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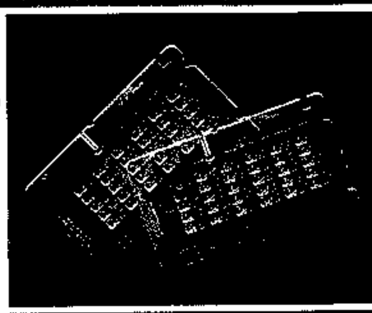

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MICROBIOLOGIA SEM 9 (1993), 1-13 **MINIREVIEW**

Modern microbiological methods for foods: Colony count and direct count methods. A review

M. R. García-Armesto, M. Prieto, M. L. García-López, A. Otero and B. Moreno*

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Summary

Over the last years methods for enumeration of microorganisms in foods are changing rapidly. Techniques based on totally new concepts as well as instruments and miniaturized systems that allow the automation and simplification of existing microbiological procedures have been developed. These rapid methodologies should satisfy the increasing requirements for effective quality assurance of foods. In the present paper we review some of the more interesting methods based on colony count or direct bacterial count.

Key words: Food microbiology, microbiological analysis of foods, rapid methods.

Resumen

En los últimos años, los métodos de enumeración de microorganismos en los alimentos han cambiado rápidamente. Se han desarrollado técnicas basadas en conceptos totalmente nuevos, así como instrumentos y sistemas miniaturizados que permiten la automatización y simplificación de los métodos microbiológicos ya existentes. Estas metodologías más rápidas permitirán satisfacer la creciente exigencia de garantía microbiológica de los alimentos. En el presente trabajo se revisan algunos de los métodos más interesantes basados en el recuento de colonias o en el recuento directo de bacterias.

Introduction

The microbial contamination of perishable commodities needs to be frequently and reliably assessed. Ideally, the methods used should be cheap, simple to perform and interpret, and provide the necessary information within defined limits of precision and repeatibility. More important, such methods need to be rapid enough to allow the efficient management of food samples.

Many modern approaches to measurement of microbial loads have been developed over the last years. These include modifications of the traditional plate count method, direct cell counts, metabolically based techniques, and techniques which directly estimate constituents of microbial

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cells (3, 32, 33, 34, 51, 52, 103, 110). In the present review we have focused our attention on the two first type of techniques, either because they are reference procedures for microbiological methods (colony count) or because they are some of the more rapid techniques at present available (direct count). This paper deals with microbiological methods used in foods. However, most of them are also applicable to other samples, such as pharmaceuticals, beberages and some clinical samples.

METHODS FOR ESTIMATING COLONY FORMING UNITS

Colony counting methods, i.e. plate counts, are conventional methods of estimating microbial populations. They are by definition not rapid because of the incubation periods necessary for the growth of colony forming units to colonies and also because they are time consuming in terms of sample preparation as well as labour intensive.

Over the last years there have been many innovations of this method addressed at saving time, effort and materials. We have classified these developments in two categories taking into account whether they represent automatization of some of the steps involved in the conventional procedure or they rely on more substantial modifications, such as miniaturization, ready-to-use commercialized systems or membrane filtration.

Automation in routine laboratory procedures

Media preparators/pourers and dispensers

Preparation and dispensing of sterile media is a time consuming and labour intensive operation. The various systems designed to automate these processes consist in bench-top sterilizers, pumps and carousels for feeding and stacking Petri dishes. Some of them also offer plate labelling and process recorders (3).

Several automatic sterilizers and plate pourers are available (3, 44), commonly with operating volumes in the range 1-10 litres (3).

Gravimetric diluter

The gravimetric diluter (Spiral Systems Inc., MD, USA) is a new instrument that automatically prepares accurate dilutions of samples (solid or semisolid). The instrument will automatically weigh the sample and then it will add the correct volume of liquid to make a desired dilution (44). Depending on the volume tested, the accuracy of delivery for most samples was found to be in the range 90-100% (62). It is an APHA (1992) recommended instrument (44).

Liquid diluters

Several automated instruments dispense and dilute liquids according to a specific program (e.g.: Dynatech SPD 3000; Hamilton Micro Lab AT Sample Transfer System; Beckman Biomek; DuPont Summit, and Tecan Sampler). Microwell plates, bead plates, or tubes can be used (44).

Spiral plating system

The spiral plate maker was developed by the US Food and Drug Administration in the early 1970's (37). The instrument dilutes automatically the sample as it is added in a spiral to an agar plate. The sensitivity of the instrument is 10^3-10^6 cfu/ml or g (31).

Since it was first introduced commercially in late 1976, several studies comparing the spiral plating system with conventional plating methods have been reported using pure cultures (20, 36, 57). Also the method has been evaluated against conventional techniques for aerobic plate counts (19, 20, 27, 35, 53, 57, 58, 81, 82, García, M. R. *et al.,* 1988. VI Reun. Cientif. Microbiol. Alim., pp. 158-159) and counts of specific groups of microorganisms (58, 82, 111, García, M. R. *et al.,* 1988. VI Reun. Cientif. Microbiol. Alim., pp. 158-159) in a range of food samples. In addition, collaborative studies have been made (38, 69). It is an approved AOAC method (1984), a recommended APHA alternate (class A2) to the standard plate count method for milk products (1985) and other foods (1992). It is also included in the FDA Bacteriologial Analytical Manual (1984) (90).

Plate loop method

The plate loop method of Thompson *et al.* (106) avoids decimal dilutions by the use of volumetrically calibrated loops (0.01 or 0.001 ml) for transferring samples.

This method is a recommended APHA alternate to the standard plate count method for milk (class O) and other foods (1985, 1992) (47, 104). Also several official standard plate loop methods have been published (17, 45, 50).

It has been widely applied for routine bacterial counting of milk for payment purposes. The sensitivity of the method is 2.5×10^3 cfu/ml for nonviscous liquids or 2.5×10^4 cfu/ml for viscous and solid foods (104).

Several methods have been developed which mechanize or automate the plate loop technique (42). The Petrifoss and mini-Petrifoss instruments (A/S N Foss Electric, Denmark) are fully automated developments (45). Other simpler instruments have also been developed, such as the Autoloop (John R. Hunter Ltd., Hamilton, New Zealand) (22a, 61).

Automated counting procedures

The automated count of colonies is made by measuring the optical differences between them and the solid media. This principle is used in the laser colony counter. This electronic method compared well with the traditional procedure (33).

The counting of colonies may be also automated using video image analysis technology. The image analysis system has been improved to solve counting problems related with large, touching and very small colonies (Aldridge, J., 1992. VIII Reun. Cientif. Grupo Microbiol. Alim., p. 62).

The most common limitations of the automated counting have been reported elsewhere (15, 25, 91a). There are several equipments commercialized which are APHA recommended (44).

Alternative colony count procedures

Microcolony techniques

They are labour and/or materials saving methods for cultivating microorganisms. Some of

those described are not recent innovations, yet they can be particularly appropriate in situations where there is growing pressure to increase the microbiological monitoring of food but insufficient funds for the high capital and/or running costs of other, more recent, rapid methods. These methods will be only briefly described.

Drop plate method

This method is a modification of the developed by Miles & Misra in 1938 (49). The sensitivity of the method is 3×10^3 cfu/ml or g (49, 104).

It is still widely applied today and has many of the advantages of speed and economy associated with the spiral plate system but without the same capital equipment cost (3, 56). It is a recommended aerobic colony count method by ICMSF (1978) and APHA (1992) (49, 104). Drawback: fast growing organisms which produce large, spreading colonies make counting difficult.

The droplet technique of Sharpe & Kilsby (92) can be considered as a development of the Miles-Misra method (3). It produces further economies in running costs by using molten agar as the diluent. The interest in its use has subsided nowadays (3). However, the method has recently been found well suited for quality control units in dairy plants and government facilities and as rapid as the fastest method for psychrotrophs enumeration (7° C for 5 days) in raw milks inoculated with lactic acid bacteria (22).

Roll tube technique

The roll tube is an early modification of the pour plate technique (3). An apparatus that combines the loop method and the roll tube method has been developed in 1974 (80). This instrument is used in the milk quality payment scheme in all regions of the Netherlands (97). Advantages and disadvantages of the technique have been reviewed thoroughly (3, 10).

Electronic counting of microcolonies

The electronic counting of microcolonies has been used as a routine method for grading producer's milk in one state of the former Federal Republic of Germany for 10 years (101).

The technique consists in preparation of milk-nutrient gelatine dilution in bottle-like tubes and solidification, overlaying with nutrient gelatine and closing of the bottles. Incubation 20 h-21° C, fixation by overlaying formaldehide hydrochloric acid mixture, liquefaction in water bath, addition of an electrolyte and electronic counting of microcolonies by Coulter Counter (101). Drawbacks of the technique have also been reported (101).

Microcolony microscopy techniques

Little plate system

This system was developed by Frost at the beginning of this century for the dairy industry (10). Many modifications to the system have been proposed since then (3). A method for enumeration of psychrotrophic bacteria in raw milk by the microscopic colony count has also been reported (54).

Microcolony epifluorescence microscopy technique

Microorganisms can be counted using the Direct Epifluorescent Filter Technique (DEFT) after a short period of incubation on agar (4-6 h) (85). The use of selective media can allow for a distinction between different bacterial groups. The sensitivity of the technique is 10^3 cfu/g or over (85, 86).

This method has been used to give a reliable estimate of the numbers of different types of bacteria in a range of foods (85). Recently, a high sensitivity has been obtained (10 cfu/100 ml) in the analysis of high quality pharmaceutical grade water by the use of a fully automated counting system (66). One of the most important drawbacks of the method is that sublethally injured cells plated onto highly selective media would not have sufficient time to repair and form microcolonies. In order to solve this problem a 3 to 5 h resuscitation step has been proposed, for frozen and heat-treated foods (86).

Recently, a combined fluorescent antibody-microcolony technique has been developed and applied to the rapid detection of salmonellas (87) and *Listeria* (96) in raw meats.

Ready-to-use commercialized systems

All these methods share the advantage of their simplicity of use, and greater economy in material and time. Common drawbacks also are the length of incubation required, and spreading of colonies because of the small surface area seeded.

Dip slide

The dip slide or contact slide is a sterile slide coated with an appropriate agar medium or with dehydrated medium. After sample contact is made, the slide is returned to its vial or chamber and is incubated as in routine plating methods. Bacteria can be counted quantitatively or semiquantitatively. The method is considered an alternative but not a replacement for routine methods (31).

Redigel

Redigel (RCR Sicentific, Inc., Indiana) is another interesting new system (32). It consists of sterile nutrients with a pectin gel in a tube. A 1 ml sample is first pipetted into the tube. After mixing, the sample is poured into a special Petri dish previously coated with a gelation material. When liquid comes in contact with the gelation material it forms a complex that swells to resemble conventional agar. After an appropriate incubation time and temperature, the colonies can be counted (32).

Collaborative studies have been made comparing Redigel with conventional methods for aerobic plate count and coliform count in different foods (88, 89). Because of these two studies, Redigel has received the Official first action of the AOAC (32).

The total cost per analysis (including materials and labour costs) of Redigel and normal Petri dish procedure have been calculated to $US 8.22 and 13.62, respectively (21).

Dry rehydratable films

The principle of Petrifilm™ (3M Co., Minneapolis, MN) method has been described before (31). Petrifilm™ units have been developed and evaluated for total bacterial count (Standard

methods agar) (2, 9, 39, 98, 99), coliforms (Violet Red Bile agar) (9, 63, 99), *E. coli* (VRBA + a beta-glucuronidase-specific indicator dye) (63), and yeasts and molds (Sabouraud agar modified, supplemented to contain 3 % of glucose) (12, 13) in different foods. Recently, Petrifilm™ Standard methods has been successfully applied for thermoduric counts (18). It has also been used for surface checks (64). A collaborative study was made for enumeration of total bacteria and coliforms in milk by Petrifilm™ methods (40).

Petrifilm™ methods have been adopted as Official first action by the AOAC (10) and are also recommended APHA alternate to the aerobic plate count method for milk (class A2) and other foods as well as for the coliform count (1987, 1992) (62a, 104).

One advantage of the Petrifilm™ method over traditional plate count procedures is that medium preparation by the user is unnecessary, avoiding temperature stress caused by molten agar. In addition, the total cost per analysis is less than with Petri dishes (21).

Membrane filtration

This classical procedure has been useful for analyzing water and various beverages when the microbial count is relatively low (10). To solve the problem of limited reliable counting range, Sharpe and Michaud in 1974, developed the hydrophobic grid membrane filter (HGMF) (93).

This is a filter with hydrophobic non toxic grids printed on it to form 1600 squares (Iso-Grid HGMF, QA Lab, Ltd., Canada) in each filter. These grids are capable of limiting physically the size and degree of spreading of bacterial colonies and make it possible the counting of inocula having a concentration range of 4 log cycles on one filter (91).

The HGMF is a most probable number device, like a single MPN test using 1600 tubes. Maximum counting limit is 1.18×10^4 cfu/filtrate. Practical maximum counting limit recommended is 4.8×10^3 cfu/filtrate (91).

The HGMF is an Official first or final action procedure of the AOAC for all foods in total viable counts (7), coliforms, fecal coliforms and *Escherichia coli* (5), and *Salmonella* (6). It is also a recommended APHA alternate to the standard plate count method for milk (class A2) and other foods (1987, 1992) (62a, 104).

In addition, HGMF procedures have been published for fecal streptococci (16), *Staphylococcus aureus* (70), lactic acid bacteria (65), *Vibrio parahaemolyticus* (30), and yeasts and moulds (60), but these have not been subjected to collaborative validation (44). A complete description and critical review of HGMF methods, including analysis for *Clostridium perfringens, Pseudomonas aeruginosa,* and *Yersinia enterocolitica* have been published (94).

DIRECT COUNT METHODS FOR ESTIMATION OF BACTERIA

They are very rapid techniques, because no incubation period is needed for the cells to metabolize and multiply.

