of sediment onto agar plates would allow earlier isolation of colonies and a quicker identification (14). This, however, seems not to be so relevant as one can also obtain diagnosis of candidemia in the first 24 h through the visualization of blastoconidia directly in the broth of the conventional method, as we have demonstrated in this study. Furthermore, identification and sensitivity tests can be done directly from broth (10).

Since concentration alone seems not improve the recovery of *Candida albicans*, the reported superiority of Isolator™ may be explained by lysis. Studies using conventional broth blood culture, supplemented with a lytic agent are therefore warranted. This will certainly be an enormous improvement for laboratories that use conventional methods.

RESUMO

Processo de concentração do sangue na detecção de *Candida albicans*

O sistema de lise-centrifugação (Isolator ™) tem sido considerado como método padrão para aumentar as taxas de diagnóstico de candidemia através de hemocultura. Neste estudo, o processo de concentração, segundo este sistema, foi comparado com métodos convencionais de cultivo em meio líquido e bifásico. Foram realizados testes "*in vitro*" utilizando *Candida albicans* em contagens de 100, 10, 1 e 0,5 células/ml de sangue. Culturas em ágar-chocolate foram realizadas a partir do sedimento obtido pela centrifugação do tubo de Isolator ™ e do caldo das culturas convencionais após sua incubação por 24 horas a 35°C. Esfregaços corados pelo Gram, preparados a partir dos métodos convencionais, também foram observados em 24 horas. Foi possível detectar *Candida albicans* independentemente do número de células ou da metodologia utilizada. O tempo para diagnóstico também não foi diferente para os métodos comparados, já que blastoconídios e crescimento foram observados no mesmo prazo de tempo. Assim, sugerimos que o processo de concentração não é o maior fator responsável pelas taxas de recuperação de *Candida albicans* a partir do sangue obtidas pelo sistema Isolator™.

Palavras-chave: candidemia, hemocultura, sistema de lise-centrifugação.

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PREVALENCE OF *H. PYLORI* INFECTION IN A POPULATION FROM THE RURAL AREA OF ARAÇUAÍ, MG, BRAZIL

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SHORT COMMUNICATION

ABSTRACT

The prevalence of *Helicobacter pylori* infection was evaluated by ELISA in a rural population in Minas Gerais, Brazil. A total of 114 among 131 adults (87.0%) and 54 among 87 children (62.0%) presented anti-*H. pylori* antibodies and the prevalence of the infection increased with age ($p < 0.001$).

Key words: *H. pylori*-epidemiology; *H. pylori*-seroprevalence; *H. pylori*-socioeconomic level

Helicobacter pylori is a spiral-shaped microaerophilic Gram-negative bacterium that colonises the gastric mucosa of human beings. The microorganism is the major agent of gastritis and plays an important role in the pathogenesis of peptic ulcer and gastric cancer (3).

Current data suggest that the overall prevalence of *H. pylori* infection is higher both in developing countries and in lower socioeconomic groups in the developed world (1). Probably these populations are exposed to conditions that favour the acquisition of the microorganism such as precarious hygiene, crowded household conditions and deficient sanitation.

The aim of this study was to determine the seroprevalence of *H. pylori* infection in a rural population living in the municipality of Araçuaí, Minas Gerais, and to evaluate the possible risk factors linked to the acquisition of the microorganism.

Araçuaí is a municipality with 33,798 inhabitants (2) located in the Jequitinhonha Valley, Northeastern region of the State of Minas Gerais, distant 680 km from the state capital. The rural population (n = 17,257) lives on subsistence agriculture and animal husbandry. The region is considered one of the poorest in Brazil.

The present study was approved by the Ethics Committee of the University Hospital, Federal University of Minas Gerais, Brazil, and informed consent to participate in the study was obtained from the children and from their parents or persons responsible and from the adults.

Serum samples were collected from 218 patients: 131 adults (115 females and 16 males; mean age 37.0

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yrs., range 20 to 78 yrs.) and 87 children and teenagers (53 girls and 34 boys; mean age 13.7 yrs., range 10 to 19 yrs.) residing in the rural area of Araçuaí and attending outpatient clinics linked to the University Hospital.

Data concerning age, sex, use of medication, water supply, sewage, number of rooms in the household, number of persons residing in each dwelling and monthly family income were obtained during interviews with the adults and with parents or persons responsible for the children.

Serum samples were assayed for anti-*H. pylori* IgG using the Cobas Core anti-*H. pylori* EIA (Roche Diagnostic Systems, Switzerland) which had been previously validated for Brazilian adults (7) and children over 10 years old (6) in terms of culture, preformed urease test and stained smear, showing 95.4% and 95.2% sensitivity and 100% and 100% specificity, respectively.

Categorical variables were analysed using the Chi-square test with Yates' correction or the two-tailed Fisher exact test. The linear tendency of the proportions of positivity to anti-*H. pylori* antibodies in different categories of exposure was analysed by the Chi-square test for trends. The level of significance was set at $p < 0.05$.

The annual family income was less than U$ 3,600.00 for 92.4% of the patients studied. The population used untreated water for drinking and cooking and lived in dwellings with no sewer network. The index of crowding (SD), defined by dividing the number of people living in the household by the number of rooms (except bathroom and kitchen), was 1.9 (1.4).

A total of 114 adults (87.0%) and 54 (62.0%) children and teenagers presented anti-*H. pylori* antibodies with no significant difference in the prevalence of the infection between adult males and females ($p = 1.0$) or between boys and girls ($p = 0.8$).

The prevalence of *H. pylori* infection increased significantly (Chi-square for trend = 15.9, $p < 0.001$) with age (Table 1). No association was observed between the rate of infection and annual family income ($p = 0.8$) or between prevalence and crowding ($p = 0.3$).

The results of the present study demonstrate that adults, children and teenagers attending outpatient clinics in a rural area of Araçuaí presented high rates of *H. pylori* infection. It should be noted that the population studied lived in overcrowded homes and had very poor environmental sanitation and water supply, conditions that have been considered important determinants in the acquisition of the microorganism. Similar results were observed by us in a population of the rural area of the State of Mato Grosso with sociodemographic data very similar to those of the present population (8). However, the prevalence of the infection among children was higher than observed in a study of children of low socioeconomic level residing in overcrowded homes in the urban area of Belo Horizonte, Minas Gerais (5). It is likely that the contaminated environment, lack of proper sanitation, and lack of sufficient clean water may explain the difference observed in the prevalence of *H. pylori* infection between these populations since the children of Belo Horizonte used treated city water and had their dwellings served by a sewer network. Other factors that were not investigated in the present study may also account for these differences.

Table 1 - Seroprevalence of *H. pylori* infection by age in 218 subjects from Araçuaí, Minas Gerais.

Age (yr) range	Number	Seropositivity (%)	Odds ratio	CI 95%
10 - 19	87	54 (62.0)	1.00	-
20 - 29	49	40 (81.6)	2.72	1.09 - 6.90
30 - 39	37	35 (94.5)	10.69	2.43 - 96.30
40 - 49	20	18 (90.0)	5.50	1.18 - 51.29
> 50	25	21 (84.0)	3.21	0.95 - 13.88

CI, confidence interval; $p < 0.001$

It should be pointed out that most adults studied were females. The reason for this fact is that the serum samples were collected from a predominantly female population of adults attending outpatient clinics. However, several studies have demonstrated that there is no difference in prevalence of *H. pylori* infection between males and females which allows to extrapolate these data to the general population.

The increase in the prevalence of the infection with age observed in the present study is considered by some investigators to be due to a continuous risk of infection throughout life and by others to be due to a cohort effect. It is also possible that, as suggested by Mitchell *et al.* (4), there are probably two major periods of acquisition of the microorganism in developing countries: the first one in childhood when the chance of infection is quite elevated and the second in adulthood with a small but constant chance of infection.

In conclusion, the prevalence of *H. pylori*

infection in a rural area of Araçuaí is very high and similar to that observed in other poor regions of Brazil and in other developing countries

The high prevalence of *H. pylori* infection observed in the present study probably reflects the precarious hygiene, the crowded living conditions and the absence of sanitation of this population.

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RESUMO

Prevalência de infecção por *H. pylori* em uma população da área rural de Araçuaí, MG, Brasil

A prevalência da infecção por *Helicobacter pylori* foi avaliada em uma população rural de Minas Gerais, Brasil, empregando uma reação de ELISA. Cento e quatorze de 131 adultos (87,0%) e 54 de 87 crianças (62,0%) apresentaram anticorpos anti-*H. pylori*. Foi observado aumento da prevalência da infecção com a idade ($p < 0,001$).

Palavras-chave: *H. pylori*-epidemiologia; *H. pylori*-prevalência; *H. pylori*-nível sócio-econômico

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CATABOLISM OF CAFFEINE AND PURIFICATION OF A XANTHINE OXIDASE RESPONSIBLE FOR METHYLURIC ACIDS PRODUCTION IN *PSEUDOMONAS PUTIDA* L

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ABSTRACT

Caffeine catabolism and a xanthine oxidase involved in the alkaloid breakdown were studied in *Pseudomonas putida* L, a strain displaying high ability to grow on this substrate. Cells cultured with unlabelled caffeine and ^{14}C labeled caffeine and xanthine showed that this alkaloid was broken-down via theobromine/paraxanthine -> 7-methylxanthine -> xanthine -> uric acid -> allantoin -> allantoic acid. Methyluric acids were formed from the oxidation of theobromine, paraxanthine and 7-methylxanthine, although no bacterial growth was observed on these compounds, indicating that this might be due to a wide substrate specificity of xanthine oxidase. This was confirmed by activity staining in PAGE where activity was observed with theophylline and 3-methylxanthine, which are not involved in the alkaloid breakdown. A single band of activity was detected without addition of NAD^+, showing an oxidase form of the enzyme. The enzyme optimum temperature and pH were 30°C and 7.0, respectively. The determined K_m was 169 µM, and the pI 3.1 - 4.0. The molecular weight determined by side by side comparison of activity staining of the enzyme in PAGE and PAGE of BSA was 192 kDa, which was coincident with the sum (198.4 kDa) of three subunits (71, 65.6 and 61.8 kDa) of the purified protein.

Key- words: caffeine, methylpurines, methyluric acids, *Pseudomonas*, xanthine oxidase.

INTRODUCTION

Woolfolk (15) was the first to investigate caffeine degradation by *Pseudomonas putida*. A previous comprehensive review on the degradation of purine and structure-related compounds by microorganisms (14) did not indicate any other report. However, it is worth mentioning that Dikstein *et al.* (5) observed that 3-methylxanthine, but not 1-methylxanthine, was dehydrogenated to the corresponding methyluric acid in protein preparations from fluorescent *Pseudomonas*.

Woolfolk (15) suggested that caffeine was hydrolytically *N*-demethylated, with the production of free xanthine and methanol. The alcohol was further oxidized to CO_2 and xanthine to uric acid. Dimethylxanthines and monomethylxanthines would be also converted to their respective methyluric acids.

The metabolism of caffeine by a *P. putida* strain was investigated by Blecher and Lingens (4). The

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bacteria was isolated in a complex medium, containing different sources of carbon and nitrogen, supplemented with the alkaloid. The complete pathway for caffeine degradation was established (Fig. 1) by culturing the bacteria with several substrates in mineral salts medium (without any nitrogen or carbon source) and the subsequent identification of the products released in the culture media. It was also shown that instead of methanol, the methyl groups were splitted off as formaldehyde. A similar pathway was also observed to occur in *Serratia marcescens* isolated from soil cultivated with coffee (9).

Ten years latter this subject was retaken by Glück and Lingens (6), but now focussed on the production of demethylated caffeine derivatives using the same *P. putida* isolated by Blecher and Lingens (4). Similarly, Asano *et al.* (1) isolated a *P. putida* strain that, cultivated in the presence of Zn^{2+}, accumulated theobromine formed from caffeine, with a yield of 92 %.

The *Pseudomonas* strains used by Woolfolk (15), Blecher and Lingens (4) and Asano *et al.* (1) in their studies were also isolated after several transfers in complex media suplemented with caffeine. Middelhoven and Lommen (10) also used the same procedure to isolate a *P. putida* which grew in mineral salts medium supplemented with 5 g/l of caffeine. Recently we have isolated a *P. putida* strain from soil collected under coffee trees, without such long term enrichment, suggesting its adaptation to continuous exposure to the caffeine released from the coffee trees (18). Our strain could grow in mineral salts liquid and solid media, containing 20 g/l and 50 g/l of caffeine.

Since this strain showed an considerable higher ability to grow on caffeine than previous reports in the literature, the aim of this work was to study the pathway for the alkaloid degradation, and the characterization and purification of the xanthine oxidase responsible for the oxidation of methylxanthines and xanthine produced during the alkaloid demethylation. To our knowledge, this enzyme has never been studied in caffeine degrading microorganisms.