They have been divided into two groups according to the principle they are based on: microscopic examination of samples or particle counting systems (cell-by-cell analysis).

Microscopic methods

Conventional brightfield illumination

The dried film process was originally developed for milk by Breed (14) but similar methods have application for numerous other foods (100).

This method is recommended by the APHA (1985) as a rapid technique for determining the extent of bacterial contamination of samples of raw milk or cream (class O) and pasteurized milk and cream (class D) (67). Also a similar method has been recommended by the AOAC (1984) for liquid and frozen eggs (79, 100). In addition to the dried film method, the APHA (1992) recommends microscopic methods involving counting chambers for enumeration of bacteria in foods, and special methods for mould counts have also been recommended for foods (100).

Whilst direct microscopic method is very rapid (less than 15 min/sample) and simple to perform, its low sensitivity (> 3×10^5 cfu/ml) (45a) must be considered its major drawback (41). In addition, the accuracy and reproductibility of the microscopic method depend largely upon the training and skill of the technician (67).

Fluorescence microscopy

In order to enhance the differentiation of cells and improve the sensitivity and accuracy of microscopic methods, fluorescent staining and concentration of bacteria by filtration or centrifugation, have been proposed.

Direct epifluorescent filter technique (DEFT)

With this technique, microorganisms are collected on membrane filters, stained with fluorescent dyes (usually, acridine orange), and then counted with an epifluorescence microscope. Acridine orange binds to DNA and RNA, and produces a green or orange-red fluorescence, respectively. This technique cannot be considered a viable count method but an actively growing cells count.

The DEFT was originally developed for the rapid enumeration of bacteria in milk (74) but was later modified for application to other foods including meat (75). It has also been used for a variety of applications as estimation of microbial populations on food contact surfaces (46, Kearney, L., and Holah, J. T., 1992. 61st. Ann. Meet. Summer Conf. The Soc. Appl. Bacteriol., ix), equipment rinses (48), pharmaceutical products (66), and urine (24). This method has also been applied to selective enumeration of bacteria (85), detection of spoilage yeasts (84), bacterial spores (55), moulds (77), psycrotrophs by selective pre-incubation (105), and irradiated foods (11).

Recently, the DEFT method was used to examine samples of raw milk (23), raw fish, ham and minced meat (1), chilled, cured canned pork hams and shoulders (59), and meat and poultry (95). Also a new method of separating bacteria from beef mince has been described (109).

The DEFT is also recommended by the APHA (1992) for enumeration of microorganisms in foods (44, 100) and water and wastewater (44).

Normally a rough differentiation of the microflora is possible via DEFT on the basis of morphology. A gram-negative bacterial DEFT count has been developed which does introduce a further degree of selectivity (83). A better differentiation between the types of bacteria present has been approached recently, by using the selectivity of commercially available microbiological media to grow microcolonies of the selected bacterial type (73, 85). See previous section.

The main advantage of the DEFT method is its rapidity (the technique take less than 30 min to complete) (73). Improved rapidity has also been achieved (83).

The method can detect 10^3-10^4 bacteria/ml or g, with a correlation coefficient of 0.91 (fresh meat, fish and raw milk) (71). Anomalous results have been described with heated and irradiated cells (8, 11).

A totally automated DEFT system has recently been developed, COBRA (Biocom, France), which permits process > 100 samples/h and operator (76).

Currently the DEFT is being used mainly for raw milk but its use for other foods, most notably meat, is increasing (71). It is considered one of the most important methods developed to date for directly and rapidly obtaining results equivalent to total plate counts.

A detailed evaluation of the technique and its applications have been published elsewhere (71, 73).

Bactoscan

The Bactoscan instrument (Foss Electric, Denmark) is a fully automated instrument, in which the bacteria are separated from the milk by centrifugation, stained by a fluorescent dye and counted in a continuous flow fluorescent microscope (102).

Although the Bactoscan has been used for several years to count bacteria in raw milk, the technique is still under development (102). Different versions have been commercialized during the last years. The latest generation (Bactoscan III/8000) has a capacity of 80 samples per hour and the results are obtained in about 15 min (10 min preheating of the milk + 5 min Bactoscan procedure) (102). The limit of determination is 4×10^4-8×10^4 cfu/ml (102).

Particle counting systems

Particle counting can be used to enumerate directly microbial cells in liquids by monitoring the effect of microorganisms on an electric field as they traverse that field or by optically-based techniques which can provide cell-by-cell analysis.

Electronic particle count

The electronic counter is based on the principle that cells are poor electrical conductors as compared to an electrolyte solution. A diluted suspension of cells in saline or other suitable electrolyte is drawn through a minute aperture conducting an electric current between two electrodes. Each cell passing through the aperture displaces an equal volume of the electrolyte solution and causes a momentary increased impedance to the flow of electric current. The resulting voltage pulse is amplified and counted (10). Drawbacks have also been reported (52).

Although electronic particle counters are used successfully to count somatic cells in milk, blood cells, and mammalian cells, much work needs to be done before they are useful in determining microorganisms in foods (10). Particle counting is made by the Coulter Counter and Orbec instruments (Orbec Ltd., Surrey) (52, 103).

Flow cytometry

Flow cytometry (FCM) is an emerging technology (43) that permits the rapid characterization of a cell population based on a number of structural and functional parameters such as morphology, nucleic acid content, surface antigenicity, membrane integrity, etc. Up to six parameters can be measured simultaneously on the same cell (60a). Cells are treated with appropriate stains and passed rapidly in suspension on a cell-by-cell basis through a laser beam. By analyzing the interactions of each cell (light scatter, fluorescence) with the beam, a representation of the distribution of the desired parameter within the population is acquired (26). Computerized graphic programs allow presentation of multiple analyses of the data (44).

Early work suggested the potential of the FCM for the rapid counting and identification of bacteria in clinical, food, and environmental samples (108).

Recently flow citometry has been investigated as a rapid counting method for bacteria in pure cultures (68, 78) and in foods (meat, paté, milk) (68) by using two-parameter detection scheme (particle size and nucleic acid content).

The sensitivity of the technique was found to be between 10^2-10^3 cfu/ml for pure cultures and results were achieved in a few minutes (68, 78). When applied to meat samples FCM gave a good correlation with plate counts which allowed enumeration to be made with a sensitivity of 10^5 cfu/g. However, with samples of paté and milk, sensitivity was reduced (68).

Discrimination between different species in heterogenous populations was investigated by the use of light scatter profiles of bacteria (dual parameter) combined with DNA content of the cells to enhance the discrimination between the organisms (4).

A recently developed and marketed instrument (Chemflow system, Chemunex, S. A., France) based on the measure of only a single fluorescence parameter has been evaluated for the rapid detection of yeasts, directly after addition in a range of soft drinks (72) and after preenrichment in dairy products (28). The instrument has a high capital cost but its analysis time is short (approximately 2 min) and it can detect as few as 100 yeast/ml (29).

Other microbiological applications of flow cytometry include differentiation of bacteria by determination of C + G content (108), the detection of *Legionella* spp in cooling water towers (107), and *Listeria monocytogenes* in enrichment cultures from milk (26).

The results obtained suggest that FCM shows great promise as a method for the rapid discrimination and identification of bacterial populations (4). The rapid and reliable detection and quantification of bacteria in raw meats with minimal sample preparation and processing, indicate that further work could expand the usefulness of this approach to other foods (68).

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Incidencia de la contaminación bacteriana en la ría de Ares-Betanzos (NW España)

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Summary

The presence of bacterial indicators of fecal pollution and *V. parahaemolyticus* in the estuary of Ares-Betanzos (ría de Ares-Betanzos, NW of Spain) was investigated. Resistance patterns of coliform bacteria to eight antibacterial agents were also determined. In general, high numbers of indicator bacteria were found; for instance, heterotrophic bacteria ranged between 1.82×10^2 to 1.9×10^4 CFU/ml and up to 4.6×10^3/100 ml fecal coliforms in surface waters and 1.2×10^4/100 ml fecal streptococci in sediment could be found. Surface waters of sampling points 2 and 7, located at the inner part of the estuary, were more polluted than the corresponding ones in the mouth (sampling points, 1, 3, 4 and 9), whereas the sediment showed just the opposite distribution. An 88.5% of isolated coliforms were resistant to one or more antibacterial agents. The MAR index points to urban wastewaters as the probable origin of pollution. The low incidence of *V. parahaemolyticus* and the lack of correlation with any of the fecal indicator bacteria determined, discard its use as indicative of fecal pollution in marine environments.

Key words: Water pollution, bacterial indicators, antimicrobial, resistance.

Resumen

Se analizó la presencia de bacterias indicadoras de contaminación fecal y *V. parahaemolyticus* en la ría de Ares-Betanzos, la incidencia de bacterias resistentes a 8 agentes antimicrobianos y su relación con el origen de la contaminación. Los resultados de nuestro estudio muestran la presencia de altos niveles de bacterias heterótrofas ($1{,}82 \times 10^2$ a $1{,}9 \times 10^4$ CFU/ml), y bacterias indicadoras de contaminación fecal, llegando a detectarse valores de $4{,}6 \times 10^3$ coliformes fecales/100 ml, en aguas de superficie, y $1{,}2 \times 10^4$ estreptococos fecales/100 ml, en sedimento. Además, se observó una mayor contaminación en el agua superficial de las estaciones 2 y 7, situadas en el interior de la ría, mientras que aquellas situadas en su desembocadura (estaciones 1, 3, 4 y 9) presentaron mayor contaminación a nivel del sedimento. Se detectó un 88,5% de bacterias resistentes a uno o más antibióticos, si bien la mayoría lo fueron a un bajo número de antibacterianos. Por otra parte, la aplicación del índice MAR correspondiente a

(*) A quien debe dirigirse la correspondencia.

área, reveló el probable origen urbano de la contaminación. La escasa incidencia de *V. parahaemolyticus* en la ría de Ares-Betanzos, junto con los altos niveles de bacterias indicadoras, demuestra la ineficacia del empleo de este microorganismo como indicador de contaminación fecal en este ecosistema.

Introducción

La práctica generalizada del vertido, sin tratamiento previo, de desechos de origen industrial, animal o humano en los márgenes costeros, puede producir graves alteraciones de estos ecosistemas marinos, al tiempo que ocasiona graves perjuicios económicos a aquellas industrias o empresas cuyas actividades están directa o indirectamente relacionadas con el mar. Así, se ha comprobado una mayor frecuencia de enfermedades en la población animal residente en aguas contaminadas, así como un descenso en el número de peces y fauna bentónica (9).

Desde el punto de vista sanitario, no cabe duda que esta práctica constituye un grave riesgo para la salud pública, especialmente en lo que se refiere al consumo de algunos moluscos, tal como ostras y mejillones, que filtran y concentran en su aparato digestivo bacterias y virus presentes en el agua, entre los que indudablemente pueden encontrarse gérmenes potencialmente patógenos para el hombre (8, 14). Este problema se ve agravado por la progresiva incidencia en ambientes acuáticos de bacterias resistentes a antibióticos y otros agentes quimioterápicos (23), como consecuencia del uso indiscriminado de estos agentes en el campo sanitario, agrícola y ganadero (19).

Por otra parte, investigaciones recientes han puesto de manifiesto que la supervivencia de estas bacterias en ecosistemas acuáticos ha sido subestimada y que organismos dañados pueden permanecer viables durante largo tiempo, conservando además intacto su poder patógeno (10).

Galicia tiene 1.200 km de costa y rías de una gran riqueza biológica, de los que tradicionalmente obtiene gran parte de sus recursos. Sin embargo, la ubicación de los principales grupos de la población e industrias en los márgenes costeros provoca un importante aporte de residuos de origen industrial y urbano directamente al mar, sin que se haya evaluado suficientemente su impacto sobre el medio marino. Nuestro objetivo ha sido conocer la incidencia y distribución de la flora contaminante en una de las rías gallegas, la ría de Ares-Betanzos.

Materiales y métodos

Area estudiada y toma de muestras

Se analizaron un total de 27 muestras procedentes de 9 estaciones de la ría de Ares-Betanzos (Fig. 1). En cada estación se tomaron 2 muestras de agua [superficial (S) y profundidad media (Pm)], utilizando botellas tipo Van Dorn estériles, y una de sedimento (Sed), mediante una draga de la cual se trasladaron en condiciones asépticas a botellas estériles. Se determinaron *in situ* los siguientes parámetros fisicoquímicos: temperatura, salinidad y pH (Tabla 1).

Desde su recogida, las muestras se mantuvieron a 4° C, siendo procesadas inmediatamente después de su llegada al laboratorio. Las muestras de agua homogeneizadas se sembraron directamente en los medios de cultivo, mientras que de las muestras de sedimento se tomó la cantidad suficiente para enrasar a 100 ml una probeta con 80 ml de buffer fosfato (BF 1M, pH 7,5) (1); tras su homogeneización y sedimentación, el sobrenadante se sembró de manera análoga a las muestras de agua.