MATERIALS AND METHODS

Bacteria and chemicals - All chemicals and solvents were purchased from Sigma or Merck. [^{14}C-2]Caffeine was produced by methylation of [^{14}C-2]xanthine (1.94 Gbq/mmol, Sigma St. Louis, USA) (7). *P. putida* strain L isolated by Yamaoka-Yano and Mazzafera (18) was used in this study. The bacteria was stored in skin-milk at -20°C and, when used, it was plated on solid M9 medium (12) with caffeine (0.3 g/l) as the only source of carbon and nitrogen (M9-CAF). The plates were kept at 28°C for optimum growth.

Detection and distribution of caffeine catabolites and proposed pathway of caffeine degradation - The starter culture was produced growing the bacteria in liquid M9-CAF (1 g/l) for 18 h at 28°C. Two hundred microliters of this starter culture was used to inoculate Eppendorf tubes containing 800 µl of M9-CAF (0.3 g/l) and 25 kBq of [^{14}C-2]caffeine or 29 kBq of [^{14}C-2]xanthine. Other set of Eppendorf tubes contained the same mixture, except that labeled caffeine was replaced by unlabeled at 1 g/l concentration. The tubes were held horizontally in an orbital shaker (200 rpm, 28°C). For those tubes where only unlabeled caffeine was present, aliquots were taken after 9, 12, 27, 33, 36 and 48 h of incubation. When labeled compounds were added, only one sample was taken after 27 h. The cells were harvested by centrifugation at full speed in a bench-top centrifuge (Eppendorf, model 5415C) and the supernatants kept at -20°C until analysis. The compounds released in the medium were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detection (270 nm), as described by Berthou *et al.* (3), using a Pharmacia HPLC system. For the assay with labeled caffeine 5 µg of each methylxanthines (7-methylxanthine, 3-methylxanthine, 1-methylxanthine, 3,7-dimethylxanthine - theobromine, 1,3-dimethylxanthine - theophylline, 3,7-dimethylxanthine - paraxanthine and 1,3,7-trimethylxanthine - caffeine), methyluric acids (7-methyluric acid, 3,7-dimethyluric acid, 1,3-dimethyluric acid, 1,7-dimethyluric acid), xanthine and uric acid were added to the samples and the peaks were collected directly in scintillation vials, dried at 60°C and the radioactivity determined after addition of scintillation fluid. Alternatively, for some samples assayed with labeled caffeine, the detection was performed in a radioactivity monitor (model 1208 Betacord, Reeve Analytical, Glasgow, UK). In the assays with labeled xanthine, the compounds were separated by RP-HPLC with the same column indicated above, using 0.5 % aqueous acetic acid (V/V) as isocratic solvent at a flow rate of 1 ml/min. Internal standards (5 µg) of allantoic acid, allantoin, xanthine and uric acid were added to the samples. In

this case two runs were carried out, the first for the detection of xanthine and uric acid (270 nm) and the second for allantoin and allantoic acid (210 nm). The peaks were collected, dried and the radioactivity determined after addition of scintillation fluid.

Characterization and determination of kinetic parameters of xanthine oxidase - Crude protein extracts were used for the characterization and determination of the kinetic parameters. Proteins were extracted from cells of 12 h cultures grown in Luria Broth medium containing 1 g/l caffeine. The cells were pelleted by centrifugation (4,400 X g / 10 min, 4°C), resuspended in a small volume of 25 mM Na-phosphate buffer, pH 7.5, containing 10 mM β-mercaptoethanol. The cells were disrupted by sonication (5 x 15 sec, with 2 min intervals on ice) and the debris eliminated by centrifugation (28,200 X g / 20 min, 4°C). The supernatant was filtered on PD-10 G-25 Sephadex minicolumns (Pharmacia), using 25 mM Na-phosphate buffer pH 7.5 containing 10% glycerol for elution, and the protein concentration was determined with a ready-to-use BioRad protein reagent. Increasing amounts of protein (20 - 200 µg) were separated by native discontinuous polyacrylamide electrophoresis (PAGE) with 5% and 10 % of polyacrylamide in the stacking and in the main gel, respectively, and incubated with or without addition of NAD^+ (13), for determination of its dependence. Alternatively, in order to detect NADH-diaphorase activity, NADH was included in the incubation buffer. As a blank, one lane was not loaded with protein. A single blue band, formed by the reduction of nitrobluetetrazolium salt, appearing only in the gel that was not incubated with NAD^+ or NADH, characterized the enzyme as a xanthine oxidase (E.C. 1.2.3.2, type O enzyme), here on referred to as XO.

The substrate specificity was determined by PAGE as above. The activity was tested with caffeine, theobromine, theophylline, paraxanthine, monomethylxanthines (1-, 3- and 7-methylxanthine) and hypoxanthine. In some cases the gels were left to develop for several hours.

The best protein concentration for *in vitro* activity was obtained in a reaction mixture containing protein, obtained as above, 150 µM of xanthine and 1.1 kBq of [^{14}C-2]xanthine, with the final volume adjusted to 200 µl with 25 mM Na-phosphate buffer, pH 7.5. The incubation proceeded by 30 min at 30°C in Eppendorf tubes. The reaction was stopped by addition of 5 µl of 1 M zinc acetate and the supernatant recovered after centrifugation in a bench-top centrifuge was analyzed by RP-HPLC for the production of labeled uric acid. This compound was separated by RP-HPLC with the same column indicated above, using 0.5 % aqueous acetic acid (v/v) as isocratic solvent at a flow rate of 1 ml/min. Internal standard (5 µg) was added to the samples and the corresponding peak at 270 nm was collected, dried and the radioactivity determined after addition of scintillation fluid.

The optimum reaction time, temperature and pH were determined in *in vitro* assays as described above. The K_m for xanthine was determined in a Lineweaver-Burk plot with data from *in vitro* assays where xanthine was added at different concentrations.

The molecular weight of XO was determined by PAGE. One lane was loaded with the bacterial protein extract and the other with bovine serum albumin (BSA). After electrophoresis, the gel was cut in two halves. The one with the BSA was stained with Coomassie Blue GR-250 and the other was used for XO activity (13). The first gel had its size restored to the size of the gel for enzyme activity by addition of water in the destaining solvent, and by side-by-side comparison of the gels it was possible to determine the Rf of XO and compare it with those of BSA.

The isoelectric point of XO was determined by chromatography focusing of crude protein extracts, as described in the next item, and by electrofocusing using a Rotofor electrophoresis (BioRad). The fractions collected from the Rotofor had the pH measured and XO activity detected in PAGE, after increasing the pH of the fractions to near 6 with 250 mM Tris-HCl, pH 7.

Purification of XO - XO was purified by liquid chromatography and UV detection (280 nm) in a Pharmacia HPLC system. During all the purification steps, the activity of XO was tested in PAGE, since several fractions could be rapidly assayed at the same time. In most of the cases the activity reaction was developed after the running front (bromophenol blue) had run half way along the main gel. The purification was carried out at 4°C and all buffers used contained 10 % glycerol.

Proteins were extracted from the pellet obtained from 25 L culture growing in 1:1 LB/M9-CAF (1 g/l) medium for 48 h. The extraction buffer was the same used for the characterization and kinetic parameters. The crude extract was desalted in Sephadex G-25 columns (70 ml bed volume) and the proteins separated in an anionic exchanger column (DE-52, Beckman, 3 cm x 12 cm), using a gradient of 0 - 0.5 M NaCl in 25 mM Na-phosphate buffer, pH 7, at a

flow rate of 3 ml/min was carried out in 60 min. Three minutes (9 ml) fractions were collected, the protein concentration determined with the BioRad reagent and assayed for XO activity in PAGE. The fractions showing the highest activities were pooled, desalted on Sephadex G-25 and the proteins separated in a Mono Q HR5/5 column (Pharmacia), using a gradient of 0 - 0.5 M NaCl in 25 mM Na-phosphate buffer, pH 7, at a flow rate of 0.75 ml/min, developed in 40 min. Fractions were collected every 2 min (1.5 ml) and after determination of protein concentration they were assayed for XO in PAGE. The fractions with the highest activities were pooled, desalted and the proteins separated in a Mono P HR 5/5 column (Pharmacia). The proteins were eluted from the column with a 30 min pH gradient from 7.1 to 4 using Polybuffer PBE74 (LKB-Pharmacia). Two-minute fractions (1.5 ml) were collected for additional 20 min after the end of the gradient. The pH and the protein concentration of each fraction were determined and XO assayed by PAGE. The same fraction was subject again to PAGE using larger combs. Part of the gel was stained with Coomassie Blue GR250 (30 min of staining at 30°C and 2 x 15 min destaining) and the other half for XO activity. Despite the presence of other proteins, by side-by-side comparison of the gels it was possible to see a distinct band in the Coomassie Blue stained gel. This was excised from the gel, transferred to an Eppendorf tube and ground with a rod after addition of 100 µl of 50 mM Tris-HCl buffer, pH 7.9, containing 200 mM NaCl. The tube was left shaking at 4°C for 48 h, and the polyacrylamide residues removed by centrifugation in a 0.22 µm Amicon Micropure separator. An aliquot of the eluted protein was subjected to RP-HPLC, in order to verify its purity. The protein was separated in a C_{18} Bio-Sil ODS column (Bio-Rad, 250 mm x 4.6 mm, 5µm) with UV detection at 220 nm. Solvent A was aqueous 0.1% trifluoroacetic acid and solvent B was 30% of solvent A in acetonitrile, and the gradient was 0 - 100% of solvent B in 60 min. The remaining protein eluted from the PAGE gel was subjected to denaturing electrophoresis (SDS-PAGE, 10 % polyacrylamide in the main gel) and stained with $AgNO_3$ (11).

The XO purification was repeated twice, with identical results.

RESULTS

Caffeine degradation pathway - The results of the experiment where cells were cultivated only with unlabeled caffeine (1 g/l) are shown in Table 1. After 9 h cultivation 20 % of the caffeine had been degraded, showing the high ability of the *P. putida* L to use this alkaloid as the only source of C and N. After 33 h very few caffeine was detected in the medium. A transient increase was observed for the other compounds detected by RP-HPLC. It is interesting to observe that highest concentrations of paraxanthine and theobromine, which are the first degradation products of caffeine according to Blecher and Lingens (4), were observed at 12 h of cultivation. This occurred later (27 h) for 7-methylxanthine, 7-methyluric acid and xanthine, suggesting that they are formed from demethylation and oxidation of the dimethylxanthines. The monomethyluric acid remained at the same concentration after 27 h. Even by increasing the sensitivity of the UV monitor or injecting larger samples in the HPLC we were not able to detect other caffeine derivatives.

Table 2 shows the results when labeled and unlabeled caffeine were included in the culture medium. We choose 27 h of culture here taking into account the results obtained in the previous experiment. Until the elution of uric acid, the eluate

Table 1. Caffeine and other methylxanthine concentrations (mg/l) in the culture media supplemented with 1g/l of caffeine.

	Incubation time					
	9 h	12 h	27 h	33 h	36 h	48 h
Xanthine	0.18	0.24	0.34	0.19	0.09	nd*
7-Methylxanthine	4.04	25.00	52.13	0.11	nd	nd
7-Methyluric acid	0.96	3.84	7.84	9.11	8.98	9.06
Theobromine	43.82	81.00	72.43	nd	nd	nd
Paraxanthine	0.86	1.68	1.59	nd	nd	nd
Caffeine	792.59	634.22	238.13	0.26	nd	nd

* nd = not detected

from the column was collected and the radioactivity also determined (solvent front), probably representing the compounds derived from uric acid degradation. The data presented in Table 2 show the percent recovery of the total radioactivity detected in the fractions collected from the column, on average 59.7% of the radioactivity given (25 kBq). This difference may probably be due to the degradation of caffeine to CO_2. Compounds that were not detected in the assays with unlabeled caffeine could be observed here, such as 3,7- and 1,7-dimethyluric acids, formed by the oxidation of theobromine and paraxanthine, respectively. Using a radioactivity monitor, whose sensitivity was above 0.02 kBq, it was not possible to detect any other peak of radioactivity.

Table 2. Percent distribution of radioactivity in caffeine/xanthine metabolites in the culture media supplemented with labeled caffeine/xanthine and unlabeled caffeine. Means of two independent experiments are presented.