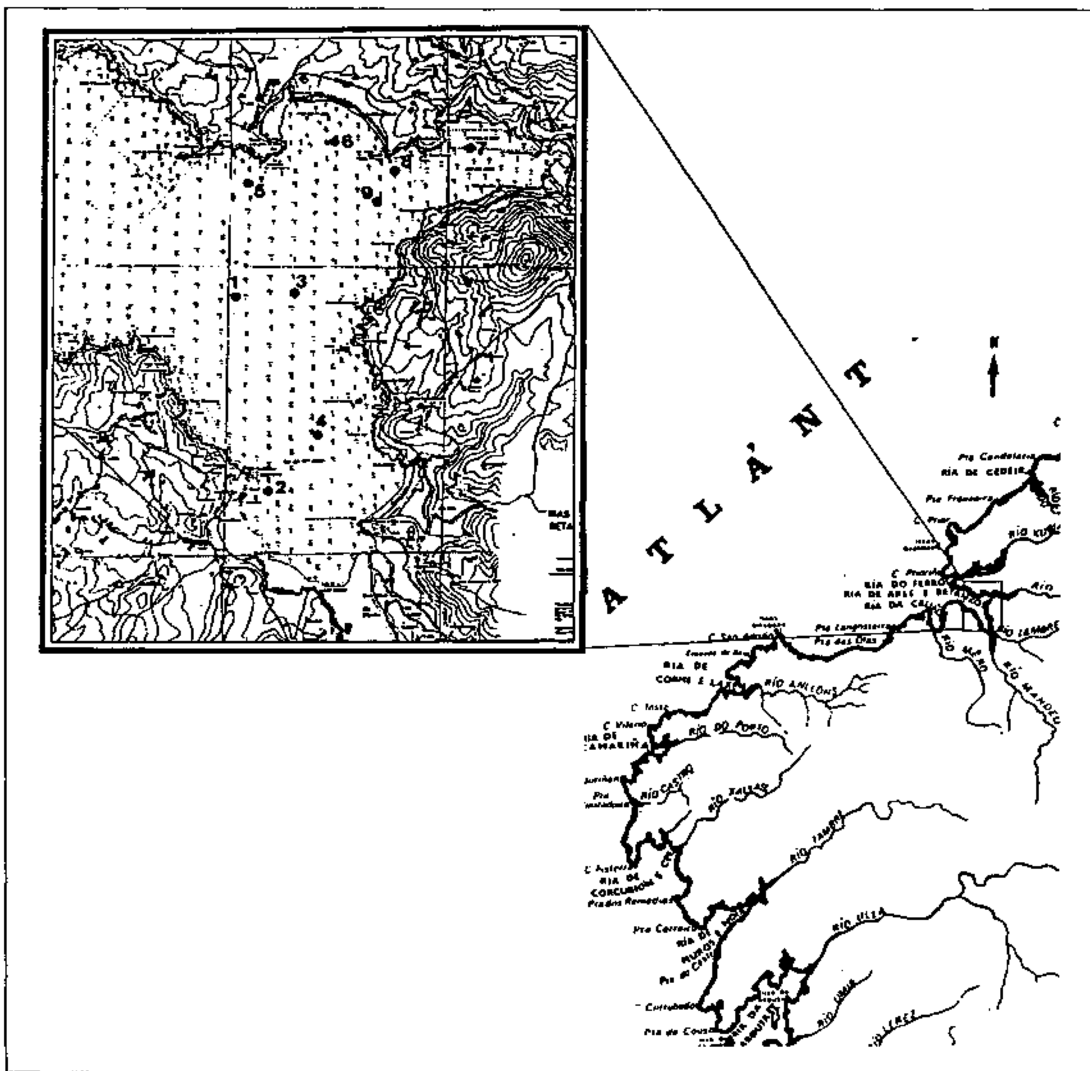

Fig. 1. Estaciones de muestreo en la ría de Ares-Betanzos.

Determinación de los microorganismos e indicadores

El recuento de coliformes totales (CT), coliformes fecales (CF) y estreptococos fecales (SF) se efectuó siguiendo la técnica del número más probable (NMP) (1), empleando 3 series de 5 tubos. El NMP de *E. coli* se determinó según lo establecido por Grunnet y Grundstrup (18). Los aislados seleccionados en medio Bacto-Levine Agar se identificaron mediante el sistema API 20-E.

La cuantificación de clostridios sulfito reductores (CSR) se efectuó siguiendo el procedimiento desarrollado por Wilson y Blair modificado (7), siendo expresado el resultado como el número de colonias con coloración negra por 100 ml de muestra.

El recuento de bacterias heterótrofas se realizó por duplicado mediante siembra por extensión de 0,1 ml de la muestra o diluciones adecuadas en placas de medio Bacto-Marine Agar, tras incubarlas a 22° C durante 72 h. El resultado se expresó como unidades formadoras de colonias (UFC) por mililitro de muestra.

TABLA 1
CARACTERISTICAS FISICOQUIMICAS DE LAS MUESTRAS DE AGUA Y SEDIMENTO

	Estaciones de muestreo								
	1	2	3	4	5	6	7	8	9
Superficial									
Prof. (m)	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5
pH	7,5	7,3	8,0	8,0	8,0	7,7	7,7	8,2	8,2
Temp. (° C)	10,2	11,0	13,0	13,4	14,3	14,0	14,5	15,0	15,0
Salinidad (%)	3,0	2,5	3,0	3,0	3,2	3,0	2,2	2,5	2,5
Prof. media									
Prof. (m)	6,5	2,7	5,5	4,5	3,8	3,5	2,0	1,7	3,2
pH	7,6	7,5	8,0	8,0	8,0	7,9	7,9	8,1	8,3
Temp. (° C)	10,4	11,4	13,0	14,0	14,3	14,3	14,7	15,0	15,0
Salinidad (%)	3,4	3,1	3,4	3,2	3,3	3,2	3,5	3,0	3,0
Sedimento									
Prof. (m)	13,0	5,5	11,0	9,0	7,5	7,0	4,0	3,5	6,5
pH	7,6	7,8	7,8	8,5	8,3	8,1	6,9	7,5	8,0
Temp. (° C)	11,0	12,4	14,0	14,0	15,0	15,0	15,0	15,0	15,0
Salinidad (%)	3,4	3,5	3,5	3,2	3,5	3,3	3,5	3,5	3,5

El NMP de *V. parahaemolyticus* se determinó según las indicaciones de Roberts y Seidler (25). La identificación de las cepas en agar TCBS (Oxoid Ltd.) se realizó en función de los caracteres morfológicos, fisiológicos y bioquímicos, de acuerdo con lo establecido en el *Bergey's Manual of Systematic Bacteriology* (6).

Determinación de sensibilidad a agentes antibacterianos

Se determinó la sensibilidad a 8 agentes antimicrobianos mediante el método de difusión en disco de Barry y Thornsberry (5) sobre medio Agar Mueller-Hinton (Oxoid Ltd.), empleando los siguientes antibióticos y agentes quimioterápicos (μg/disco; BBL Microbiology Systems): ampicilina (Am, 10), estreptomicina (Sm, 10), gentamicina (Gm, 10), eritromicina (Er, 15), cloranfenicol (C, 30), tetraciclina (Tc, 30), nitrofurantoína (Nf, 300) y sulfadiazina (Sf, 300).

El índice de resistencia múltiple a antibióticos (índice MAR) en *E. coli* para cada uno de los aislados se calculó como la relación entre el número de antibióticos al que cada aislado es resistente y el número total de antibióticos probados. El índice MAR correspondiente al área se calculó como la relación entre el número de antibióticos al que todos los aislados son resistentes y el número de antibióticos probados × número de aislados (20).

Resultados y discusión

Los resultados de nuestro estudio muestran la presencia de altos niveles de bacterias alóctonas que oscilaron entre $1,82 \times 10^2$ UFC/ml (Pm, estación 9) y $1,9 \times 10^4$ UFC/ml (Sd, esta-

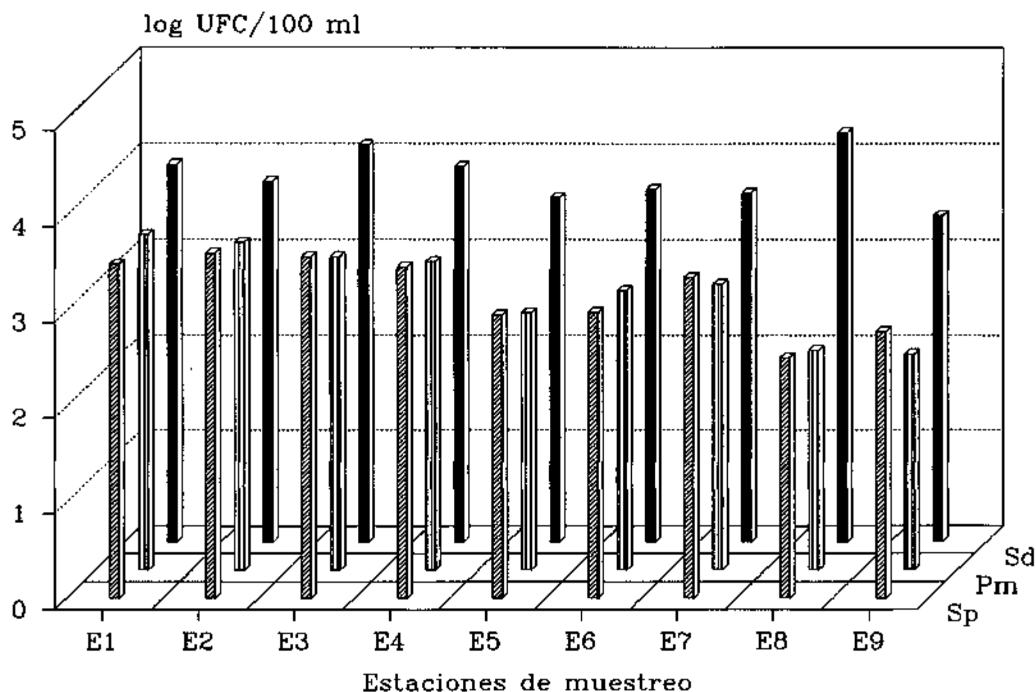

Fig. 2. Distribución del recuento de bacterias heterótrofas en 9 estaciones de la ría de Ares-Betanzos a 3 niveles de profundidad. Sp: Superficial. Pm: Profundidad media. Sd: Sedimento.

ción 8), obteniéndose por lo general valores superiores en el sedimento que en la columna de agua (Fig. 2).

Se detectó la presencia de indicadores de contaminación fecal en todas las estaciones muestreadas (Figs. 3-7). Los mayores recuentos de CT se obtuvieron en las estaciones 2 ($4{,}6 \times 10^4$/100 ml), 7 y 9 ($1{,}1 \times 10^4$/100 ml) a nivel de superficie, 6 y 7 ($4{,}6 \times 10^3$/100 ml) en profundidad media, y 7 ($5{,}5 \times 10^4$/100 ml) y 9 ($1{,}2 \times 10^4$/100 ml) a nivel de sedimento (Fig. 3). CF y *E. coli* alcanzaron los mayores niveles en las estaciones 2 ($4{,}6 \times 10^3$/100 ml) en aguas de superficie, 6 ($1{,}1 \times 10^3$/100 ml) en profundidad media, y 7 ($2{,}3 \times 10^3$/100 ml y $4{,}65 \times 10^2$/100 ml, respectivamente) en sedimento (Figs. 4 y 5).

En cuanto a su distribución vertical se obtuvieron mayores recuentos de CT en el sedimento que en algunos de los niveles de la columna de agua en las estaciones 1, 4, 7, 8 y 9 (Fig. 3). CF y *E. coli* presentaron una mayor distribución en sedimento en las estaciones 1, 3, 4, 7 y 9 (Figs. 4 y 5). Unicamente se obtuvieron valores superiores en la columna de agua en las estaciones 2 para CT; 2 y 6 para CF, y 2, 6 y 8 para *E. coli.*

Los mayores recuentos de SF en la columna de agua se obtuvieron en la estación 9 ($4{,}6 \times 10^3$/100 ml), y en el sedimento de las estaciones 2 y 7 ($1{,}2 \times 10^4$/100 ml) observándose, por otra parte, recuentos más elevados o similares en sedimento que en la columna de agua (Fig. 6). En el caso de CSR se observó, en todos los casos, una mayor distribución a nivel de sedimento (Fig. 7).

Esta mayor distribución de microorganismos indicadores en sedimento fue también observada por otros autores como Goyal *et al.* (17) y Gerba *et al.* (15), probablemente como consecuencia de los procesos de adsorción de las bacterias a partículas y su posterior sedimentación, que tienden a llevar y concentrar los microorganismos en el sedimento (27), donde pueden permanecer viables durante largo tiempo.

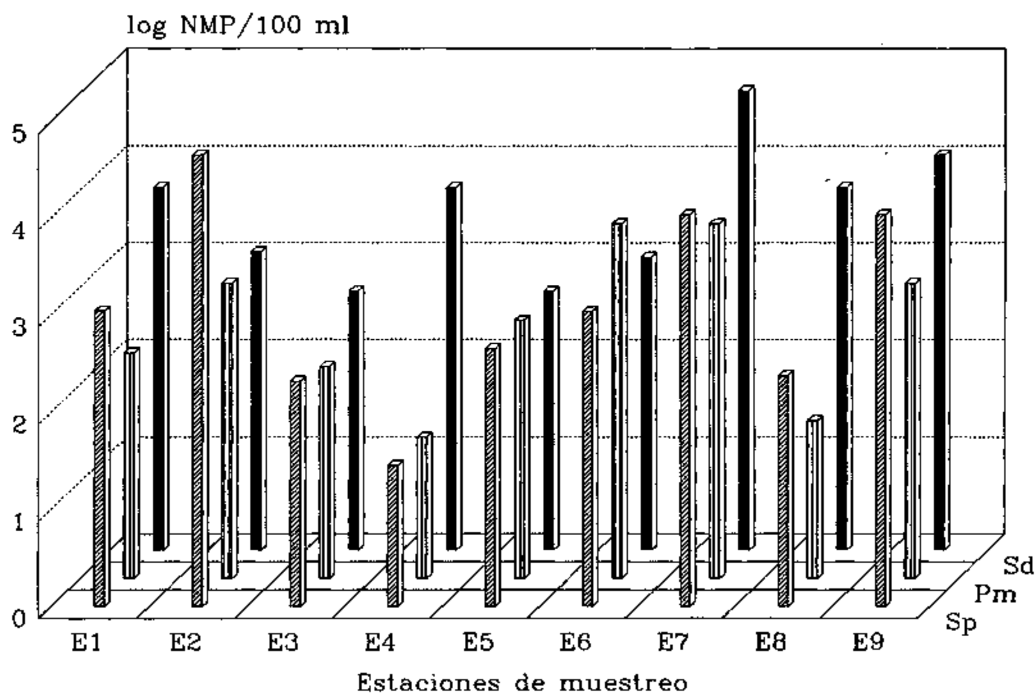

Fig. 3. Distribución de CT en 9 estaciones de la ría de Ares-Betanzos a 3 niveles de profundidad. Sp: Superficial. Pm: Profundidad media. Sd: Sedimento.