	Radioactivity Distribution (%)
	Labeled caffeine
Solvent front	93.7 ± 1.00
Uric acid	4.90 ± 0.81
Xanthine	0.20 ± 0.02
7MU*	0.85 ± 0.14
7MX	0.20 ± 0.05
3,7DMU	0.06 ± 0.01
1,7DMU	0.10 ± 0.01
Theobromine	0.04 ± 0.00
	Labeled xanthine
Solvent front	99.6 ± 0.05
Allantoin	0.12 ± 0.00
Allantoic acid	0.20 ± 0.05
Uric acid	0.09 ± 0.01
Xanthine	0.05 ± 0.01

* = 7-methyluric acid = 7MU, 7-methylxanthine = 7MX, 3,7-dimethyluric acid = 3,7DMU, 1,7-dimethyluric acid = 1,7DMU. ± = Standard error

When labeled xanthine was included in the media (Table 2) we could detect radioactivity in xanthine, uric acid, allantoin and allantoic acid. The data presented in Table 2 were calculated as previously for labeled caffeine. The total radioactivity collected was 71.0 % of the radioactivity given (29 kBq). Higher radioactivities were found in the ureides allantoin and allantoic acid, confirming at least up to this point that the degradation pathway in our *P. putida* strain is similar to that found by Blecher and Lingens (4) in their studies.

Characterization and kinetic parameters of XO - The enzyme responsible for xanthine oxidation was established as an oxidase. Substrate specificity was tested in PAGE and, besides the natural substrates xanthine and hypoxanthine, bands of activity in the same position in the gels were observed only for 3-methylxanthine and theophylline (data not shown), which are not involved in the caffeine degradation pathway. Whereas 10 - 15 min were needed for the appearance of the bands with xanthine and hypoxanthine, almost 1 h was necessary with 3-methylxanthine and 3 h with theophylline. For the latter, a very weak band was observed.

XO activity was linear between 20 and 60 min. The optimum protein concentration for *in vitro* assays, temperature and pH were, respectively, 14 - 28 µg protein, 30°C and pH 7.0. Good activities were also observed with pH 8.0. Based on these results all further assays were carried out with 30 µg of protein, 30 min, 30°C and Na-phosphate buffer, pH 7.0.

The K_m determined for XO was 169 µM, and the apparent molecular weight, determined by PAGE, was 192 kDa (Fig. 3C). The pI of XO determined in the Rotofor and Mono P column were 3.1 and 4, respectively.

Purification of XO - XO was purified after two anionic (DE52 and Mono Q) and one chromatofocusing columns (Fig. 2A-C). More intense XO activity was found in the first column in three fractions, between 0.35 and 0.41 M of the NaCl gradient (Fig. 2A). These fractions were applied in the Mono Q column (Fig. 2B), most of the activity being eluted from this column in two fractions, between 0.35 and 0.37 M NaCl. These fractions were loaded in the Mono P column and only one fraction from this column, corresponding to pH 4, showed XO activity (Fig. 2C). This fraction was submitted again to PAGE with a continuous comb and half of the gel was stained with Coomassie Blue GR250 and the other half for XO activity. Side-by-side comparison of the gels revealed a distinct band in the Coomassie Blue stained gel (Fig. 3A). This band was eluted from the gel and an aliquot of the eluted protein was subjected to RP-HPLC, a very sensitive technique, in order to evaluate its purity. The Fig. 4 shows that a single peak was detected, indicating that elution from the PAGE gel yielded a pure protein. The eluted protein was also subjected to SDS-PAGE with silver staining (Fig. 3B). We found three subunits (71, 65.6 and 61.8 kDa), whose sum (198.4 kDa) is in agreement with the molecular weight estimated by PAGE (192 kDa).

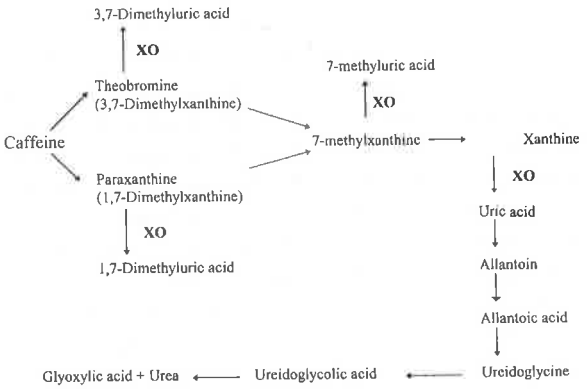
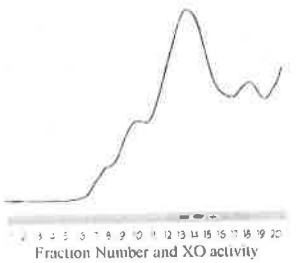
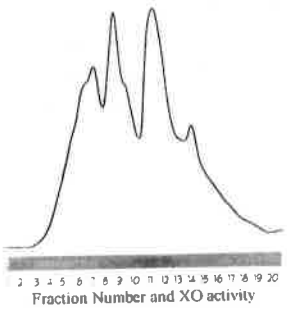


Figure 1. Caffeine degradation pathway in *Pseudomonas putida*. XO indicates the reactions mediated by xanthine oxidase.





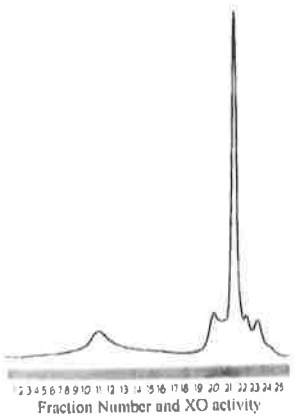
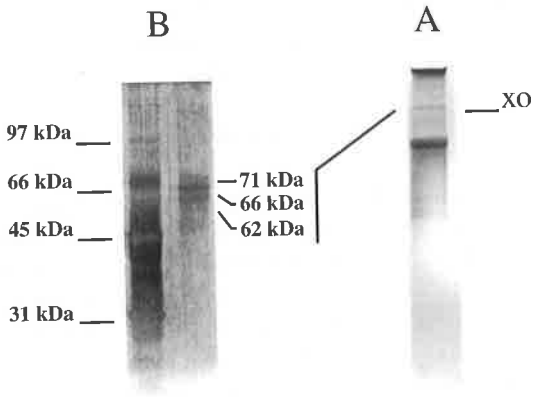


Figure 2. Protein elution profiles from chromatographies in DEAE-cellulose (A), Mono Q (B) and Mono P (C) columns. XO activity in the fractions was detected in PAGE and they are shown at the bottom of each chromatography.



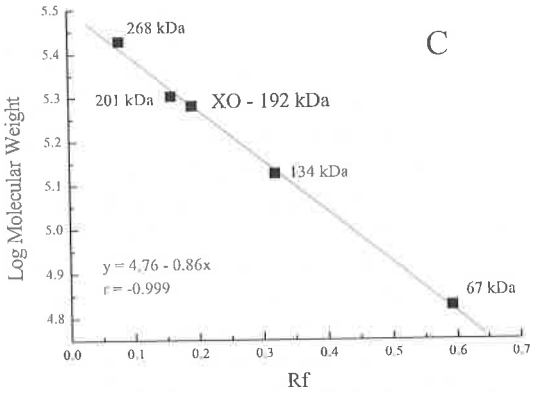


Figure 3. Coomassie blue protein profile staining of PAGE of the fraction from the Mono P column, where a band corresponded to the band of activitity of a parallel active staining gel (A), SDS-PAGE of the eluted band from the coomassie blue stained PAGE gel (B), and M_r determination on PAGE gel using paralleled gels for active staining and coomassie blue staining of BSA non denatured (C). Molecular markers are indicated in the left lane of B.

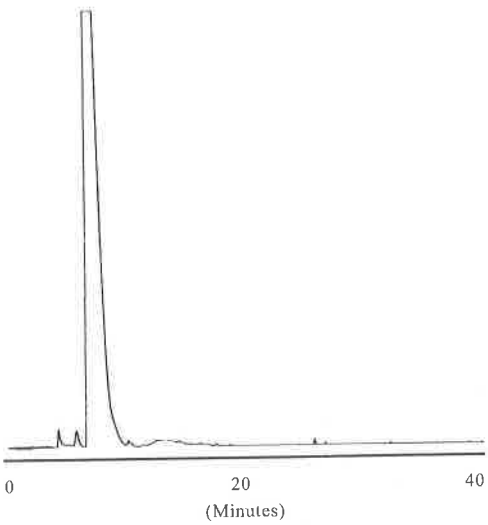


Figure 4. Reversed-phase high-performance liquid chromatography of XO eluted from the PAGE gel.

Although an accurate native molecular weight can not be determined by one polyacrylamide concentration the data obtained are very similar.

DISCUSSION

Previous studies carried out with induced mutants for caffeine degradation showed that dimethylxanthines and monomethylxanthines, formed by sequential caffeine demethylation, might be oxidized to their respective methyluric acids (4), as seen in Fig. 1. Recently, we have isolated *P. putida* strains displaying a very high ability to degrade caffeine (18) and experiments carried out here with the strain L, with non labeled and labeled caffeine, showed the same caffeine catabolic pathway. Therefore, the higher ability of our strain to grow on very high caffeine concentrations is not related to an alternative catabolic route but probably due to its own metabolic efficiency in breaking down the alkaloid.

Blecher and Lingens (4) observed that during the caffeine degradation, very small amounts of methyluric acids were released in the medium (3 - 5 mg/l for 7-methyluric acid, 1 mg/l for 3,7-dimethyluric acid). For the detection of 1,7-dimethyluric acid, it was necessary to grow the bacteria in medium containing 1,7-dimethylxanthine as the sole source of carbon and nitrogen, instead of caffeine. Even using this approach, they still obtained an amount enough only for thin layer chromatography identification by co-chromatography with an authentic sample. The other methyluric acids were characterized additionally by infrared and mass spectroscopy. In the three cases they started from 1 to 10 L culture media.

Here, using more sophisticated techniques (HPLC and labeled caffeine), which enabled us to work with low volumes of media, we could also show that, in addition to the similar caffeine catabolic pathway, the *P. putida* L also accumulated more 7-methyluric acid than 3,7-dimethyluric and 1,7-dimethyluric acid, which was not further degraded. Similar to the results of Blecher and Lingens (4), there was no bacterial growth and decrease in concentration when these methyluric acids were separately included in media culture as sole sources of carbon and nitrogen, indicating the high substrate specificity of uricase. However, our data from growing *P. putida* L with labeled caffeine showed higher radioactivity in 1,7-dimethyluric acid than 3,7-dimethyluric acid.

Xanthine oxidase have a wide substrate specificity. Active staining of PAGE electrophoresis showed that XO from *P. putida* L was able to oxidize xanthine, hypoxanthine, 3-methylxanthine and theophylline. Woolfolk (15) prepared cell-free extracts from xanthine and caffeine grown cells of induced mutants of *P. putida* for caffeine degradation and used them in a ferricyanide-linked dehydrogenases assay against several methylxanthines. For both extracts higher activities were observed with xanthine, 3-methylxanthine and 1-methylxanthine. Activities with 7-methylxanthine, theobromine, theophylline and paraxanthine were observed only with caffeine grown cell extracts, however, much lower than those previously mentioned methylxanthines. Although Woolfolk (15) had observed that molecular oxygen was essential for active methylxanthine degradation in cell suspension experiments, it is not possible to suggest an oxidase activity since further work (2,8) showed that different cytochrome P450 activities, an enzyme dependent of oxygen, were involved in the demethylation of caffeine, theobromine and 7-methylxanthine. However, studies with *S. marcescens*, which presented a very similar caffeine catabolic pathway with *P. putida*, showed xanthine dehydrogenase nonspecificity against several methylxanthines (9).

Our data show that for the *P. putida* strain L isolated by Yamaoka-Yano and Mazzafera (18) a xanthine oxidase is responsible for the oxidation of xanthine, and probably, methylxanthines, however with much less efficiency. Woolfolk and Downard (17) examined protein extracts from a diverse collection of xanthine-metabolizing bacteria with respect to their ability to oxidize xanthine, 1- and 3-methylxanthine in the presence of NAD or oxygen as electron acceptors. They found that among the gram-negative bacteria, *Pseudomonas* seemed to have two activities, a particulate oxidase and a soluble dehydrogenase, as already suggested by these authors in a previous work (15). In the particulated fraction an oxygen-dependent xanthine oxidase was detected, and in the soluble fraction a NAD-dependent and a ferricyanide-linked xanthine dehydrogenase were found. The particulate fraction was obtained from a second centrifugation at higher speed with the supernatant obtained from a first lower centrifugation force with sonicated cells. Interestingly, the particulate activity was found exclusively in cells grown with xanthine. All activities remained in the supernatant in extracts prepared from caffeine-grown cells during the preparation of the particles (15).

In a following work Woolfolk (16) separated these

two activities and purified a ferricyanide-linked xanthine dehydrogenase concluding that the apparent molecular weight of the enzyme was 255 kDa, a trimer/tetramer of 77 kda subunits. Active staining on PAGE of the enzyme showed two main bands, somewhat overlapping.

Here protein extracts were obtained from cells grown exclusively in caffeine. Therefore one might suggest that we were dealing with the three enzymes detected by Woolfolk in his studies. However, since NAD^+ was not needed for detection of activity in PAGE with active staining and in the *in vitro* assays, we probably have purified the oxygen-dependent enzyme form. Moreover, during the purification in the anionic (DE52 and Mono P) chromatofocusing columns we have always observed only one peak of activity, represented by a single sharp band in PAGE electrophoresis. In addition, a single protein peak was detected with RP-HPLC and the molecular weight determined was consistent with the sum of the three subunits detected by SDS-PAGE with silver staining.

The metabolic and physiological role of the enzyme purified is debatable. We did not observe any bacterial growth when methyluric acids were added as the sole source of carbon and nitrogen in the medium as well as the concentration of 7-methyluric acid was stable even after caffeine depletion of the medium. With respect with the other methyluric acids, low levels of radioactivity in 3,7- and 1,7-dimethyluric acid were observed in the assays with labeled caffeine, after 27 h of culture, suggesting a very low degradation rate or, supported by the lack of bacterial growth in their presence, accumulation in very small amounts. Consequently, we might presume that XO has a higher specificity for 7-methylxanthine among the methylxanthines whose methyluric acids detected here. Therefore, the formation of these compounds may be considered as a consequence of the wide substrate specificity of XO. This is reinforced by the fact that activity in PAGE gels were also observed with 3-methylxanthine and theophylline, which are not metabolites of caffeine degradation in *P. putida*, as observed here and by Blecher and Lingens (4).

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RESUMO

Catabolismo de cafeína e purificação de xantina oxidase responsável pela produção de ácidos metilúricos em *Pseudomonas putida* L

O catabolismo de cafeína e a enzima xantina oxidase, envolvida na sua degradação, foram estudados em *Pseudomonas putida* L, uma linhagem com alta capacidade para utilizar este substrato como fonte de energia. Células crescidas na presença de cafeína e xantina marcadas com ^{14}C, e cafeína não marcada, mostraram que este alcalóide foi degradado via teobromina/paraxantina -> 7-metilxantina -> xantina -> ácido úrico -> alantoína -> ácido alantóico. Ácidos metilúricos foram formados a partir de teobromina, paraxantina e 7-metilxantina, embora nenhum crescimento bacteriano ter sido observado quando estes compostos foram usados como substratos, indicando que a xantina oxidase possui um ampla especificidade para substratos. Isto foi confirmado por detecção de atividade em gel não desnaturante (PAGE), tendo sido observada atividade para teofilina e 3-metilxantina, que não estão envolvidas na degradação de cafeína. Uma única banda de atividade foi observada em (PAGE) quando NAD^+ não foi incluído no meio de incubação, indicando ser a enzima uma oxidase. A temperatura ótima e o pH ótimo da reação para a enzima foram 30°C e 7,0, respectivamente. O K_m determinado foi de 169 µM, e o pI 3.1 - 4.0. A massa molecular determinada através da comparação lado a lado de gel de atividade em PAGE e PAGE de padrão de albumina de soro bovino foi de 192 kDa, que foi coincidente com a soma (198,4 kDa) de três subunidades (71, 65,6 e 61,8 kDa) da enzima purificada.

Palavras-chave: ácidos metilúricos, cafeína, metilpurinas, *Pseudomonas*, xantina oxidase.

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CHARACTERIZATION OF FUSION PRODUCTS FROM PROTOPLASTS OF YEASTS AND THEIR SEGREGANTS BY ELECTROPHORETIC KARYOTYPING AND RAPD

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ABSTRACT

In order to characterize fusion products from yeast protoplasts and their segregants, with important features to the wine making industry, electrophoretic karyotyping and RAPD (Random Amplified Polymorphic DNA) were utilized. Electrophoretic karyotyping was performed by the CHEF ("contour-clamped homogeneous electric field electrophoresis") method, which allowed the detection of chromosomal band complementation in fusion products and the presence of patterns of both parental and intermediary strains in segregants. By utilizing two primers, an amplification pattern of DNA fragments was obtained. While fusion products (diploid) showed a pattern of complementary bands, segregants showed bands of either parental strains or even intermediary bands.

Key words: Protoplast fusion, electrophoretic karyotyping, RAPD.

INTRODUCTION

Industries, especially those that make use of yeasts in the production of alcoholic drinks, are very traditional, reflecting the conservative attitude that men, in general, have towards the nature of the food and beverage they produce. Besides that, industries of alcoholic drinks have shown recent innovations by introducing benefits from reengineering and genetic manipulation. Beer and wine making industries have faced many challenges over the past years, such as, improving yeast resistance to ethanol, temperature and carbon dioxide as well as eliminating or diminishing the production of compounds which interfere with the quality of beer and wine.

In order to obtain strains showing more suitable properties, genetic manipulation methods have been used. However, due to the aneuploid, diploid or polyploid nature of most strains used in beer and wine making, traditional crossing techniques have not been very successful. Thus, the use of new technologies was necessary, such as protoplast fusion and transformation. New genotypes were obtained by protoplast fusion, which showed recombinant features, while the transformed strains showed heterologous genes (17, 7, 13).

Other applications of genetic techniques are the identification, characterization and monitoring of strains used in the production of alcoholic drinks by electrophoretic karyotyping (10, 2, 20, 18, 4) and RAPD (Random Amplified Polymorphic DNA) markers (5).

Yeasts used for producing non-distilled drinks need additional properties, such as low levels of H_2S

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production (16, 7). H_2S is an undesired malodorous compound which seriously depreciates beer and wine quality (21). Flocculation, another interesting feature in strains used by beer and wine making industries, has recently been introduced by genetic changes in these microorganisms (19). This phenomenon has been detected in some yeast strains, which, under certain circumstances, tend to cluster and form flakes, which are deposited on the bottom of the fermenter.

The objective of this paper is, therefore, to genetically characterize parental strains used in protoplast fusion, fusion products (FP) and their segregants (S) by means of electrophoretic karyotyping and RAPD markers.

MATERIALS AND METHODS

Biological Material: for this study, two yeast strains (*Saccharomyces cerevisiae*) and hybrids obtained from protoplast fusion of these two strains and their recombinants were used. The strain identified as IZ 987, unable to produce H_2S, was provided by the laboratory at the *Departamento de Ciência e Tecnologia Agroindustrial (Department of Agroindustrial Science and Technology)* at the *Escola Superior de Agricultura "Luiz de Queiroz"* ("Luiz de Queiroz" Agriculture School) */USP* (University of São Paulo). The other, ABXR 11B, highly flocculent, was provided by the *Laboratório de Genética de Leveduras do Departamento de Genética da Escola Superior de Agricultura "Luiz de Queiroz"* (Yeast Genetic Laboratory, Department of Genetics, "Luiz de Queiroz" Agriculture School)/ *USP* (University of São Paulo).

Pulsed-field Electrophoresis for yeast chromosomal DNA separation: strains were incubated in Erlenmeyer flasks with 100 ml YEPD (2% glucose, 2% peptone, 1% yeast extract), at 28°C under constant agitation (150 rpm), to half-grown phase. Samples were harvested by centrifugation (10 minutes at 5000g) and washed three times with 0.05 M EDTA, pH 8.0. After washing, the cells were resuspended at a concentration of approximately $5x10^9$ cells/ml in EDTA (1). Cell suspension was heated at 42°C, and, then, combined with 1.4 % LGT (low gelling temperature) agarose (1:1 solution), and immediately transferred into plug moulds. For plugs solidification, moulds were refrigerated. After gel solidification, the plugs were transferred (with spatula) into tubes containing NDS (0.5 M EDTA, pH 5.8/ 10 mM Tris-HCl, pH 9.5/ Sodium N-lauroyLsarcosinate), proteinase K (1 mg/ml) and incubated at 50°C for approximately 15 hours (9).

Following incubation, the plugs were washed four times, the first three times with 0.05 M pH 8.0 EDTA, and the last one with TBE 0.5X running buffer. After the last washing, the plugs were stored in the same TBE 0.5X running buffer at 4°C. The samples of integral yeast DNA were applied in 1% agarose gel and sealed with same agarose. The gel was transferred into the CHEF-DR II (Bio Rad) Apparatus chamber, containing TBE 0.5X buffer, previously cooled at 14°C (14).

The chromosomal DNA separation run was carried out within 23 hours, at 200 V and at 14°C, the first 15 hours with 60-second pulses, and the remaining eight hours with 90-second pulses (15). At the end of the run, the gel was stained with ethidium bromide (10 µl/ml) for approximately 30 minutes and observed and photographed (Polaroid 6FP 67 or Fuji FP 699 film) in UV transilluminator. The chromosomal bands molecular weight of the strains studied and obtained was estimated by the diagram drawn by employing the molecular weight log (Mb) of the *Saccharomyces cerevisiae* pattern against the gel migration distance (cm).

Random Amplified Polymorphic DNA (RAPD): amplifications were conducted in a total volume of 25 µl containing 20 mM Tris-HCl, pH 8.4, 4.50 mM KCl, 3.75 mM $MgCl_2$, 100 µl of each deoxynucleotide (d NTP's), 30 ng primer oligonucleotide, 40 ng DNA and 1.5 U Taq DNA Polimerase (6). The experiment was conducted with OPB-12 and OPX-10 primers.

Pre-denaturation was conducted at 92°C for 2 minutes followed by 40 1-minute cycles at 92°C, 1 minute at 37°C and 2 minutes at 72°C, total time of 3 minutes at 72°C in MJ Research Inc. PTC-100™ thermocycler.

The products amplified were electrophoretically separated, in 1.3% agarose gel at 2.9 V/cm, for approximately 3 hours and 30 minutes. Lambda DNA cleaved with Hind III restriction enzyme was utilized as molecular weight marker. The gel was stained in ethidium bromide solution for 30 minutes and destained in water for further observation and photography (Polaroid 6FP 67 or Fuji FP 699 film) in UV transilluminator, with orange filter.

Yeast DNA Isolation for RAPD: isolation was conducted according to Johnston *et al.* (11), with the

exception of not utilising RNAse. DNA samples were quantified by spectrophotometer (BECKMAN DU 640 [λ 260]) and DNA integrity was analysed by DNA electrophoresis in 0.8% agarose gel for 1 ½ hour at 50 V. Following electrophoresis, gel was stained in ethidium bromide and observed in UV transilluminator.

Dendrograms: the data obtained by RAPD were analysed by NTSYS-PC (Applied Biostatistics, Inc.). This program produced a similarity matrix by means of a coefficient of similarity, and a dendrogram was devised based on the analysis of the matrices by the UPGMA SAHN-clustering method. This dendrogram grouped the strains showing the level of similarity among each other.

RESULTS AND DISCUSSION

Pulsed-field yeast chromosomal DNA separation: in order to verify the taxonomic relationship of parental strains submitted to protoplast fusion and to analyse fusion products and recombinants obtained, yeast was submitted to electrophoretic karyotyping.

Table 1 shows flocculation and H_2S production, characteristic of parental strains ABXR.11B and IZ 987, fusion products FP 67 and FP 70, and segregants S26, S15 and S18 obtained from them.

Table 1: Characteristics of strains selected for electrophoretic karyotyping and RAPD.

Strain	Remark	Flocculent	H_2S Production
ABXR.11B	Parental Strain 1	+	+
IZ 987	Parental Strain 2	-	-
FP 67	Fusion Product	+	-
FP 70	Fusion Product	-	+
S26	Segregant	+	-
S15	Segregant	-	-
S18	Segregant	+	+/-

(+) indicates presence of the studied character;
(-) indicates absence of the studied character.

Individualized bands do not necessarily represent a chromosome since chromosomes with same or close molecular weight can be contained in the same band. Thus, the term chromosomal band was used to designate the bands visualised. The electrophoretic patterns of parental strains ABXR.11B and IZ 987, fusion products FP 67 and FP 70, and segregants S26, S15 and S18 are shown in Fig. 1. Fig. 2 shows the schematic representation of the electrophoretic pattern of each strain. Table 2 contains the molecular weight of: parental strains ABXR.11B and IZ 987, fusion product FP 67 and segregant S15, obtained from the molecular weights of the pattern utilised (*S. cerevisiae*).