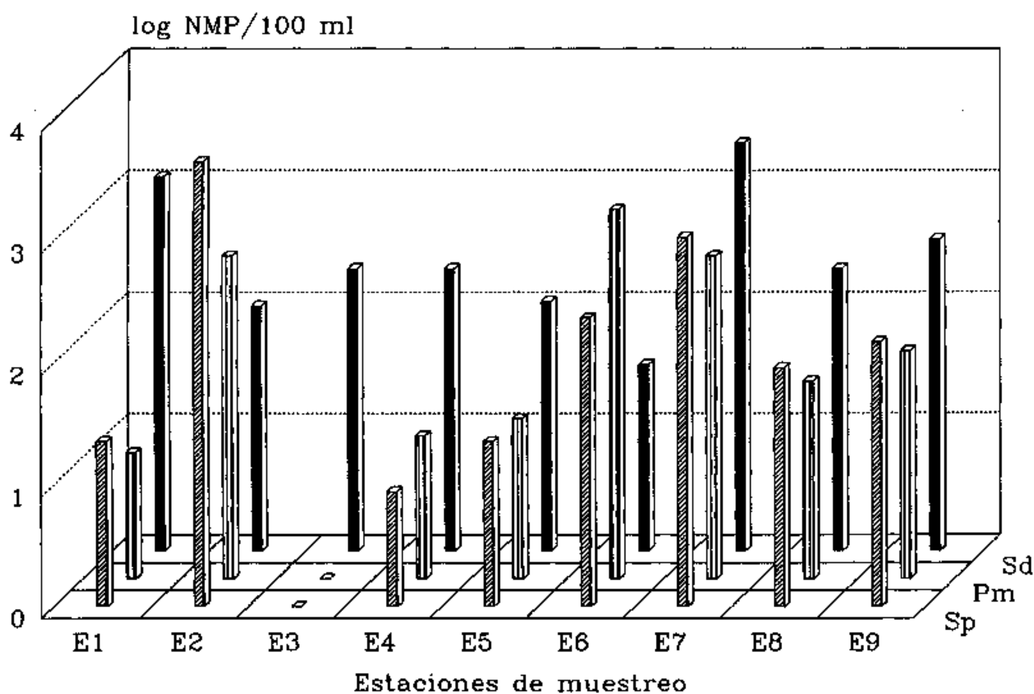

Fig. 4. Distribución de CF en 9 estaciones de la ría de Ares-Betanzos a 3 niveles de profundidad. Sp: Superficial; Pm: Profundidad media. Sd: Sedimento.

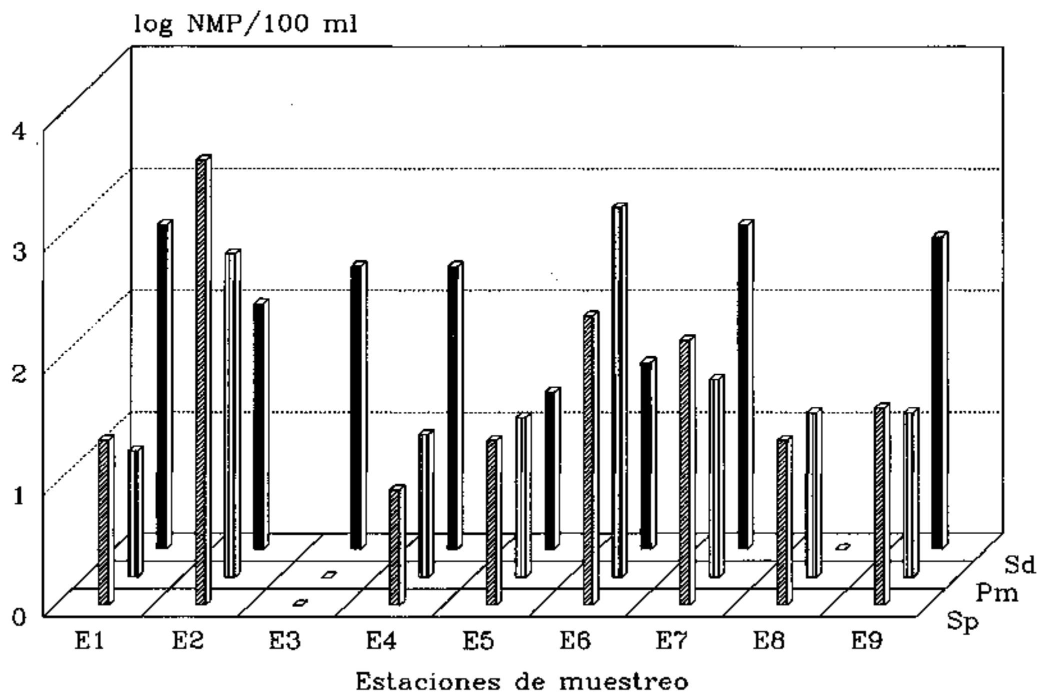

Fig. 5. Distribución de *Escherichia coli* en 9 estaciones de la ría de Ares-Betanzos a 3 niveles de profundidad. Sp: Superficial. Pm: Profundidad media. Sd: Sedimento.

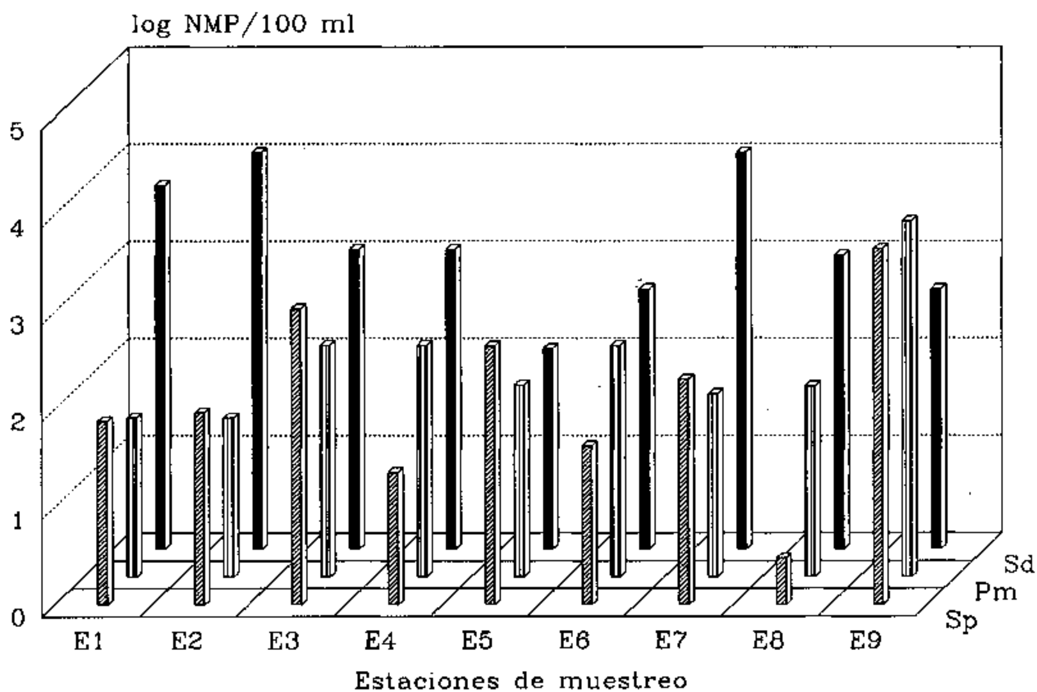

Fig. 6. Distribución de SF en 9 estaciones de la ría de Ares-Betanzos a 3 niveles de profundidad. Sp: Superficial; Pm: Profundidad media. Sd: Sedimento.

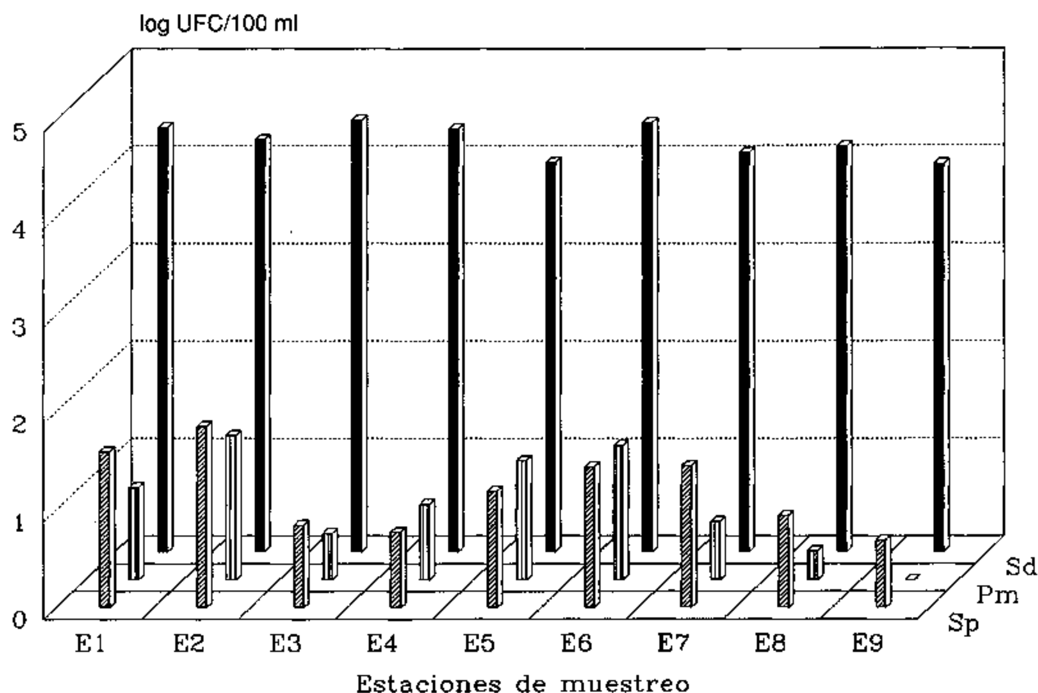

Fig. 7. Distribución de CSR en 9 estaciones de la ría de Ares-Betanzos a 3 niveles de profundidad. Sp: Superficial. Pm: Profundidad media. Sd: Sedimento.

La obtención en algunas estaciones de mayores recuentos de coliformes en la columna de agua que en el sedimento podría explicarse por la disposición a nivel superficial de las aguas residuales a su llegada al estuario, como consecuencia de su menor densidad con respecto al agua de mar (24). Este sería el caso de la estación 2, situada en el interior de la ría de Betanzos, y una de las más contaminadas, y de la 6, situada en las proximidades de Ares. En este sentido, Sayler *et al.* (28) y González *et al.* (16) obtuvieron en general mayores recuentos a nivel de la columna de agua que en sedimento, si bien especifican que obtuvieron mayores recuentos a nivel de sedimentos en las estaciones más cercanas al mar.

A nivel global se observó una disminución de coliformes y un aumento de SF, y principalmente CSR, en el sedimento (Fig. 8). Esta distribución podría explicarse por la mayor supervivencia de SF, y sobre todo de CSR, frente a los coliformes, pese a la mayor concentración de los coliformes en el agua (12).

El porcentaje de CF respecto a CT (Tabla 2), que refleja el grado de contaminación fecal en ecosistemas acuáticos, muestra que en superficie las zonas que presentan mayor grado de contaminación fecal son las del interior de las rías (estaciones 2 y 4 en la ría de Betanzos, y 6, 7 y 8 en la de Ares), mientras que en sedimentos se encuentran en la zona más próxima a la boca de las rías (estaciones 1, 3 y 5).

En la mayoría de las estaciones se obtuvo un mayor grado de contaminación fecal (%CF/CT) a nivel de la columna de agua que en el sedimento, resultados que concuerdan con los obtenidos previamente por Goyal *et al.* (17) y González *et al.* (16). Sin embargo, se observó lo contrario en las estaciones 1, 3 y 5, situadas en la desembocadura de las rías, posiblemente como consecuencia de una mayor protección de las bacterias frente a factores desfavorables a nivel de sedimento que en la columna de agua (27), efecto que podría ser más evidente en aquellas estaciones más alejadas del foco de contaminación. Por otra parte, al tratarse de las estaciones más cercanas al

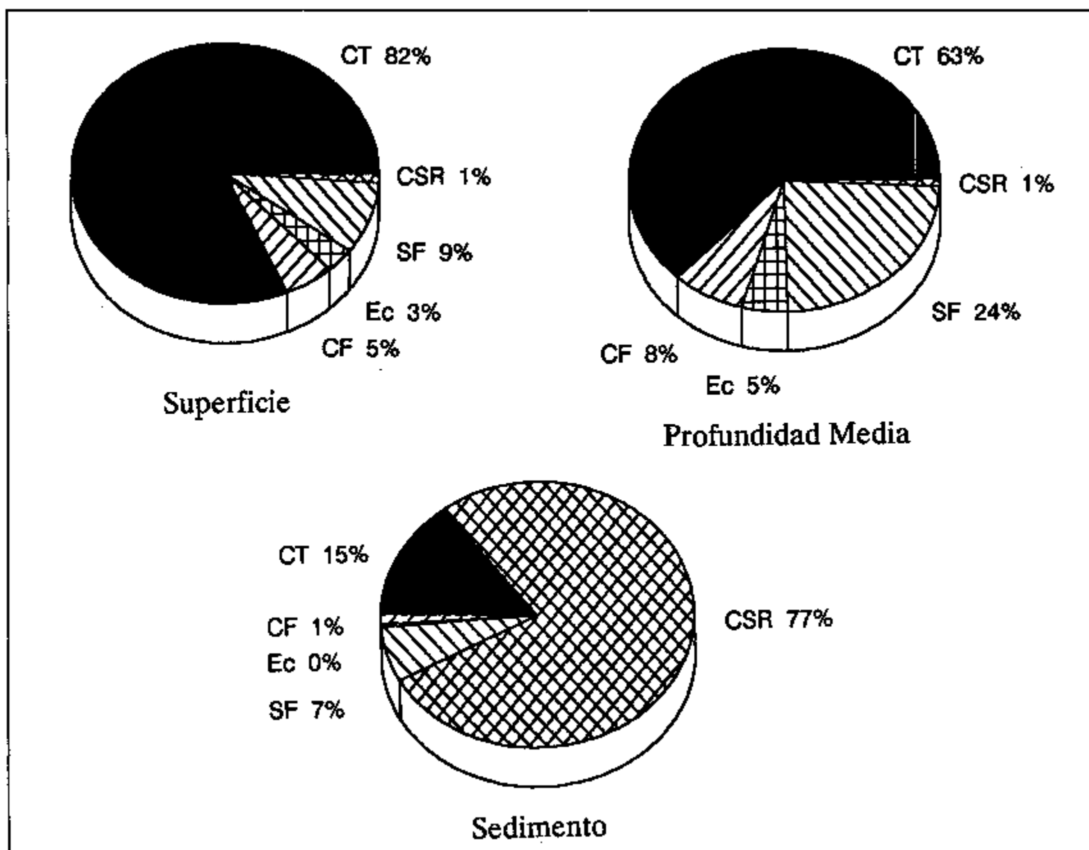

Fig. 8. Porcentaje de los indicadores en los distintos niveles de profundidad en las 9 estaciones muestreadas.

mar, es probable que el efecto de dispersión de los contaminantes sea más acusado que en las zonas del interior de la ría y más a nivel de la columna de agua que del sedimento.