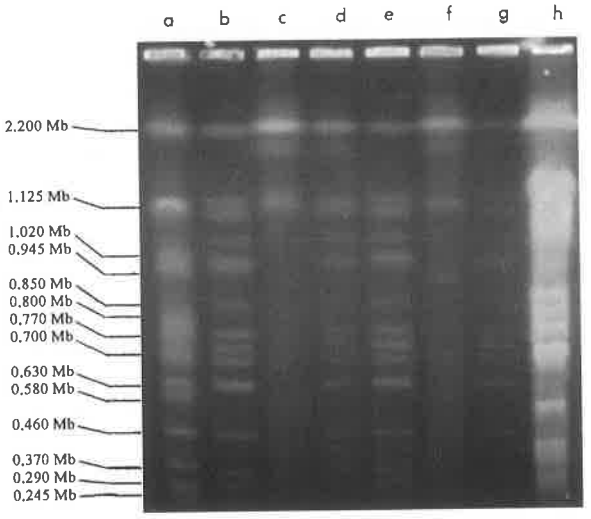

Figure 1: Chromosomal bands resolution of parental strains, of fusion products and of segregants. (a) *Saccharomyces cerevisiae* pattern; (b) ABXR.11B; (c) IZ 987; (d) FP 67; (e) S26; (f) S15; (g) S18; (h) FP 70.

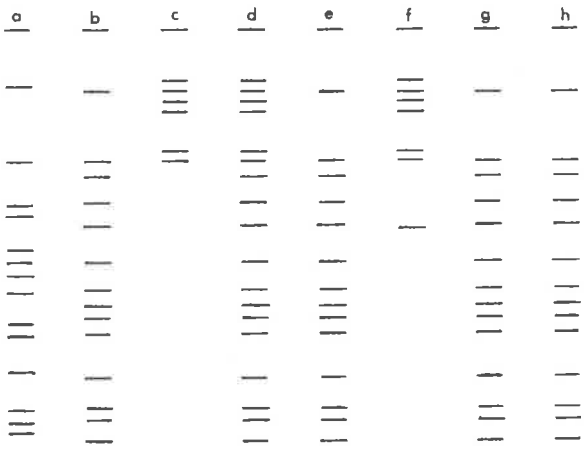

Figure 2: Diagram of the chromosomal bands resolution of parental strains, of fusion products and of segregants. (a) *Saccharomyces cerevisiae* pattern; (b) ABXR.11B; (c) IZ 987; (d) FP 67; (e) S26; (f) S15; (g) S18; (h) FP 70.

It can be observed that the electrophoretic patterns of parental strains are very different, strain IZ 987 showed only 6 chromosomal bands ranging from 2.3 to 1.125 Mb, while strain ABXR.11B showed 14 chromosomal bands ranging from 2.09 to 0.215 Mb. Parental strain IZ 987 shows 2 chromosomal bands of molecular weight similar to parental strain ABXR.11B (2.09 to 0.215 Mb), band 1.125 Mb is also present in *S. cerevisiae* pattern. Parental strain IZ 987 still shows the first chromosomal band with molecular weight very close to the first band of the pattern. Besides that, it can be observed that the resolution pattern of chromosomal bands of parental strain ABXR.11B is similar to the *S. cerevisiae* pattern.

By studying the data of chromosomal DNA separation of several yeast species analysed by the OFAGE Apparatus and obtained by Jonge *et al.* (12), a great difference in the band patterns among the different genera and species is observed. In addition, a difference in the electrophoretic pattern among strains of same species, but with a similar number and positioning of bands, can be observed.

Johnston and Mortimer (10), by using the OFAGE Apparatus, found variations in the electrophoretic karyotyping of several strains of commercially- and scientifically-used *Saccharomyces*, as well as in strains of other species. The most interesting observation was the presence of only three chromosomal bands in *Saccharomyces kluyveri*, against 14 in *Saccharomyces carlsbergensis*, 14 in *Saccharomyces uvarum* and 17 in *Saccharomyces bayanus*. Based on these results, the authors suggested a reclassification of *Saccharomyces kluyveri* as another genus. These authors still detected that the species *Candida albicans*, *Candida utilis*, *Kluyveromyces lactis*, *Pichia (Hansenula) canadensis* and *Schwanniomyces occidentalis* have a small number of chromosomes, all of them larger than 1000 kb.

Naumov *et al.* (15), investigating the genetic homology of 3 species akin to the *Saccharomyces* stricto senso genus by means of electrophoretic karyotyping, concluded that the 3 species analysed had 16 chromosomes, and that the electrophoretic karyotype of *S. cerevisiae* and *S. paradoxus* wild strains are practically identical, while *the S. bayanus* strains have species-specific karyotypes.

Table 2: Molecular weight of parental strains ABXR.11B and IZ 987, of fusion product FP 67 and of segregant S15.

Band #	*S. cerevisiae* pattern		ABXR.11B		IZ 987		FP 67		S15	
	Molec. Weight (Mb)	Rf (cm)	Molec. Weight (Mb)	Rf (cm)	Molec. Weight (Mb)	Rf (cm)	Molec. Weight (Mb)	Rf (cm)	Molec. Weight (Mb)	Rf (cm)
1	2.200	1.15	2.090	1.25	2.300	1.05	2.300	1.05	2.300	1.05
2	1.125	2.60	1.125	2.60	2.090	1.25	2.090	1.25	2.090	1.25
3	1.020	3.45	1.090	2.90	1.900	1.45	1.900	1.45	1.900	1.45
4	0.945	3.65	1.050	3.40	1.750	1.65	1.750	1.65	1.750	1.65
5	0.850	4.30	0.940	3.85	1.250	2.40	1.250	2.40	1.250	2.40
6	0.800	4.55	0.800	4.55	1.125	2.60	1.125	2.60	1.125	2.60
7	0.770	4.80	0.710	5.10	-	-	1.090	2.90	0.930	3.90
8	0.700	5.15	0.680	5.40	-	-	1.050	3.40	-	-
9	0.630	5.75	0.670	5.65	-	-	0.940	3.85	-	-
10	0.580	6.00	0.600	5.95	-	-	0.800	4.55	-	-
11	0.460	6.70	0.450	6.80	-	-	0.710	5.10	-	-
12	0.370	7.45	0.375	7.40	-	-	0.680	5.40	-	-
13	0.290	7.70	0.310	7.65	-	-	0.670	5.65	-	-
14	0.245	7.90	0.215	8.05	-	-	0.600	5.95	-	-
15	-	-	-	-	-	-	0.450	6.80	-	-
16	-	-	-	-	-	-	0.375	7.40	-	-
17	-	-	-	-	-	-	0.310	7.65	-	-
18	-	-	-	-	-	-	0.215	8.05	-	-

The results above possibly indicate that the differences in band patterns obtained through chromosomal DNA separation in strains ABXR.11B and IZ 987 are due to a great taxonomic distance among these strains, strain ABXR.11B electrophoretic karyotype is similar to the *Saccharomyces cerevisiae*, while the strain IZ 987 karyotype suggests another genus. This fact may explain the high instability of fusion products obtained from it.

Moreover, fusion product FP 67 shows chromosomal band complementarily, while segregants (S26 and S18) of a fusion product show the same pattern of parental strain ABXR.11B. In contrast, segregant S26 shows the characteristic H_2S and segregant S18 shows an intermediary phenotype as to the H_2S production (low H_2S production). On the other hand, segregant S15 shows a chromosomal DNA separation intermediary pattern, with all bands of parental strain IZ 987, plus a band with molecular weight (0.930 Mb) different from parental strain ABXR.11B.

The band with molecular weight 0.930 Mb that appears in segregant S15 may have resulted from the loss of a patch of chromosome during mitotic permutation between the fusioned nuclei. Heluane *et al.* (8), separating hybrid chromosomes obtained through protoplast fusion between *Pachysolen tannophilus* and *Saccharomyces cerevisiae*, detected that the fusion products showed altered genomes compared to parental strains. This change was detected by the presence of 4 bands as in the first parental strains, but with mobility of 3 bands larger than the referred to parental strain.

Random Amplified Polymorphic DNA (RAPD): for such reaction, DNA from the strains specified in Table 1 was isolated. By utilizing two primers, an amplification pattern of DNA fragments for the strains under investigation was obtained, as demonstrated in Fig. 3. The two selected primers produced 11 polymorphic bands, and the combined analysis of these primers allowed the construction of a similarity matrix which prompted a dendrogram of genetic similarity among parental strains, fusion products and some segregants (Fig. 4).

Based on these data (Fig. 3), fusion product FP 67 is believed to be a hybrid, for it shows a pattern of complementary bands between parental strains. This kind of result was obtained by Francis and Clair (3), who conducted the progenesis identification of *Pythium ultimun* strain crossings. These authors, who also identified the F_1 hydrides through band

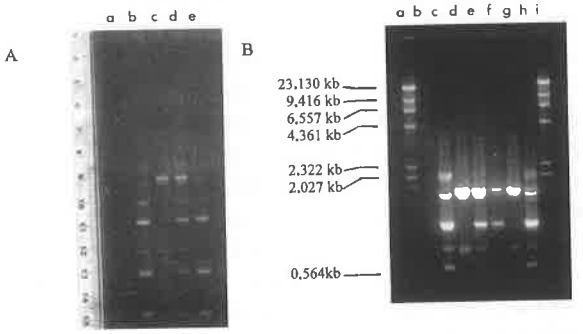

Figure 3: Amplification pattern of DNA fragments. (A) OPB-12, clockwise: ABXR.11R, IZ 987, FP 67, FP 70; (B) OPX-10, clockwise: molecular weight marker, blank, ABXR.11B, IZ 987, FP 67, S18, S15, FP 70, molecular weight marker.

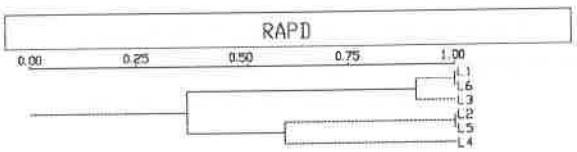

Figure 4: Dendrogram obtained from the genetic similarity matrix among parental strains, their fusion products and their segregants, based on data obtained through RAPD, where L1 stands for parental strain ABXR.11B; L2 for IZ 987; L3 for FP 67; L4 for segregant S18; L5 for segregant S15, and L6 for FP 70.

complementation, obtained a well-defined band in each parental strain, and the hydrides showed both bands. It can be verified that FP 70 shows an amplification pattern identical to parental strain ABXR.11B. However, when viewed through the amplification pattern obtained from OPX-10 primer (Fig. 3B), it can be verified that the strain S15 is similar to parental strain IZ 987 and segregant S18 shows a new pattern.

Gomes (5), by comparing yeast identification methods, detected that RAPD is the most sensitive and most efficient method in identifying very similar strains, which show a high similarity coefficient. Based on these findings, we can suggest that the low similarity coefficient observed among parental strains leads to a large genetic divergence among them, emphasising the hypothesis that the strain does not belong to the *Saccharomyces* genus.

CONCLUSIONS

Based the results of this study, we can conclude that: (a) protoplast fusion was efficient in obtaining

new genotypes from taxonomically distant strains, as observed in strains ABXR.11B and IZ 987; (b) strain IZ 987 may not belong to the *Saccharomyces* genus, as having an electrophoretic karyotype profile very different from all karyotypes obtained and presented by the literature about this genus. The low similarity coefficient viewed in the dendrogram obtained from RAPD data also leads to this fact.

In conclusion, we suggest that the recombinant strains obtained in this study, which present high flocculation and H_2S non-production, should be analysed as to their both technological and industrial viability.

RESUMO

Caracterização de produtos de fusão de protoplastos de leveduras e seus segregantes via cariotipagem eletrodorética e RAPD

Com o objetivo de caracterizar os produtos de fusão de protoplastos de leveduras com características de importância para a indústria vinícola e seus segregantes, foram empregadas as técnicas de separação de bandas cromossômicas por eletroforese e de RAPD (amplificação ao acaso de DNA polimórfico). O cariótipo eletroforético foi realizado pelo método CHEF ("contour-clamped homogeneous eletric field eletrophoresis"), constatando-se a complementação de bandas cromossômicas no produto de fusão e padrões de ambos os parentais e padrões intermediários nos segregantes. A análise do padrão de amplificação dos fragmentos de DNA com dois *primers* evidenciou um padrão de bandas complementares nos produtos de fusão (diplóide) e padrão de bandas de um e de outro parental ou mesmo bandas intermediárias nos segregantes.

Palavras-chave: fusão de protoplastos, cariótipo eletroforético, RAPD.

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SCREENING OF BASIDIOMYCETES FOR THE PRODUCTION OF EXOPOLYSACCHARIDE AND BIOMASS IN SUBMERGED CULTURE

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ABSTRACT

Fifty-six strains of Basidiomycetes, including native Brazilian fungi isolated from different ecosystems and edible mushrooms, were screened for production of exopolysaccharides and biomass in submerged culture. *Agaricus* sp. (CCB 280) and *Oudemansiella canarii* (Jungh.) Hohn (CCB 179) were the highest exopolysaccharide producers (6.01 and 3.54 g dry w./l respectively) after 7 days of incubation. The best producer of biomass was *Schizophyllum commune* Fr.:Fr. (CCB 473) with 16.68 g dry w./l in 14 days of incubation. When the culture filtrate was submitted to freezing prior to polysaccharide precipitation, a gelatinous fraction was formed.

Key words: Basidiomycetes, exopolysaccharide, biomass, submerged culture

INTRODUCTION

Following previous work regarding collection, identification and isolation of native Brazilian Basidiomycetes in pure culture, investigations were carried out concerning their utilization in biotechnological processes such as lignin and recalcitrant substances degradation, soil bioremediation, edible fungal biomass and metabolites production.

Basidiomycetes have been studied extensively for their capacity of degradation. The so-called white rot fungi, which degrade lignin, have this peculiar capacity that leads to research on degradation of xenobiotics. In addition to enzymes, there is evidence that the extracellular polysaccharides produced by these lignocellulolitic fungi play an important role in the process (6, 16). These exopolysaccharides can immobilize the exocellular enzymes. According to Catley (3), the gel formed by these biopolymers prevents the hyphal dehydration, permits cell adherence to other cells or to surfaces and could possibly select molecules from the environment.

A practical aspect of the study and characterization of fungal exopolysaccharide is the availability of data for the investigation of its physiological and ecological importance. In addition, this biopolymer may have potential industrial applications. An example is the exopolysaccharide known as schizophyllan that is produced by the Basidiomycete *Schizophyllum commune*. This polymer is a β- (1→3), (1→6)-glucan, soluble in water, that forms a viscous solution with high thermal stability. It is already used in commercial areas.

Another possible application of these biopolymers is in human health. There is intensive research on fungal polysaccharides as antitumor agents (8, 9).

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The fungal biomass can have various uses, which is an advantage as far as the fermentation is concerned because the process residue is reduced (14). Possible uses for this biomass are food or feed in the form of protein supplement or source of lipids. It can also be used for the extraction of flavours (10) and other metabolites, such as enzymes and polysaccharides. The most recent utilization of fungal biomass is for wound healing. According to Hamlyn and Schmidt (7), chitin, that has a healing capacity, is already in the fibrous form when extracted from the fungal cell wall. This might facilitate its manipulation.

The aim of this work was to screen 56 strains of Basidiomycetes for exopolysaccharide and biomass production in submerged culture contributing to the study of the potentiality of the Brazilian mycobiota.

MATERIALS AND METHODS

Microorganisms. 48 strains of native Brazilian Basidiomycetes and 8 strains of commercial edible mushrooms, corresponding to 51 different species belonging to 42 genera, were screened. The pure cultures came from the Culture Collection of Basidiomycetes (CCB) of the Instituto de Botânica - São Paulo - Brazil, and are shown in Table 1.

Liquid culture medium (g/l): Peptone 1.0; yeast extract 2.0; K_2HPO_4 1.0; $MgSO_4.7H_2O$ 0.2; $(NH_4)_2SO_4$ 5.0; glucose 39.0; pH 6.0. This medium was selected in preliminary studies as adequate for exopolysaccharide production by Basidiomycetes (4).

Erlenmeyer flasks containing 100 ml of sterilized culture medium were inoculated with the suspension in sterile water of fungal mycelium grown on two potato dextrose agar slants. Incubation was done at 25°C on shaker at 150 rpm.

Screening. For the screening, the incubation times were 7 and 14 days. The culture was filtered to separate fungal biomass, which was washed twice with distilled water and quantified as dry weight (105°C to constant weight). Isopropanol was added to the culture filtrate (1:1 v/v) and after 24 h at 4°C the precipitated biopolymer was separated by centrifugation (8,000 rpm for 10 minutes) and also quantified as dry weight.

Glucose assay. The residual glucose content of the culture filtrate was determined with a colorimetric method (17).

Chemicals used were produced by: E. Merck GmbH, Darmstadt, Germany; BDH Chemicals Ltd, Poole, England; Boehringer Manheim GmbH; Fluka Chemie AG., Buchs, Switzerland; DIFCO Laboratories, Detroit, U.S.A. and A. Constantino & C. s.p.a., Favria, Italy.

Table 1 - Results of the screening for the production of exopolysaccharide (P_p) and biomass (P_x), with the conversion yield of glucose in polymer ($Y_{p/s}$), in biomass ($Y_{x/s}$) and the specific yield (Y_c).

CCB	STRAIN	DAY	P_x g dry w./l	$Y_{x/s}$	P_p g dry w./l	$Y_{p/s}$	Y_c
041	*Agaricus xanthodermus* *	7	0.88	0.098	1.61	.0179	1.830
		14	0.92	0.137	1.39	0.207	1.511
280	*Agaricus* sp.	7	1.64	0.208	6.01	0.761	3.665
		14	3.09	0.322	1.36	0.142	0.440
211	*Agrocybe platensis*	7	8.37	0.406	1.00	0.048	0.120
		14	10.18	0.318	1.33	0.042	0.131
392	*Antrodiella ginestae*	7	6.28	0.262	0.49	0.020	0.078
		14	7.15	0.248	0.64	0.022	0.090
045	*Auricularia fuscosuccinea* *	7	1.20	0.200	0.54	0.090	0.450
		14	3.62	0.266	1.10	0.081	0.304
173	*Calvatia cyathiformis* *	7	1.10	0.157	2.05	0.293	1.864
		14	0.70	0.082	0.72	0.085	1.029
191	*Climacodon pulcherrimus*	7	0.34	0.046	0.75	0.101	2.206
		14	0.63	0.066	1.03	0.107	1.635

(continuação...)

CCB	STRAIN	DAY	P_x g dry w./l	$Y_{x/s}$	P_p g dry w./l	$Y_{p/s}$	Y_e
111	*Coprinus comatus*	7	5.18	0.395	1.11	0.085	0.214
		14	7.56	0.450	1.53	0.091	0.202
513	*Flammulina velutipes*	7	4.86	0.273	1.05	0.059	0.216
		14	8.28	0.213	1.74	0.045	0.210
214	*Fomitopsis spraguei*	7	2.91	0.239	0.21	0.017	0.072
		14	2.38	0.165	0.28	0.019	0.118
168	*Ganoderma australe*	7	14.75	0.382	2.64	0.068	0.179
		14	15.02	0.387	2.04	0.053	0.136
323	*Ganoderma lipsiensis*	7	7.29	0.361	0.98	0.049	0.134
	*	14	13.24	0.404	2.75	0.084	0.208
177	*Gloeophyllum striatum*	7	5.70	0.533	0.20	0.019	0.035
		14	8.60	0.422	0.53	0.026	0.062
188	*Gloeophyllum striatum*	7	4.56	0.356	0.19	0.015	0.042
		14	8.06	0.593	0.15	0.011	0.019
249	*Gymnopilus sp.*	7	2.98	0.339	0.41	0.047	0.138
		14	7.00	0.432	1.08	0.067	0.154
289	*Hydnopolyporus fimbriatus*	7	2.40	0.189	0.26	0.020	0.108
		14	3.71	0.277	0.51	0.038	0.137
160	*Hypochnicium sp.*	7	11.24	0.420	1.50	0.056	0.133
		14	10.30	0.380	1.20	0.044	0.116
207	*Inonotus ludovicianus*	7	0.99	0.116	0.64	0.075	0.646
		14	2.56	0.194	1.32	0.100	0.515
196	*Irpex lacteus*	7	12.46	0.398	2.49	0.080	0.200
	*	14	15.65	0.404	2.01	0.052	0.128
157	*Lachnocladium sp.*	7	12.86	0.331	1.83	0.047	0.142
	*	14	12.74	0.328	2.12	0.055	0.166
072	*Lentinula edodes*	7	2.24	0.311	0.49	0.068	0.219
	*	14	4.70	0.331	1.18	0.083	0.250
162	*Lentinus strigosus*	7	4.74	0.373	0.11	0.009	0.023
		14	6.26	0.252	0.51	0.021	0.082
268	*Lentinus velutinus*	7	6.37	0.344	1.46	0.079	0.229
	*	14	9.30	0.332	1.76	0.063	0.189
110	*Lepista sp.*	7	5.82	0.485	2.68	0.223	0.460
	*	14	13.48	0.709	2.09	0.110	0.155
279	*Macrolepiota procera*	7	1.93	0.179	0.52	0.048	0.269
		14	3.94	0.281	0.88	0.063	0.223
361	*Marasmius cladophyllus*	7	9.40	0.490	0.70	0.036	0.074
	*	14	9.91	0.312	0.56	0.018	0.056

(continuação...)

CCB	STRAIN	DAY	P_x g dry w./l	$Y_{x/s}$	P_p g dry w./l	$Y_{p/s}$	Y_c
184	*Melanoporia nigra*	7	6.44	0.467	1.42	0.103	0.220
	*	14	12.16	0.316	1.90	0.049	0.156
216	*Nothopanus hygrophanus*	7	16.16	0.444	1.78	0.049	0.110
	*	14	13.56	0.361	2.20	0.058	0.162
164	*Oligoporus sp.*	7	4.54	0.286	2.71	0.170	0.597
	*	14	12.15	0.334	2.92	0.080	0.240
179	*Oudemansiella canarii*	7	13.40	0.496	3.54	0.131	0.264
	*	14	15.37	0.430	1.70	0.048	0.111
187	*Panaeolus papilionaceus*	7	7.86	0.624	1.38	0.110	0.176
		14	5.91	0.296	1.70	0.085	0.288
204	*Peniophora cinerea*	7	13.22	0.472	2.58	0.092	0.195
	*	14	13.72	0.352	3.04	0.078	0.222
379	*Perenniporia piperis*	7	6.86	0.408	1.18	0.070	0.172
	*	14	12.06	0.520	1.90	0.082	0.158
190	*Phellinus gilvus*	7	8.30	0.506	0.61	0.037	0.074
	*	14	10.32	0.266	1.23	0.032	0.119
078	*Pholiota nameko*	7	2.96	0.302	1.64	0.167	0.554
	*	14	5.18	0.454	0.66	0.058	0.127
394	*Pleurotus flabellatus*	7	5.94	0.381	0.81	0.052	0.136
	*	14	8.50	0.362	2.00	0.085	0.235
004	*Pleurotus ostreatus*	7	4.06	0.366	0.57	0.051	0.140
	*	14	4.50	-	0.32	-	0.071
016	*Pleurotus ostreatoroseus*	7	8.56	0.408	2.20	0.105	0.257
	*	14	9.00	0.280	2.38	0.074	0.264
017	*Pleurotus sajor-caju*	7	11.47	0.484	1.85	0.078	0.161
	*	14	10.39	0.299	1.72	0.049	0.166
001	*Pleurotus* sp. "florida"	7	11.02	0.510	2.85	0.132	0.259
	*	14	11.72	0.480	1.36	0.056	0.116
259	*Psilocybe castanella*	7	8.96	0.498	1.18	0.066	0.132
	*	14	9.80	0.315	1.52	0.049	0.155
224	*Psilocybe subcubensis*	7	2.92	0.243	0.58	0.048	0.199
		14	4.96	0.359	0.59	0.043	0.119
113	*Pycnoporus sanguineus*	7	6.10	0.295	1.04	0.050	0.170
		14	7.83	0.201	0.87	0.022	0.111
277	*Pycnoporus sanguineus*	7	0.36	0.047	0.74	0.096	2.056
		14	0.43	0.090	0.82	0.171	1.907
334	*Rigidoporus microporus*	7	4.70	0.402	0.81	0.069	0.172
	*	14	16.04	0.685	0.83	0.035	0.052

(continuação...)