El porcentaje de *E. coli* con respecto a CF (Tabla 3) fue del 100% en la mayoría de los casos, mientras que se alcanzaron valores muy bajos en las estaciones 7, 8 y 9, a excepción del sedimento de esta última estación, en el que se obtuvo un porcentaje del 100%. Por otra parte, se alcanzaron valores igualmente bajos en el sedimento de las estaciones 1 y 5.

TABLA 2

INDICE DE CONTAMINACION FECAL (% CF RESPECTO CT) EN 9 ESTACIONES DEL ESTUARIO DE LA RIA DE ARES-BETANZOS

	Estaciones de muestreo								
	1	2	3	4	5	6	7	8	9
Superficial	2,0	10,0	—	31,0	5,0	21,8	10,0	38,7	0,8
Prof. media	5,2	41,8	—	51,7	4,5	23,9	10,0	100,0	6,8
Sedimento	21,8	8,7	46,2	3,9	24,7	3,3	0,4	3,9	3,1

CF: Coliformes fecales. CT: Coliformes totales.

TABLA 3
PORCENTAJES DE *E. COLI* CON RESPECTO A CF EN LAS 9 ESTACIONES DE MUESTREO A NIVEL DE SUPERFICIE, PROFUNDIDAD MEDIA Y SEDIMENTO

	Estaciones de muestreo								
	1	2	3	4	5	6	7	8	9
Superficial	100,0	100,0	—	100,0	100,0	100,0	13,6	24,7	28,7
Prof. media	100,0	100,0	—	100,0	100,0	100,0	9,3	53,5	30,5
Sedimento	38,7	100,0	100,0	100,0	17,4	100,0	20,2	2,3*	100,0

CF: Coliformes fecales. * Calculado considerando como 1 el recuento de *E. coli* (ver Fig. 3).

Estos resultados confirman, en gran medida, que la determinación de CF es altamente indicativa de la presencia de *E. coli* en muestras ambientales. En este sentido, Lamka *et al.* (21) encontraron que *E. coli* representaba un 73 % de los CF en aguas de pozos. Sin embargo, teniendo en cuenta el carácter no estrictamente fecal de algunos CF, tal como *Klebsiella pneumoniae,* los bajos porcentajes de *E. coli* respecto a CF, así como el alto porcentaje de aislamiento de *Klebsiella* en las estaciones 7, 8 y 9, demuestra que gran parte de las bacterias alóctonas en estas estaciones son de origen no fecal. En este sentido, diversos autores pusieron de manifiesto la presencia de altos niveles de *Klebsiella,* en zonas con altos contenidos en nutrientes de origen vegetal, debido a la proximidad de bosques o industrias madederas (22), lo que sería el caso de las estaciones 7 y 8, próximas a zona forestal, por lo que es posible que tenga lugar un aporte importante de klebsielas al estuario. Nuestros resultados ratifican, por otra parte, la conveniencia de determinar los niveles de *E. coli* en ambientes naturales con objeto de poder establecer el posible origen fecal de la contaminación y ponen en evidencia el alto grado de contaminación fecal de la ría de Ares-Betanzos, dado el carácter estrictamente fecal de *E. coli* (11), así como su escasa supervivencia en agua de mar en relación con otros microorganismos indicadores (4).

A nivel global podemos considerar que las estaciones que presentaron mayores niveles de contaminación fueron, a nivel de superficie, la 2 y 7, a nivel de profundidad media la 2, 6 y 7 y a nivel de sedimentos la 1, 7 y 9, estaciones que por su proximidad a importantes núcleos de población (las estaciones 7 y 9 están situadas en las proximidades de Puentedeume y la estación 2 se encuentra próxima a Sada y en el interior de la ría de Betanzos) pueden recibir grandes cantidades de residuos urbanos sin tratar o insuficientemente depurados.

V. parahaemolyticus, microorganismo marino ligeramente halófilo, ha sido aislado de aguas de estuarios y del litoral de diversas partes del mundo. Robinson y Tobin (26) propusieron su

TABLA 4
V. PARAHAEMOLYTICUS/100 ml EN LAS 9 ESTACIONES DE MUESTREO A NIVEL DE SUPERFICIE, PROFUNDIDAD MEDIA Y SEDIMENTO

	Estaciones de muestreo								
	1	2	3	4	5	6	7	8	9
Superficial	< 3	< 3	< 3 (4)	4	< 3	< 3	4 (9)	4 (23)	4 (28)
Prof. media	< 3	< 3 (3)	3	3	3 (43)	3	< 3	4	3 (23)
Sedimento	< 3 (3)	< 3 (3)	< 3	< 3	3	< 3	3	< 3	< 3 (4)

( ) Resultados obtenidos en la prueba presuntiva.

TABLA 5
ESPECIES DE *G. VIBRIO* IDENTIFICADAS Y ABUNDANCIA RELATIVA EN LAS 9 ESTACIONES DE LA RIA DE ARES-BETANZOS A NIVEL SUPERFICIAL, PROFUNDIDAD MEDIA Y SEDIMENTO

	Estaciones								
	1	2	3	4	5	6	7	8	9
Superficial									
V. alginolyticus	—	—	3	2	—	—	3	3	2
V. hollisae	—	—	1	—	—	—	—	—	—
V. mimicus	—	—	1	2	—	—	1	1	2
V. parahaemolyticus	—	—	—	1	—	—	1	1	1
Prof. media									
V. alginolyticus	—	—	2	3	2	2	—	2	1
V. hollisae	—	2	—	1	1	2	—	—	1
V. mimicus	—	2	2	—	1	—	—	2	2
V. parahaemolyticus	—	—	1	1	1	1	—	—	1
V. vulnificus	—	1	—	—	—	—	—	—	—
V. damsella	—	—	—	—	—	—	—	1	—
Sedimento									
V. alginolyticus	2	2	3	—	1	—	2	—	3
V. hollisae	3	3	—	—	3	—	2	—	2
V. mimicus	—	—	2	—	1	—	—	—	—
V. parahaemolyticus	—	—	—	—	—	—	1	—	—

empleo como indicador de contaminación, en base a las mayores densidades observadas en presencia de elementos nutritivos aportadas al medio marino por las aguas residuales, así como a su mayor supervivencia con respecto a las bacterias alóctonas empleadas habitualmente como indicadoras de contaminación fecal.

Los resultados obtenidos en nuestro estudio muestran, por una parte, el alto número de falsos positivos en la prueba presuntiva, como puede observarse en la Tabla 4. Por otra parte, la escasa incidencia de *V. parahaemolyticus* en la ría de Ares-Betanzos (3-4/100 ml en 7 de las 9 estaciones muestreadas) contrasta con los altos niveles de microorganismos indicadores, lo que demuestra la ineficacia de *V. parahaemolyticus* como indicador de contaminación fecal en un ecosistema de estas características. Estos bajos recuentos podrían explicarse por las bajas temperaturas del agua durante la época del muestreo (10-15° C) y por los altos niveles de salinidad (2,2-3,5 %), si tenemos en cuenta que los rangos óptimos de temperatura y salinidad son 17-35° C y 0,5-2,5 %, respectivamente (29). En este sentido, también otros autores observaron ausencia o bajos niveles de *V. parahaemolyticus* en ambientes marinos con bajas temperaturas (2). Por otra parte, la identificación de los aislados mostró una mayor abundancia relativa de *V. alginolyticus* y *V. mimicus* en la ría de Ares-Betanzos (Tabla 5).

La identificación de los aislados en medio Levine demostró un alto aislamiento de *E. coli* (77 %) con respecto a los restantes coliformes, así *Klebsiella* spp. constituyó el 11,5 %, *Citrobacter* spp. el 2,6 % y *Enterobacter* spp. el 0,88 %. Por otra parte, se identificaron enterobacterias no coliformes pertenecientes a los *G. Proteus* (3,5 %) y *Salmonella* (2,6 %) y no enterobacterias pertenecientes al G. *Bacillus* y estreptococos, aunque en muy baja frecuencia (0,88 %).

La determinación de la sensibilidad a 8 agentes antibacterianos reveló que, de un total de

TABLA 6
PATRONES DE RESISTENCIA A LOS AISLADOS EN LA RIA DE ARES-BETANZOS

Bacterias	Patrones de resistencia							N.°	Indice MAR
E. coli	Er	Am	Tc	Sm	C	Sf		1	0,75
	Er	Sm	Tc	C	Sf			2	0,63
	Er	Am	Tc	Sf				3	0,50
	Er	Am	Sm	Gm				2	0,50
	Er	Am	Sm	Nf				3	0,50
	Er	Am	Tc	C				1	0,50
	Er	Am	Tc					7	0,38
	Er	Am	Sf					9	0,38
	Er	Tc						12	0,25
	Er	Am						14	0,25
	Er	Nf						4	0,25
	Er							23	0,13
Klebsiella	Er	Am	Tc	Sm	C	Sf		1	
	Er	Am	Tc	Sm	C			1	
	Er	Tc	Nf					1	
	Er	Am	Tc					1	
	Er	Tc	Sf					1	
	Er	Am						2	
	Er	Tc						1	
	Er	Sf						1	
	Er							1	
Enterobacter	Er	Am	Tc	Sm				1	
Citrobacter	Er	Tc	Nf	Sf				1	
	Sf							1	
Proteus	Er	Am	Tc	Sm	Gm	C	Sf	2	
	Er	Am	Gm	Nf				1	
	Er	Am	Gm					1	
Bacillus	Er							1	
Streptococcus	Er	Tc	Sm					1	

Ampicilina (Am), estreptomicina (Sm), gentamicina (Gm), eritromicina (Er), cloranfenicol (C), tetraciclina (Tc), nitrofurantoína (Nf) y sulfamida (Sf).

113 enterobacterias, un 88,5 % eran resistentes a uno o más antibacterianos, siendo un 65,5 % de ellos multirresistentes (2 o más antibióticos). La mayoría de las cepas de *E. coli* fueron resistentes a Er (93,1 %) y con menor frecuencia a Am (45,9 %) y Tc (29,8 %). Similares resultados se obtuvieron con las cepas de *Klebsiella*, que fueron resistentes a Er (76,9 %), Tc (46,2 %) y Am (38,5 %). Los patrones de resistencia más frecuentes para *E. coli* fueron Er (23 cepas), Er Am (14 cepas) y Er Tc (12 cepas) (Tabla 6).

El índice de resistencia a antibióticos (índice MAR) en los aislados de *E. coli* osciló entre 0,75 y 0,13, si bien la mayoría de ellos presentaron índices de 0,25 ó 0,13 (Tabla 6), siendo el índice MAR correspondiente al área de 0,263. En este sentido, Kaspar *et al.* (19) observaron un amplio rango en los índices MAR de los aislados procedentes de áreas urbanas que oscilaron entre 0,091 y 0,818, observándose valores más bajos y menor variedad entre los aislados procedentes

de fuentes rurales que oscilaron entre 0,091 y 0,545. En cuanto a los índices correspondientes a áreas, estos autores observaron valores más bajos en las áreas de influencia rural (entre 0 y 0,091) que en áreas de influencia urbana (entre 0,086 y 0,133). Sin embargo, los resultados obtenidos en la ría de Ares-Betanzos muestran que la mayoría de los aislados presentaron índices MAR bajos y un escaso margen de variación, mientras que el índice correspondiente al área fue elevado, es decir, la mayoría de los aislados fueron resistentes a un bajo número de antibióticos, pero, sin embargo, se observó un alto número de bacterias resistentes en esta área, lo que sugiere una mayor importancia del índice MAR correspondiente al área en el establecimiento del posible origen de la contaminación y, por consiguiente, a considerar como más probable el origen urbano de la contaminación en este ecosistema. Por otra parte, contrariamente a lo observado por Karpar *et al.* (19), no se observó en general el mismo perfil de resistencias para aquellos aislados que presentaron el mismo índice MAR, lo que les había llevado a considerar que aislados con el mismo índice MAR podrían presentar un origen común; sin embargo, dado el alto número de bacterias que presentaron un mismo perfil de resistencias, parece más probable que este criterio pueda ser más útil en la determinación de su origen.