CCB	STRAIN	DAY	P_x g dry w./l	$Y_{x/s}$	P_p g dry w./l	$Y_{p/s}$	Y_e
467	*Ripartitella* cf. *brasiliensis*	7	6.15	0.521	0.86	0.073	0.140
	*	14	11.80	0.371	1.50	0.047	0.127
368	*Schizophyllum commune*	7	7.22	0.185	1.85	0.047	0.256
		14	6.02	0.154	0.91	0.023	0.151
473	*Schizophyllum commune*	7	13.84	0.416	1.32	0.040	0.095
		14	16.68	0.429	1.76	0.045	0.106
474	*Schizophyllum commune*	7	10.84	0.386	1.01	0.036	0.093
		14	16.10	0.415	1.97	0.051	0.122
202	*Trametes versicolor*	7	10.20	0.313	1.51	0.046	0.148
	*	14	7.43	0.191	2.34	0.060	0.315
165	*Trametes villosa*	7	6.86	0.279	1.73	0.070	0.252
	*	14	10.07	0.259	2.65	0.068	0.263
213	*Trametes villosa*	7	7.24	0.234	1.99	0.064	0.275
	*	14	9.02	0.232	2.12	0.055	0.235
203	*Trichaptum byssogenum*	7	9.00	0.232	1.35	0.035	0.150
	*	14	7.86	0.203	1.48	0.038	0.188
082	*Tricholoma crassum*	7	11.15	0.791	2.20	0.156	0.197
	*	14	15.90	0.646	3.23	0.131	0.203
390	*Trogia buccinalis*	7	5.74	0.279	1.03	0.050	0.179
	*	14	7.86	0.273	1.60	0.056	0.204
193	*Tyromyces pseudolacteus*	7	8.34	0.323	1.19	0.046	0.143
		14	6.50	0.227	0.77	0.027	0.118

* Formation of gel due to the freezing of the culture filtrate

P_x = g dry weight biomass/ l culture
$Y_{x/s}$ = g dry weight biomass/ g consumed glucose
P_p = g dry weight biopolymer/ l culture
$Y_{p/s}$ = g dry weight biopolymer/ g consumed glucose
Y_e = specific yield

RESULTS AND DISCUSSION

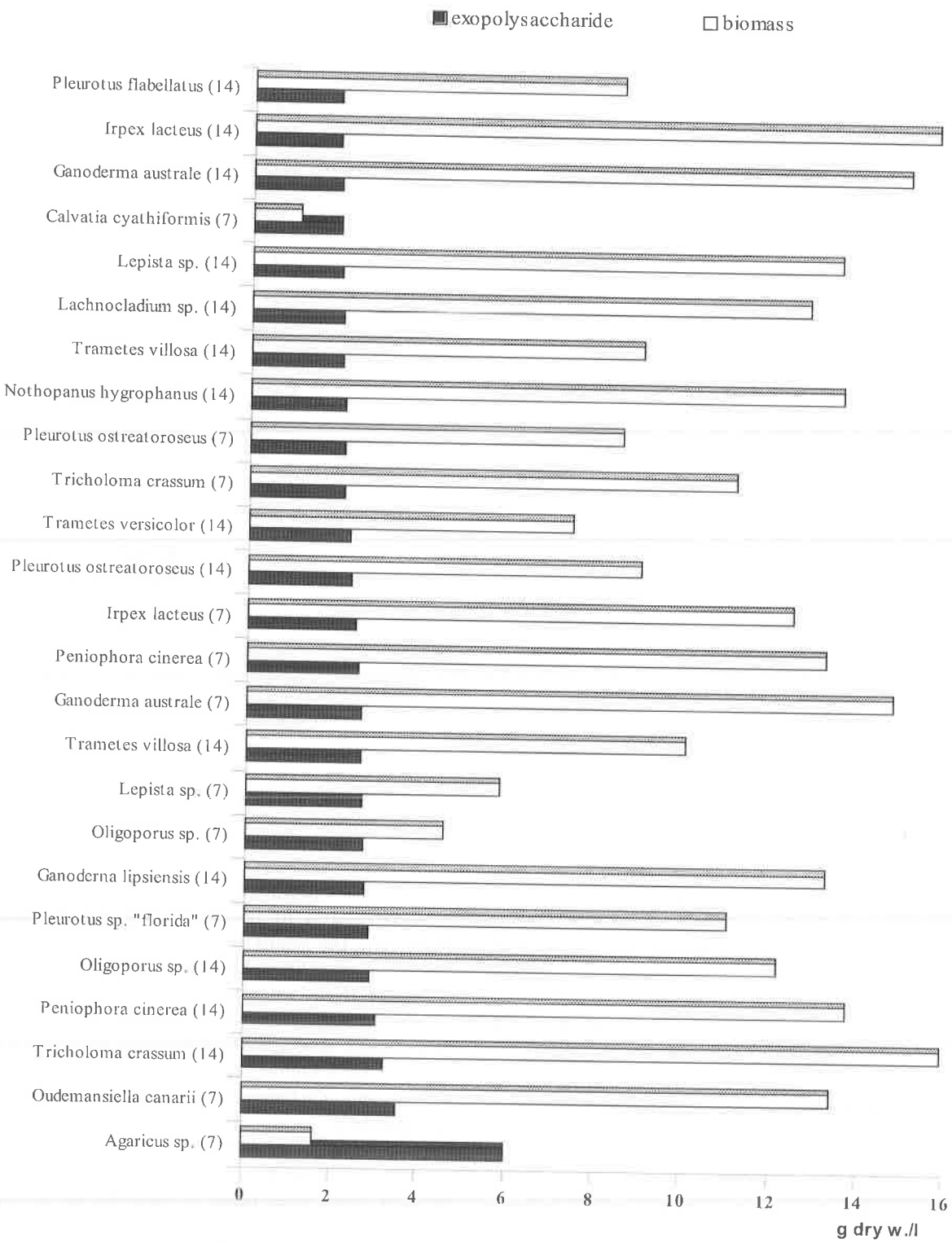
Almost all the strains produced exopolysaccharide in different quantities (Table 1). The best yield was produced by *Agaricus* sp., with 6.01 g dry w./l (conversion yield, $Y_{p/s}$ = 0.761) and *Oudemansiella canarii* with 3.54 g dry w./l ($Y_{p/s}$ = 0.131) after 7 days of incubation. *Tricholoma crassum* had a similar production (3.23 g dry w./l) with conversion yield of 0.131, but after 14 days of incubation.

About 30% of the strains produced more exopolysaccharide after 7 days incubation; however 70% produced more after 14 days, which indicates that an accurate study of each strain with growth and product kinetic profiles should be carried out if there is a possibility of its utilization for polymer production. The strains that produced more than 2.0 g dry w./l of exopolysaccharide are shown in Fig. 1.

There is no relation between biomass and exopolysaccharide production and in some cases a considerable decrease of biopolymer was observed after 14 days incubation *(Agaricus* sp., *Calvatia cyathiformis*, *Oudemansiella canarii* and *Pleurotus* sp. "florida").

The conversion yield of glucose as polymer varied between 0.020 and 0.100 for 75% of the strains and the best yields were those of *Agaricus* sp. (0.761) and *Calvatia cyathiformis* (0.293).

Figure 1. Strains that produced more than 2.0 g dry w./l of polymer after 7 and 14 days.



Different strains of *Schizophyllum commune*, *Pycnoporus sanguineus* and *Trametes villosa* showed different results not only for biomass, but also for polymer production. These data confirm the diversity of exopolysaccharide production among different strains in submerged culture. Another strain of *Schizophyllum commune* was submitted to a similar screening by Cavazzoni and Adami (4) with the same growing conditions used here. The polymer production was higher (5.3 g dry w./l).

An interesting observation was made concerning the formation of an insoluble gel when the culture filtrate was frozen prior to polysaccharide precipitation. In the Table 1 these strains are marked. This peculiar characteristic could aid polymer separation, since there is no need of an organic solvent such as isopropanol, ethanol or acetone for the precipitation of the polymer, thus increasing the process viability. Moreover, it is important to observe that the product obtained by solvent precipitation cannot be considered pure polysaccharide because proteins and salts present in the medium coprecipitate. The data obtained from this screening are just indicative for selecting strains for further investigations on exopolysaccharide production.

Some of the strains studied here were submitted to a lignin degradation activity test (2). All strains that produced more than 2.0 g dry w./l of exopolysaccharide showed good lignin degradation activity (24.8-65.4%) at 25°C and 60 days of incubation. For *Irpex lacteus* the result was higher at 30°C with 78.4% of substrate lignin degradation. Okino (15) studied some of these strains for laccase and peroxidase production and all of them showed enzyme activity.

Biomass production ranged from 0.34 to 16.68 g dry w./l. Some strains, such as *Agaricus xanthodermus*, *Calvatia cyathiformis* and *Climacodon pulcherrimus*, had a slow growth rate in these culture conditions. Others, such as *Schyzophyllum commune*, *Rigidoporus microporus*, *Oudemansiella canarii*, *Irpex lacteus* and *Nothopanus hygrophanus* produced more than 15.00 g dry w./l of biomass.

Among the edible strains, those that produced more biomass after 7 days incubation were *Pleurotus sajor-caju* (11.47 g dry w./l), *Pleurotus* sp. "florida" (11.02 g dry w./l), and *Agrocybe platensis* (10.18 g dry w./l). After 14 days incubation, the best biomass producer was *Lepista* sp. (13.48 g dry w./l).

The conditions used for the submerged culture could be considered adequate for biomass production. Data presented in literature (1, 5, 11, 12) showed lower production for *Pleurotus* species with other culture parameters.

During estimation of polymer and biomass produced it is important to consider that exopolysaccharides adherent to the hyphae are also entrapped into the pellets formed during the submerged culture (1), which means that the dry weight of biopolymer which precipitated from the culture filtrate does not correspond to the total exopolysaccharide and that the biomass can be overestimated. To minimize this problem biomass was washed twice with distilled water.

During the screening it was observed that the submerged cultures showed different characteristics according to the fungal species. The pellets formed can be regular or irregular in form and size. The form varies from spherical to cylindrical and the size from 1 to 20 mm. In some cases the formation of pellets was not observed, but rather a mycelial agglomeration without a defined form (13).

The pellets were smooth, hairy (with looser outer zones) or with fringes of aggregated hyphae that give the pellet a star form. The color and consistency were also different, as well as the flavour. In the case of *Auricularia fuscosuccinea* the pellet had a gelatinous consistency. Sometimes the culture filtrate was very clear, other times was turbid and very viscous. In most of the cultures the presence of crystals with different forms was observed, which could indicate, in some cases, the presence of excreted metabolites.

When there is a depletion of glucose in the medium it was observed that pellets begin to become darker and break up. The dead hyphae are decomposed and the resulting substances are reabsorbed by the mycelium.

Results showed that most of the Basidiomycetes strains screened are potential exopolysaccharide producers. The possibility of using these biopolymers for medical application promises a large opportunity to improve the study of such group of fungi. Besides the Brazilian mycobiota has been scarcely investigated although its great potentiality.

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RESUMO

Triagem de basidiomicetos para a produção de exopolissacarídeos e biomassa em cultura líquida

Este trabalho diz respeito à produção de exopolissacarídeos e biomassa por basidiomicetos em cultura líquida. O "screening" foi realizado com 56 linhagens incluindo fungos nativos de diferentes ecossistemas do Brasil e de fungos comestíveis. *Agaricus* sp. (CCB 280) e *Oudemansiella canarii* (Jungh.) Hohn (CCB 179) foram os melhores produtores de exopolissacarídeo (6,01 e 3,54 g peso seco/l respectivamente), em 7 dias de incubação. O melhor produtor de biomassa foi *Schizophyllum commune* Fr.:Fr. (CCB 473) com 16,68 g peso seco/l em 14 dias de incubação. Quando o filtrado cultural foi submetido à congelamento antes da precipitação do polissacarídeo, formou-se uma fração gelatinosa.

Palavras-chave: Basidiomiceto, exopolissacarídeo, biomassa, cultura líquida

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OCCURRENCE OF AFLATOXINS IN PEANUTS AND PEANUT PRODUCTS CONSUMED IN THE STATE OF SÃO PAULO/BRAZIL FROM 1995 TO 1997

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ABSTRACT

One hundred and thirty seven samples of peanuts and peanut containing foods were collected in markets in the State of São Paulo, Brazil, between January 1995 an December 1997. Most of the samples were collected by the Inspection Service of São Paulo Secretary of Health. The foods included raw peanuts, peanut candies ("paçoca" and "pé de moleque"), peanut butter, fried/roasted salted peanuts, "torrone", chocolate coated peanuts and salt-coated peanuts. The samples were analyzed for aflatoxins using a thin-layer chromatographic method. About 45% of the samples were positive for aflatoxins and 27% exceeded the limits of the Brazilian legislation (30.0 $\mu g.kg^{-1}$ for aflatoxins B_1+G_1). The aflatoxins were confirmed by derivatization with trifluoroacetic acid. The 90th percentile was 110.0 in 1995, 60.0 in 1996 and 118.0 $\mu g.kg^{-1}$ in 1997. The aflatoxins concentration in the raw peanut samples ranged from 5.0 to 382.0 $\mu g.kg^{-1}$ and 27.1% were above the legal limits. Contamination in peanut candies was above the limit in 32.8% of the samples and the aflatoxins levels ranged from 6.0 to 494.0 $\mu g.kg^{-1}$. Contamination of salty peanuts was less frequent, around 10% of the samples and the toxin levels were usually below 10 $\mu g.kg^{-1}$. The maximum level of contamination, 536.0 $\mu g.kg^{-1}$, was found in a sample of peanut with a salty coat ("amendoim japonês"). Results of previous studies in peanuts and peanut products in the city of São Paulo from 1980-1987 had 68.75% of the samples with levels greater than the limit 30.0 $\mu g.kg^{-1}$ and the 90th percentile ranged from 42.0 to 333.0 $\mu g.kg^{-1}$. In 1994, 36.0% of the samples showed results above the limit and the 90th percentile was 489 $\mu g.kg^{-1}$. The results show that aflatoxins contamination in peanuts is decreasing but it is still a serious problem in Brazil, a country where the climate, the agricultural practices and storage conditions favour fungal growth.

Key words: aflatoxins, peanuts, peanut products, thin-layer chromatography

INTRODUCTION

Aflatoxins were discovered almost forty years ago and aflatoxin B_1 is the most toxic substance of the group. It can cause liver damage, impaired productivity and reproductive efficiency in different animal species (12).

Aflatoxin contamination is the main problem in peanuts and peanut products in Brazil (2, 5, 6, 8, 9, 10). The World Health Organization recommends a

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systematic control of aflatoxin level in the population diet, mainly in countries located in tropical and sub-tropical areas, where the climatic conditions favour the growth of aflatoxin-producing fungi (13). Given the necessary conditions for mould growth and toxin production, agricultural commodities may be contaminated in the field or after harvest during storage, processing and transport. Once the commodities are contaminated they become not only a public health hazard but also a financial loss (4).

Since 1976 the Brazilian regulatory limit in food is 30.0 μg. kg^{-1} for the sum of the concentrations of aflatoxin B_1 (AFB_1) and aflatoxin G_1 (AFG_1) (1). More recently MERCOSUL, a group for common market among Argentina, Brazil, Paraguay and Uruguay, through the Resolution 56/94, established the maximum limit of 20.0 μg.kg^{-1} for the sum of the aflatoxins B_1, B_2, G_1 and G_2. This resolution was already internalized by the Brazilian Ministry of Agriculture (Portaria nº 183 of March 21,1996) and the same is expected to happen by the Brazilian Ministry of Health.

The Instituto Adolfo Lutz monitors the levels of aflatoxins in peanuts and peanut products consummed in São Paulo area through a joint program with The Sanitary Guidance of the Secretary of Health of São Paulo State. The present work reports the results from the 1995-1997 inspection. The study aims to collect data in order to furnish the State government adequate information, so it can act accordingly.

MATERIALS AND METHODS

One hundred and thirty seven samples of raw peanuts and peanut containing foods, collected from January 1995 to December 1997 in São Paulo markets, were inclued in this study.

About 3.0 kg of sample of the lot under inspection were collected and divided into three 1 kg sub-samples designated 1, 2 and 3. One of them was finely ground in a blender (Waring - Model 31BL.91) and homogenized. A sub-sample of 50 g was taken for the aflatoxins determination.

Aflatoxins B_1 and G_1 were determined by thin-layer chromatography (TLC), according to the method described by Soares and Rodriguez-Amaya (11). It involved extraction with a mixture of methanol and 4% KCl (9+1,v/v), followed by a clarification step with 10% $CuSO_4$ and partition to chloroform. The limit of quantification obtained was 5.0 μg.kg^{-1}. The chemical confirmation of AFB_1 and AFG_1 was performed with trifluoroacetic acid (7).

RESULTS AND DISCUSSION

From 137 samples, 45.3% (62 samples) were positive for aflatoxins and 27.0% (37 samples) presented levels of AFB_1 + AFG_1 above the maximum limit allowed by the Brazilian Legislation (30.0 μg.kg^{-1}) (Tables 1 and 2). The 90th percentile was 110.0 μg.kg^{-1} in 1995, 60.0 μg.kg^{-1} in 1996 and 118.0 μg.kg^{-1} in 1997 (Table 1). Comparing these values with the results obtained in the 1980-1987 study (9), which ranged from 42.0 to 333.0 μg.kg^{-1}, the present results were lower but still of concern. In the present study the aflatoxins concentration in the raw peanuts samples ranged from 5.0 to 382.0 μg.kg^{-1} and 27.1% were above the regulatory limits while 32.8% of the peanuts candies samples were above the limit and the concentration of aflatoxins B_1+G_1 ranged from 6.0 to 494.0 μg.kg^{-1}. Contamination in salted peanuts samples were around 10.0%. The toxins concentrations were mostly below 10.0 μg.kg^{-1} except for one sample of salt coated peanut ("amendoim japonês") that presented a level of 536.0 μg.kg^{-1}.

Table 1. Levels of AFB_1 and AFG_1 in samples of peanuts and peanut containing foods (μg.kg^{-1})

Year	nº. of samples	> 30 μg. kg^{-1}	< 30 μg. kg^{-1} > 5 μg. kg^{-1}	median	90 th percentile	maximum level (μg.kg^{-1})
1995	58	18 (31.0%)	10 (17.3%)	<5	110	494 [a]
1996	50	09 (18.0%)	08 (16.0%)	<5	60	469 [a]
1997	29	10 (34.5%)	07 (24.1%)	<5	118	536 [b]
TOTAL	137	37 (27.0%)	25 (18.3%)	<5	123	536

a - sample of candy made with peanut ("paçoca")
b - sample of peanut with salty cover ("amendoim japonês")

Table 2. Distribution of aflatoxins B_1 and G_1 in peanuts and peanut containing food samples according to the present Brazilian legislation (%).

year	raw peanut unshelled		peanut containing food			
			candies		salted	
	App.	Cond.	App.	Cond.	App.	Cond.
1995	68.7	31.3	60.6	39.4	100.0	0.0
1996	89.5	10.5	72.7	27.3	88.9	11.1
1997	53.8	46.2	75.0	25.0	75.0	25.0
TOTAL	72.9	27.1	67.2	32.8	90.9	9.1

App. Aproved
Cond. Condemned

Sabino (8) analyzed 572 samples of food products including peanuts in the period 1971 to 1975 and reported higher levels such as 7,800 $\mu g.kg^{-1}$ and a mean of 1131.0 $\mu g.kg^{-1}$. In the 1980-1982 period, Scussel and Rodriguez-Amaya (10) analyzed 241 samples of peanuts and peanut products. They found 128 to be positive, of which 92 exceeded the maximum limit of 30.0 $\mu g.kg^{-1}$. Sabino *et al.* (9) analyzed 1,374 samples during the period 1980-1987 and found 68.7% of the samples of peanuts and its products with concentrations of aflatoxins $B_1 + G_1$ greater than the limit allowed. Prado *et al.* (6) analyzed 400 samples of rawpeanuts consumed in Belo Horizonte (MG-Brazil), 61.5% were positive ranging from 4 to 1032 $\mu g.kg^{-1}$ of AFB_1. Oliveira *et al.* (5) found aflatoxins B_1 and G_1 above the maximum limit in 34.6% of 104 samples of peanuts collected in retail stores of Goiania/GO, Brazil. Brigido *et al.* (2) found 47% of 96 samples of peanuts and products from Campinas to exceed the maximum limit. All these studies detected a very high percentage of contaminated peanut samples. Specially of concern are candies made with peanuts ("paçoca" and "pé-de-moleque") because they are consummed basically by youngsters. Frequently these products are sold inside or nearby schools. According to the present regulations ($B_1 + G_1 = 30.0\ \mu g.kg^{-1}$) about 30% of the samples of candies with peanuts were found to be over the limit, this percentage rises to about 50% if the MERCOSUL limits are considered. The Codex Alimentarius Commission (3) has recommended a level on 5.0 $\mu g.kg^{-1}$ for aflatoxin in food. In the present survey, 45% of the samples were found to be contaminated with AFB_1 and AFG_1 exceeding this recommended level, whereas only 27% of the samples exceeded the present Brazilian tolerance limit. Probably when the MERCOSUL limits (20.0 $\mu g.kg^{-1}$ for the sum of aflatoxins B_1, B_2, G_1 and G_2) is internalized by the Health Ministry the percentage of condemned samples will be higher.

Suggestions to change the present situation may include: a) motivation of agriculture extension services to educate farmers on the problems of mycotoxins and to encourage farmers to use good agricultural practices in order to minimize field, harvest and post-harvest contamination; b) increasing government awareness of its responsibilities concerning the marketing and comsuption of peanut and peanut products.

RESUMO

Ocorrência de aflatoxinas em amendoim e produtos contendo amendoim consumidos no Estado de São Paulo/ Brasil no período 1995-1997

Cento e trinta e sete amostras de amendoim e produtos contendo amendoim, obtidas no período de janeiro de 1995 a dezembro de 1997, a grande parte delas coletadas pela Vigilância Sanitária da Secretaria de Saúde do Estado de São Paulo, foram submetidas à determinação de aflatoxinas. Foram incluídas amostras de amendoins cru, doces de amendoim ("paçoca" e "pé-de-moleque"), pasta de amendoim, amendoins salgados (frito e torrado), "torrone" e amendoins com cobertura de chocolate ou cobertura salgada ("amendoim japonês"). As amostras foram analisadas por cromatografia em camada delgada.

Sessenta e duas amostras (45,3%) foram positivas para aflatoxinas e 37 amostras (27,0%) apresentaram valores de aflatoxinas $B_1 + G_1$ acima do limite máximo da legislação brasileira (30,0 $\mu g.\ kg^{-1}$ para aflatoxinas B_1+G_1). A identidade destas aflatoxinas foi confirmada usando-se ácido trifluoro acético. O 90th percentil foi 110,0 em 1995, 60,0 em 1996 e 118,0 $\mu g.\ kg^{-1}$ em 1997. A concentração de aflatoxinas nas amostras de amendoim cru variou de 5,0 a 356,0 $\mu g.kg^{-1}$ e 27,1% delas acima do limite. Quanto à contaminação por aflatoxinas nas amostras de doces de amendoim 32,8% delas estavam acima do limite e as concentrações variaram de 6,0 a 536,0 $\mu g.\ kg^{-1}$. A contaminação nos amendoins salgados foi menos frequente, cerca de 10% das amostras e os níveis de toxina geralmente abaixo de 10,0 $\mu g.\ kg^{-1}$, porém uma das amostras com cobertura ("amendoim japonês") apresentou 536,0 $\mu g.\ kg^{-1}$. Comparando com os dados

de incidência de 1980-1987 em amendoim e produtos de amendoim na cidade de São Paulo, quando 68,75% das amostras mostraram valores maiores que o limite permitido e o 90th percentil variou de 42,0 a 333,0 $\mu g.kg^{-1}$, e de 1994 quando 36,0% das amostras mostraram resultados acima do limite e o 90th percentil foi 489,0 $\mu g.kg^{-1}$, os resultados deste trabalho mostram que a contaminação por aflatoxinas está diminuindo. Entretanto, mostram também que a contaminação por aflatoxinas em amendoim continua um problema sério no Brasil mas que deve-se levar em conta não somente as condições climáticas (umidade e altas temperaturas) mas também as práticas de agricultura e as condições de estocagem.

Palavras-chave: aflatoxinas, amendoim, produtos contendo amendoim, cromatografia em camada delgada

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ERRATA

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Cinnabarin synthesis by *Pycnoporus sanguineus* strains and antimicrobial activity against bacteria from food products

Elza de Fátima Albino Smânia, Artur Smânia Júnior, Clarice Loguercio-Leite

Pg. 318

The correct graphic is:

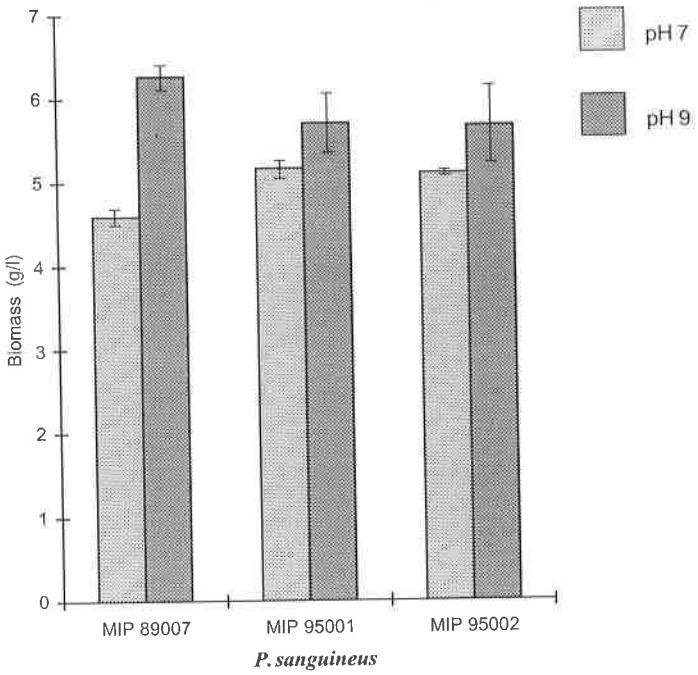

Figure 1. Prodution of biomass by three strains of *Pycnoporus sanguineus*. Error bars indicate standard deviation (triplicate samples).

Pg. 319

Title Figure 2: Substitute Pycnoporus sanguineus by *Pycnoporus sanguineus*.

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