El alto porcentaje de bacterias alóctonas resistentes a agentes antibacterianos aisladas en el estuario de la ría Ares-Betanzos y la posibilidad de transferencia a bacterias patógenas para el hombre y animales, constituye un elevado riesgo para la salud pública. Así, Gangarosa *et al.* (13) y Baine *et al.* (3) destacan la alta mortalidad asociada a epidemias provocadas por *Shigella dysenteriae* y *Salmonella typhi*, respectivamente, como consecuencia de la falta de respuesta al tratamiento con antibióticos.

Los resultados de este estudio nos llevan a considerar que el deterioro de ecosistemas de estuario como consecuencia del vertido directo e incontrolado de aguas residuales en sus márgenes no sólo se reduce a graves alteraciones ecológicas y económicas por afectar a la principal fuente de recursos pesqueros de nuestra comunidad, sino que también supone un alto riesgo para la salud pública por el alto número de bacterias alóctonas resistentes a agentes antibacterianos. Al mismo tiempo muestran la ineficacia del empleo de *V. parahaemolyticus* como indicador de contaminación fecal en ecosistemas de características similares al de la ría de Ares-Betanzos, probablemente debido a las bajas temperaturas registradas a lo largo del año.

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Storage of stock cultures of filamentous fungi at –80° C: Effects of different freezing-thawing methods

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Summary

Freezing and storage at –80° C has been applied to the preservation of nonsporulated filamentous fungi *Phytophthora, Pythium, Sclerotinia* and *Rhizoctonia*, and the results are presented. We had tested different methods of freezing and thawing, finding that the best results were obtained pre-cooling at 4° C during 1 hour followed by freezing at –80° C. The best thawing method was achieved at 37° C. The technique was found to be simple and reliable for the culture collections labours of fungi maintenance.

Key words: Filamentous fungi, freezing, thawing, freezer-storage.

Resumen

La congelación y el mantenimiento a –80° C ofrece resultados esperanzadores y permite plantear un método alternativo a la conservación en nitrógeno líquido y a la liofilización de hongos filamentosos no esporulados tales como *Phytophthora, Pythium, Sclerotinia* y *Rhizoctonia*. En el presente trabajo se han estudiado diferentes métodos de congelación y descongelación, encontrando los mejores resultados de supervivencia con una congelación a –80° C precedida por un pre-enfriamiento a 4° C durante 1 hora, y una descongelación rápida a 37° C. Esta técnica se presenta simple y útil para el mantenimiento de hongos en colecciones de cultivos.

Introduction

Several methods have been proposed for maintaining culture collections of fungi. Among these, dispersal of spores in sterile soil, sterile mineral oil overlays, distilled water at room temperature, deep freezing, ultra-low temperature freezing and lyophilization are the most usual. In the Spanish Type Culture Collection (CECT) the fungi are maintained by all theses methods excepting ultra-low freezing in liquid nitrogen.

The lyophilization is not a safe method for some nonsporulated fungi such as *Phytophthora, Pythium, Rhizoctonia* and *Sclerotinia*. Routinely, these fungi are mantained by subculturing in

(*) A quien debe dirigirse la correspondencia.

TABLE 1
VIABILITY OF DIFFERENT SPECIES STORED AT –80° C WITH 10% GLYCEROL AS CRYOPROTECTANT USING THE ULTRA-FAST FREEZING METHOD AND THE THREE DIFFERENT WARMING METHODS

Species	Fast warming				Slow warming				Room temperature warming			
	1 d	3 m	6 m	1 y	1 d	3 m	6 m	1 y	1 d	3 m	6 m	1 y
Phytophthora syringae CECT 2351	40	0	0	0	0	0	0	0	40	0	0	0
Phytophthora citrophthora CECT 2352	0	0	0	0	40	0	0	0	0	0	0	0
Phytophthora citrophthora CECT 2353	0	0	0	0	40	20	0	0	0	0	0	0
Pythium ultimum CECT 2364	0	0	0	0	0	0	0	0	0	0	0	0
Pythium ultimum CECT 2365	0	0	0	0	0	0	0	0	0	0	0	0
Sclerotinia sclerotiorum CECT 2769	100	100	100	100	80	80	60	80	100	100	100	100
Sclerotinia sclerotiorum CECT 2822	100	20	40	40	60	20	40	40	60	40	60	60
Sclerotinia sclerotiorum CECT 2823	100	100	100	100	100	100	100	60	100	100	100	100
Rhizoctonia croccorum CECT 2816	80	0	20	0	100	0	20	0	20	0	0	0
Rhizoctonia solani CECT 2815	60	20	0	0	80	20	0	0	20	20	0	0
Rhizoctonia solani CECT 2819	100	0	0	0	60	40	40	20	100	60	0	0
Rhizoctonia solani CECT 2829	100	100	100	0	100	100	60	0	100	100	40	20

(The results are expresed as the percentage of living pieces.) d: No. of days stored at –80° C. m: No. of months stored at –80° C. y: No. of years stored at –80° C.

the appropriate medium at 3 or 6-monthly intervals with storage at 4° C. With time it became clear that this method leads to errors, occasional loss of viability and an unacceptable degree of instability (5, 7). Preservation in liquid nitrogen has been found to be reliable (13), but ultra -low refrigeration and the handling of liquid nitrogen is troublesome.

As an alternative, commercial freezers with temperature ranges of –70° C to –100° C are readily obtainable. Storage at –80° C has been used mainly for a variety of bacteria (9), yeasts (11) and some mycelial Basidiomycetes (6). Consequently a systematic investigation was conducted to ascertain whether or not freezing at –80° C is applicable to long-term preservation of the nonsporulated fungi and which kind of cooling and warming method is the best.

Materials y methods

Strains

The strains tested are listed in Tables 1, 2 and 3. They belong to 4 genera and 12 species and

TABLE 2
VIABILITY OF DIFFERENT SPECIES STORED AT –80° C WITH 10% GLYCEROL AS CRYOPROTECTANT USING THE SLOW FREEZING METHOD AND THE THREE DIFFERENT WARMING METHODS

Species	Fast warming				Slow warming				Room temperature warming			
	1 d	3 m	6 m	1 y	1 d	3 m	6 m	1 y	1 d	3 m	6 m	1 y
Phytophthora syringae CECT 2351	60	0	0	0	40	0	0	0	100	0	0	0
Phytophthora citrophthora CECT 2352	1000	0	0	40	20	20	0	100	40	40	40	—
Phytophthora citrophthora CECT 2353	100	25	0	0	60	25	0	0	80	60	0	0
Pythium ultimum CECT 2364	100	100	100	100	100	100	80	100	100	100	100	100
Pythium ultimum CECT 2365	100	100	60	60	100	100	80	60	100	100	60	60
Sclerotinia sclerotiorum CECT 2769	100	100	100	100	100	100	100	100	100	100	100	100
Sclerotinia sclerotiorum CECT 2822	100	100	100	100	100	100	100	100	60	100	100	100
Sclerotinia sclerotiorum CECT 2823	100	100	100	100	100	100	100	100	100	100	100	100
Rhizoctonia croccorum CECT 2816	100	80	40	60	60	100	80	60	80	100	80	60
Rhizoctonia solani CECT 2815	100	80	40	40	80	40	60	60	100	80	80	100
Rhizoctonia solani CECT 2819	100	100	40	60	100	100	100	100	100	100	80	100
Rhizoctonia solani CECT 2829	100	100	100	100	100	100	100	100	100	100	100	100

(The results are expresed as the percentage of living pieces.) d: No. of days stored at –80° C. m: No. of months stored at –80° C. y: No. of years stored at –80° C.

were chosen because of their high sensitivy to storage by lyophilization and ultra-low temperature freezing.

Medium

Potato dextrose agar (PDA) (2) was used for growth and viability measurement.

Preparation of cultures

The strains were grown on PDA plates for 7-10 days at 26° C. Small pieces of fungal mycelium and agar were cut from the plate using a sterile scalpel, and 5 pieces were placed in each 2 ml polypropylene tube (SARSTEDT screw top cryopreservation tubes) in 1 ml of sterile 10% v/v glycerol in water (3).

TABLE 3
VIABILITY OF DIFFERENT SPECIES STORED AT –80° C WITH 10% GLYCEROL AS CRYOPROTECTANT USING THE MEDIUM FREEZING METHOD AND THE THREE DIFFERENT WARMING METHODS

Species	Fast warming				Slow warming				Room temperature warming			
	1 d	3 m	6 m	1 y	1 d	3 m	6 m	1 y	1 d	3 m	6 m	1 y
Phytophthora syringae CECT 2351	100	40	20	20	100	80	20	0	100	80	40	0
Phytophthora citrophthora CECT 2352	100	80	20	0	80	60	0	0	100	80	0	0
Phytophthora citrophthora CECT 2353	100	80	20	20	80	100	0	0	60	80	0	0
Pythium ultimum CECT 2364	100	100	100	100	100	100	100	100	80	100	100	100
Pythium ultimum CECT 2365	100	100	100	100	100	100	100	100	100	100	100	100
Sclerotinia sclerotiorum CECT 2769	100	100	100	100	100	100	100	100	100	100	100	100
Sclerotinia sclerotiorum CECT 2822	100	100	100	100	100	100	100	100	100	100	100	100
Sclerotinia sclerotiorum CECT 2823	100	100	100	100	100	100	100	100	100	100	100	100
Rhizoctonia croccorum CECT 2816	100	100	100	100	100	80	100	100	100	80	100	100
Rhizoctonia solani CECT 2815	100	100	40	20	100	100	40	40	100	100	40	40
Rhizoctonia solani CECT 2819	100	100	80	100	100	100	80	100	100	100	100	100
Rhizoctonia solani CECT 2829	100	100	100	100	100	100	80	100	100	100	80	100

(The results are expresed as the percentage of living pieces.) d: No. of days stored at –80° C. m: No. of months stored at –80° C. y: No. of years stored at –80° C.

Freezing

Freezing was carried out by different methods, using a Heraeus Sepatech freezer for the storage of the fungi.

1) Slow freezing. The samples were two-step cooled by placing them for 1 hour at 4° C, 1 hour at –20° C and stored to –80° C.
2) Medium freezing. The samples were pre-cooled at 4° C for 1 hour and stored to –80° C.
3) Ultra-fast freezing. The samples were pre-cooled at 4° C for 1 hour, after they were immersed in liquid nitrogen for 30 seconds and transfered immediately to -80° C.

Warming

Warming of samples was achieved by three different methods:

1) Fast warming. The samples were warmed and quickly thawed in a water bath regulated at 37° C.

2) Slow warming. The samples were transferred 1 hour to –20° C, 1 hour to 4° C and 1/2 hour at room temperature.

3) Warming at room termperature. The samples were kept at room temperature until thawing.

Revival

Agar blocks with fungal cultures of each cryotube were transferred to a fresh plate of PDA, facing the mycelia surface onto the medium. The cultures were incubated at 26° C for 3 weeks and were observed periodically for growth.

Results and discussion

Four genera of nonsporulated fungi have been studied —*Phytophthora, Pythium, Sclerotinia* and *Rhizoctonia*— using three methods of freezing combined with three methods of warming and the results are shown in Tables 1, 2 and 3. The ultra-fast freezing (Table 1) was the worst method to preserve the cultures, the viability decreases before 3 months. Only the strains of *Sclerotinia* survived 1 year but with loss of viability in some cases. The slow freezing presented good results except for *Phytophthora* genera (Table 2). In *Rhizoctonia* and *Pythium* the viability sometimes decreases a little, and in *Sclerotinia* the viability is always excelent. The medium freezing combined with warming at 37° C for fast thawing it seems to be the best method for maintenance of cultures (Table 3). The fast warming gave high viability after 1 year for all the genera. Even with *Phytophthora*, where the viability was low, it was better than the other methods.

Cooling rate is one of major factors affecting cell survival after freezing and thawing (4). The cells cannot lose water rapidly; thus cells shrink slightly and do not contain too many large ice crystals and maintain osmotic equilibrium (10). Usually, it is proposed a two-step method in freezing storage (12). We have obtained an one-step method with good viability results in some problematic-storage strains of nonsporulated fungi. The pre-cooling at 4° C may allow the glycerol to penetrate the cell and the survival of freezing is better (13).

As the results show, *Phytophthora citrophthora* and *P. syringae* are the most problematic species to the freezing maintenance system, but we have obtained survivals after one year; *Pythium* provided good results in contrast with the other authors (12). *Sclerotinia* genera presents high viability for all methods. Furthermore, we have obtained similar results with mycelia and sclerotia of the strain CECT 2822 (data not shown). These data may indicate the importance of the chemical composition differences in the fungal cell walls. This idea had been proposed just in yeasts (8). Occurrence of cellulose in *Phytophthora* and *Pythium* cell walls whereas presence of chitin in *Sclerotinia* and *Rhizoctonia* cell walls (1), would have some influence respect to the viability.

Results of the present study show one simple method of maintenance of fungal cultures at –80° C. It is not expensive and easily applicable to fungi not resistant to lyophilization. In this moment we are trying to improve the method, studying the effect of different cryoprotectants (8, 12).

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Two different alkali-soluble α-glucans in hyphal walls of the basidiomycete *Armillaria mellea*

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Summary

Two different polysaccharidic subfractions were separated from the alkali soluble, acid precipitated α-glucan located in the surface of the hyphal walls of the basidiomycete *Armillaria mellea*. The major α-glucan subfraction was composed of linear chains of α-(1->3) and α-(1->4) glucan associated to protein while the less represented was mainly an α-(1->3) glucan with some protein. Both polysaccharidic components could correspond with the fibrillar layer in which thick fibres are longitudinally oriented and thinner fibrils cross-arranged between the others.

Key words: α-glucans, hyphal walls, basidiomycetes, Armillaria mellea.

Resumen

Dos diferentes subfracciones polisacarídicas fueron separadas del α-glucano, soluble en álcali y precipitable por acidez, que se encuentra en la superficie de las paredes celulares de las hifas del basidiomiceto *Armillaria mellea*. La subfracción mayoritaria de α-glucano está compuesta por cadenas lineales de α-(1->3) y α-(1->4) glucano asociadas a proteína, mientras que la menos representada es mayoritariamente un α-(1->3) glucano, también con algo de proteína. Ambos compuestos polisacarídicos podrían corresponder a la capa fibrilar en la que fibras gruesas se orientan longitudinalmente y fibras más finas se cruzan entre las otras.

Introduction

The mycelial wall of basidiomycetes consists mainly of carbohydrate. This has been shown to be composed of distinct polysaccharides differing in the nature of monosaccharides and/or in the type of glycosydic linkage. The structure of the alkali-soluble (S-glucan) fraction derived from the cell wall of *Schizophyllum commune* has been intensively investigated by Siehr (25), Sietsma and Wessels (26), who described it as an α-(1->3) glucan. This fraction also contains xylose and mannose that may originate from distinct polymers. In cell walls of *Polyporus tumulo-*

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sus (2) the alkali soluble fraction contains two polysaccharides, an α-(1->3) glucan and a xylomannan. The alkali-soluble fraction from the common mushroom *(Agaricus bisporus)* cell walls has been assumed to be mainly an α-(1->3) glucan but some other monosaccharides (mannose, xylose and sometimes galactose) are associated to this fraction (10, 21). A different kind of glucan containing (1->4) (1->3) (1->6) linkages has been reported to exist in another alkali-soluble polysaccharide from the cell walls of *Coprinus macrorhizus* (5). Glucans of the α-type have been described in some cases in the form of microfibrils (11, 30) on basidiomycete cell walls.

An α-glucan fraction (32% w/w) has been identified previously by us in *Armillaria mellea* cell walls (24). Preliminary analysis indicated that it was mainly composed of neutral sugars (81.3% w/w) and protein (13.5% w/w), with minimal amounts of aminosugars. Among the neutral sugars glucose was the major constituent, but some mannose and galactose were also present. In this paper, we show the purification, chemical structure and physicochemical properties of this α-glucan fraction.

Materials and methods

Several methods used along this work, including organism and culture media, cell wall preparation and isolation of the alkali soluble, acid precipitated α-glucan fraction (FII) have been described before (24).

Purification of α-glucan. Samples (3 mg/ml), solubilized in 0.4 *M* KOH in an ultrasonic bath at 20° C for 5 min, were applied to a Sepharose CL-2B column (60 × 2.5 cm) and aliquots (3 ml) eluted with 0.4 *M* KOH at constant flow, were collected and tested for neutral carbohydrate and protein content as described below. Appropriate eluted fractions were pooled and precipitated by dialysis against distilled water.

Chemical analysis. Total neutral sugar content of unpurified α-glucan material and eluted fractions was determined by the anthrone procedure (7), with glucose as standard. Total protein was estimated according to Lowry *et al.* (18). The amino sugar content was evaluated by the method of Chen and Johnson (6) after hydrolysis of the samples with 6M HCl at 105° C for different periods in sealed evacuated ampoules. Protein concentration was monitored by absorption at 280 nm (8).

For analysis of monosaccharide components, the polysaccharides were hydrolysed with H_2SO_4 at 100° C under different concentrations and times: 0.5 *M*, 16 h; 1.3 *M*, 2.5 h; 2 *M*, 5 h; or Saeman hydrolysis (1). Neutral sugars released by those treatments were identified and quantified as their alditol acetate derivatives (23) by gas liquid chromatography (glc) on a column of 3% of SP-2340. Amino acids and amino sugars were determined in hydrolysates (6 *M* HCl at 105° C for 24 and 48 h in N_2 atmosphere-ampoules) of α-glucan fraction, using a Biotronik LC-7000 amino acid analyzer. Cystine-cysteine residues were converted to cysteic acid by performic acid oxidization (12) from equivalent samples hydrolysed and analyzed as above.

I.r. spectra were obtained by the KBr technique, using a Perkin-Elmer 1420 spectrophotometer.

Methylation analysis. A modification (13) of the Hakomori methylation method was used. The polysaccharide (10-15 mg) was dried overnight at 50° C *in vacuo* and then methylated. Methylation was judged to be virtually complete for the very weak i.r. absorption to hydroxyl groups. The methylated polysaccharide was hydrolysed sequentially at 100° C with 90% formic acid (2 h) and 0.13 *M* H_2OS_4 (16 h). The hydrolysis products were reduced with $NaBD_4$ and converted to partially methylated alditol acetates (PMAA). PMAAs were separated by glc (22, 23) on 3% of OV-225 at 170° C (for the retention times) and with a temperature program (for peak areas). For quantitative determinations, the molar response factors recommended by Sweet *et al.*

TABLE 1

COMPOSITION* (DRY WEIGHT %) OF THE SUBFRACTIONS OBTAINED FROM α GLUCAN FRACTION ON PURIFICATION THROUGH SEPHAROSE CL-6B

Subfraction	Recovery** from column (aprox.)	Neutral carbohydrates	Protein	Hexosamines
FII_1	18-20%	91.1	6.1	0.07
FII_2	80-82%	82.2	14.8	0.24

* Average value of at least four determinations. ** Expressed as dry-weight of the starting material.

(29) were used. Gas liquid chromatography-mass spectroscopy (glc-ms) was performed on a ITD Perkin-Elmer mass spectrometer coupled to a Perkin-Elmer Sigma-3 gas chromatograph, using a SP-2100 capillary column (30 m × 0.25 mm) from 160° C to 200° C at 2° C/min.

α-amylase digestion. FII_1 and FII_2 fractions were digested with α-amylase (Calbiochem) in 0.05M buffer borate-citrate-phosphate pH 5.5 for 24 h at 37° C. The reducing sugars liberated were evaluated by the method of Somogyi (28) and Nelson (20).

Chemical deglycosylation of α-glucan-protein complex. Total alkali soluble α-glucan and both α-glucan subfractions were deglycosylated with anhydrous trifluoromethanosulfonic acid (TFMS) by the method of Sojar and Bahl (27) for different periods of time (0.5-2 h). After dialysis, the remaining protein material was analyzed by Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Gel electrophoresis. SDS-PAGE was performed using the Laemmli (17) method. Mini-gels of 0.75 mm thickness and 8 × 10 cm in size were used. The resolving gel was 12% (w/v) acrylamide. Samples were boiled for 5 min in the sample buffer before loading. Proteins were stacked and resolved at 20 mA with a water-cooling device. After electrophoresis, proteins were stained by soaking gels in 0.025% (w/v) Coomassie G-250, 10% (v/v) acetic acid and 25% (v/v) isopropanol in H_2O overnight, 0.0025% (w/v) Coomassie G-250, 10% (v/v) acetic acid and 10% (v/v) isopropanol in H_2O during 6-9 h, and 0.0025% (w/v) Coomassie G-250 and 10% (v/v) acetic acid in H_2O overnight. Gels were then destained in 10% (v/v) acetic acid and 12.5% (v/v) isopropanol in aqueous solution. Apparent molecular weights of polypeptides were determined by comparison of their Rf-values with those of corresponding SDS-PAGE molecular weight standards (BioRad).

Gels were stained for carbohydrate using the Keyser method (16).

Proteolytic digestion of the extracted cell wall. Samples of mucilage-free cell walls prepared by treatment with water at 60° C were incubated for 24 h at 37° C with commercial pronase (0.5 mg/ml) in 0.1 *M* citrate-phosphate buffer pH 7.2. Toluene was added to the incubation mixture to avoid bacterial growth.

Electron microscopy. Aqueous suspensions of native cell walls and remaining sediments after each treatment (60° C water, 60° C water followed by the alkali extraction, and 60° C water plus proteolytic digestion) were dried on Forvard-coated grids, shadowed with Au-Pd and examined in a Philips 300 electron microscope.

Results

Isolation and chemical analysis of α-glucan subfractions. Figure 1 shows the elution profile of fraction FII when fractionated on the Sepharose column. Two peaks (FII_1, 18-20%; FII_2, 80-82% w/w), were apparent. As shown in Table 1, the material included under both peaks contained neutral sugars (80-90%) and protein (6-15%). Glucose was by far the major neutral sugar

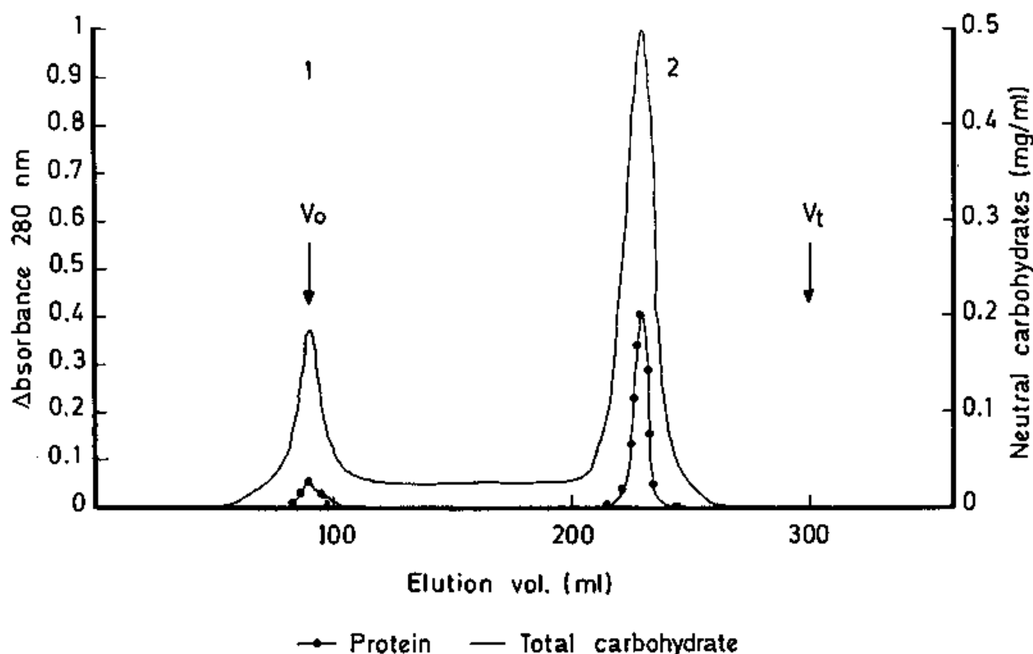

Fig. 1. Column chromatography of α-glucan fraction (FII) on Sepharose CL-2B. V_0: exclusion Vol., Vt: total vol.

found in both subfractions but mannose and galactose, which were present in minimal amounts in the crude fraction FII, where also detected.

Amino acid analysis of both subfractions showed the presence of all the usual amino acids (not shown). Glucosamine was the only aminosugar encountered.

Infrared spectra. The infrared spectra of the two α-glucan subfractions are shown in Figure 2. FII_1 showed the caracteristic band of polysaccharides having the α configuration (850 cm^{-1}) together with the band at 790 cm^{-1} feature of α-(1->3) linked glucans (4). The spectrum of FII_2 is distinguished by bands at 850 cm^{-1} (α configuration), 790 cm^{-1} [α-(1->3)] and 930 cm^{1} [α-(1->4)]. The absence of a band at 760 cm^{-1} characteristic of α-(1->4) polysaccharides suggests that this fraction FII_2 is related to nigeran, a linear glucan alternatively 1,3/1,4 α linked (4), that also does not show a band at 760 cm^{-1}.

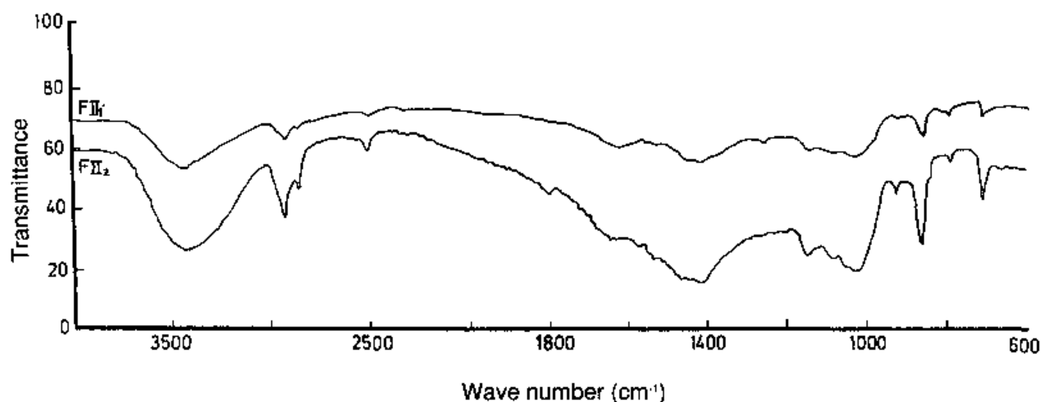

Fig. 2. Infrared spectra of the two α-glucan subfractions.

TABLE 2
GLC-MS DATA FOR THE PMAAs FROM α GLUCAN FRACTION AND ITS CORRESPONDING SUBFRACTIONS

PMAA	RRT*	Major mass-spectrum fragments (m/z)	Deduced linkage	Relative mol (%)		
				FII	FII_1	FII_2
2,3,4,6-Me_4-Glc**	1.00	45,87,88,101,102,118,129,130,161,162	Glc-(1-	1.2	tr***	0.9
2,4,6-Me_3-Glc	1.83	45,87,101,118,129,161,174,234,277	-3)-Glc-(1-	79.9	96.1	72.6
2,3,6-Me_3-Glc	2.27	45,102,118,130,162,173,233,277	-4)-Glc-(1-	18.9	3.9	26.5
2,4-Me_2-Hex	2.50	87,118,129,174,189,234	-3,6)-Hex-(1-	tr	tr	tr

* Retention time relative to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol on OV-225 at 170°. ** 2,3,4,6-Me-Glc_4=1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol, etc. *** tr = traces (< 0.1%).

Methylation analysis. The results for the fraction FII and subfractions FII_1 and FII_2 are shown in Table 2. FII_1 gave a very high percentage of 2,4,6-Me_3-glucitol, indicating that the polysaccharide consisted mainly of α-(1->3)-linked glucan. The presence of an appreciable amount of 2,3,6-Me_3-glucitol indicated (1->4)-linked glucose (3.9%). Traces of 2,3,4,6,Me_4-glucitol (coming from terminal units) as well as of 2-4 Me_2-hexitol (branch points) in the glucan backbone, suggested a high size lineal polysaccharide (> 200 KDa).

On the other hand, FII_2 gave mainly 2,4,6-Me_3-glucitol (72.6%) but with a very significant proportion of 2,3,6-Me_3-glucitol (26.5%), showing the presence of (1->3) and (1->4)-linked glucose in the polysaccharide backbone. The only traces of dimethylated hexoses indicated a very low ramification degree in this α-glucan; however, the higher proportion of terminal glucose residues (0.9%), suggested the small molecular size of this unbranched polysaccharide (≈ 20 kDa).

α amylase digestion. When incubated with α-amylase neither FII_1 nor FII_2 fractions released any reducing sugar indicating that they were free of glycogen or starch-like material.

Protein analysis. When components included in fraction FII and subfractions FII_1 and FII_2 were subjected to SDS-PAGE. They did not enter the polyacrylamide gel. However once chemically deglycosylated they migrated as a couple of rather diffuse bands (Fig. 3) which were stained only by Coomassie G-250 (estimated molecular weight about 59 and 63 kDa).

Electron microscopy. Shadowed *Armillaria mellea* native cell walls (Fig. 4a) showed the typical granular texture with underlying fibres, observed at the outer surface of basidiomycete hyphal walls (11, 21, 32). After 60° C water treatment for extracting the mucilage, loosely bound to the wall (3), thick fibres can be distinguished on the outer surface of the walls partially masked by an amorphous component (Fig. 4b). When the cell walls deprived of mucilage were digested with pronase (Fig. 4c) such shadowed fibres are more evident, suggesting the presence of proteinaceus material on the outer face. Some of the fibres seem to be mainly longitudinally interwoven but other thinner fibrils are cross-arranged between them. Treatment with KOH removed all this fibrillar material (Fig. 4d), suggesting their α-glucan nature, and give rise to a different fibrillar layer composed of chitin (11).

Discussion

Chemical analysis of alkali soluble, acid precipitated fraction from *Armillaria mellea* cell walls showed that neutral sugars [glucose, mainly linked in α-(1->3)] were the major components, with smaller amounts of protein material (24). These results are in good agreement with

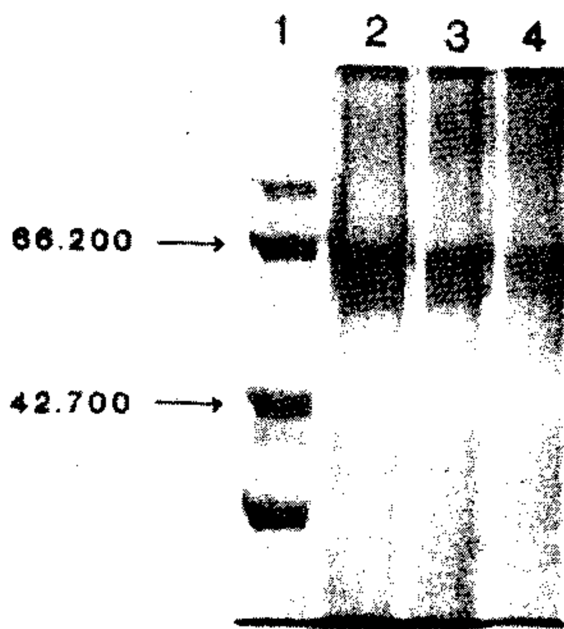

Fig. 3. SDS-PAGE of deglycosylated α-glucan fractions stained with Coomassie G-250.
1: Molecular weight standards.
2: Deglycosylated total α glucan.
3: Deglycosylated FII_2 subfraction.
4: Deglycosylated FII_1 subfraction.

those reported for α-glucan from other basidiomycete cell walls [*Schizophyllum commune* (25, 26), *Coprinus* sp. (5, 14) and *Agaricus bisporus* (10, 19, 21)].

Electron microscopy of complete and residual *Armillaria mellea* walls together with the chemical characterization of the isolated fractions can be related to the location of the different polysaccharides in the hyphal wall.

This α-glucan is located as a layer outside the wall. Our chemical studies strongly suggest that two different polysaccharides are associated in this layer: a high molecular weight α-(1->3) glucan (FII_1) and a smaller α-(1->3) (1->4) glucan (FII_2) which accounts for the larger part of this fraction (FII). The FII_2 α-glucan molecular weight calculated on the basis of chemical analysis (≈ 20 kDa) is clearly smaller than that calculated by its elution pattern in Sepharose CL-2B (estimated molecular weight: 250-350 kDa). Parallel experiments using Sepharose CL-4B and Sepharose CL-6B were in agreement with this result. It could be suggested that the FII_2 subfraction of *Armillaria mellea* α-glucan might be composed by a series of short α-(1->3) (1->4) glucan chains bound by no-covalent linkages, giving rise to a larger fibrillar structure. This organization should have a crystalline structure as previously described in *Schizophyllum commune* S-glucan subfraction not degraded by S-glucanase (9). On the other hand, the infrared spectra from *Armillaria mellea* α-glucan subfractions showed significant differences between them which could correspond to the slightly distinct fibres observed in the electron microscope.

Protein associated to α-glucan seems to be linked in some way to the polysaccharide, since it could not be removed from this glucan by gel-filtration chromatography on Sepharose CL-2B as well as CL-4B and CL-6B; moreover, SDS-PAGE carried out before deglycosylation showed a single band at the origen. SDS-PAGE of deglycosylated α-glucan subfractions gave rise in all cases to two bands (63 and 59 kDa) indicating the presence of two different proteins. Bottom and Siehr (5) suggested covalent unions between α-glucan and protein by 2-acetamide-2-deoxyglucose or its dimer in *Coprinus macrorhizus* S-glucan. If this is so in *Armillaria mellea* α-glucans, the presence of two polypeptide bands of close molecular weight could be due to a partial effect of TFMS (even in

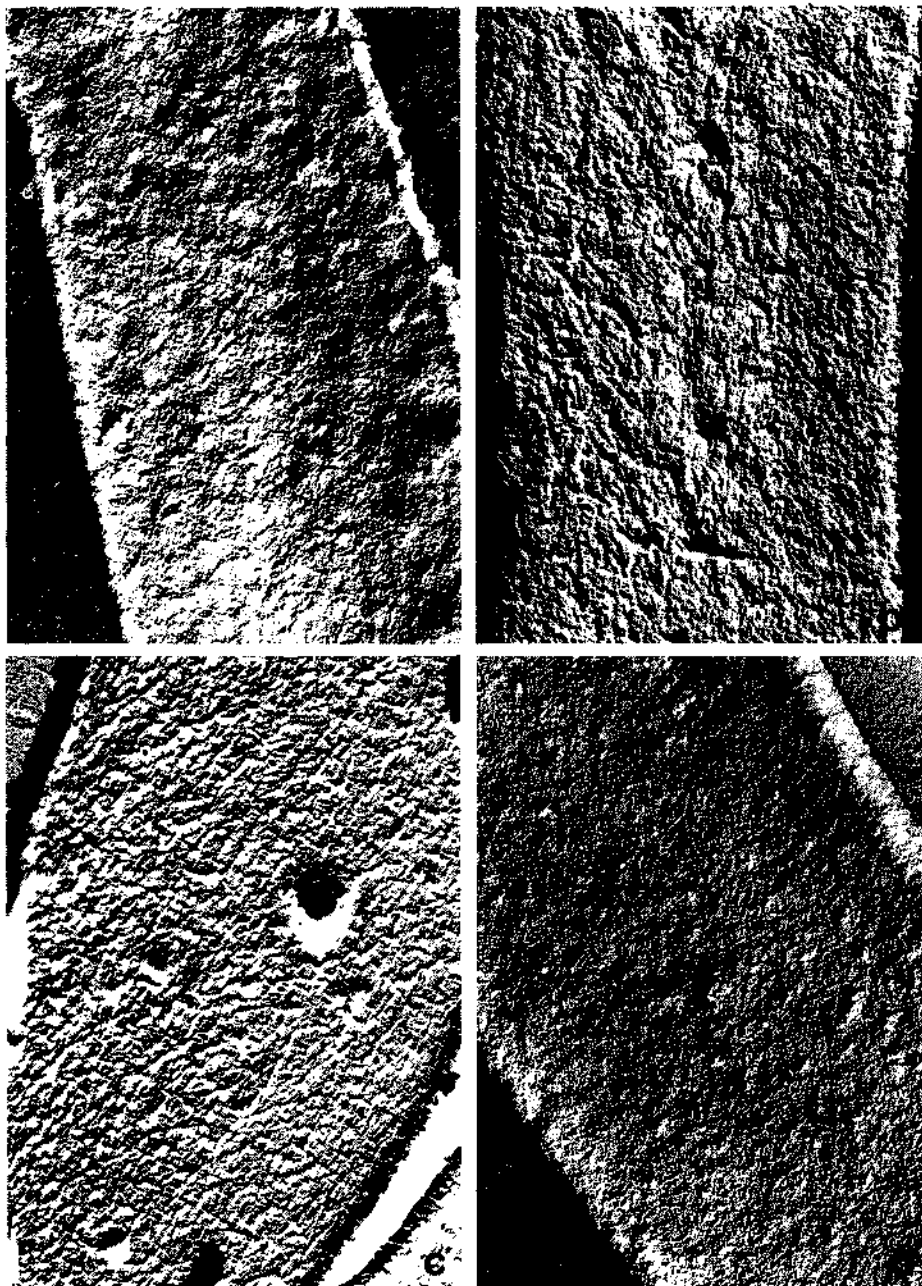

Fig. 4. Electron microscopy of shadowed *Armillaria mellea* mycelial walls. a: Native cell wall (× 12.430). b: Cell wall after mucilage extraction (×13.680). c: Cell wall after mucilage extraction and digestion with pronase (×15.860). d: Cell wall after cold alkali treatment (×16.950).

the strongest conditions) on the bridge between glucan and protein. In fact, glycosidic linkages can be broken by TFMS, but peptide linkages cannot. If modified sugars (N-acetylglucosamine oligomers) are involved in the glucan-protein linkage, as described by Bottom and Siehr (5), the TFMS breakage could be partial, and the polypeptide would retain in some cases these sugars.

On the other hand, electron microscopy showed that there was a thin pronase-sensitive layer outside the α-glucan which may correspond with that described in *Schizophyllum commune* cell walls (31), where a glycoprotein layer partially masked the α-glucan fibrils. The presence of protein material accesible to pronase action, suggests that protein chains would be located externally to the α-glucan fibrils.

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Recuento de fagos en aguas del Canal Imperial de Aragón y del río Ebro en Zaragoza

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Summary

Zaragoza city supply channel and the river Ebro (up and downstream urban sewage) were studied for the presence of coliphages and *B. fragilis* phages and their relationship with the bacterian faecal indicators.

In the supply channel the coliphages geometric mean was of 130 ufp/100 ml, and showed no corelation with faecal and total coliforms, but it showed indirect correlation with ambiental temperature.

In the river Ebro the coliphages geometric mean ranged from 290 to 8,000 ufp/100 ml; the relationship with total and faecal coliforms and faecal streptococci was high, but they were temperature independent.

With the methodology utilized *B. fragilis* phages only were recovered in samples with faecal coliforms levels $> 1 \times 10^4$ ufc/100 ml.

Key words: Coliphages, B. fragilis *phages, faecal indicators, supply channel, river.*

Resumen

En aguas del canal de abastecimiento de Zaragoza y del río Ebro (antes y después de los vertidos urbanos) se estudió la presencia de colífagos y fagos de *B. fragilis* y su relación con algunos indicadores bacterianos.

En el canal de abastecimiento la media geométrica de colífagos fue de 130 ufc/100 ml. No existe correlación con coliformes fecales ni coliformes totales y sí una correlación inversamente proporcional a la temperatura ambiental.

En el río Ebro los colífagos, con recuentos medios entre 290 y 8.000 ufp/100 ml, se relacionan con el número de coliformes totales y fecales y con el de estreptococos fecales, siendo independientes de la temperatura.

Con la metodología utilizada únicamente se detectaron fagos de *B. fragilis* en aguas con niveles de coliformes fecales $> 1 \times 10^4$ ufc/100 ml.

(*) A quien debe dirigirse la correspondencia.

Introducción

La vigilancia del grado y el origen de la contaminación fecal de las aguas superficiales es necesaria para poder evaluar los posibles riesgos para la salud y en su caso elegir las medidas correctoras adecuadas.

Estamos haciendo un estudio prospectivo de larga duración sobre la contaminación fecal de las aguas del entorno de Zaragoza: el canal de abastecimiento de la ciudad (Canal Imperial de Aragón) y el río receptor de los vertidos (Ebro). Analizamos la distribución espacial y estacional de la contaminación y caracterizamos la carga microbiana aportada por la ciudad al río. En este estudio hemos querido incluir posibles indicadores de contaminación viral de origen fecal humano.

Como ya se ha recogido en amplias revisiones, los coliformes termotolerantes y otros grupos de bacterias utilizadas como indicadores de contaminación bacteriana en las aguas, difieren considerablemente de los virus patógenos humanos, en cuanto a su ecología y resistencia a los tratamientos de descontaminación. Puesto que la determinación directa de tales virus no puede realizarse de forma rutinaria, se han estudiado diversos marcadores, tanto bacterianos como virales. Entre los marcadores que parecen más adecuados se encuentran los bacteriófagos (1, 5, 6, 9, 10, 11, 13, 19).

Los colífagos (fagos somáticos de *Escherichia coli*), aunque presentan problemas diversos (heterogeneidad, diversa resistencia a las condiciones ambientales, presencia en aguas superficiales sin contaminación (9, 17) son los más universalmente utilizados (5, 6, 10, 18, 19). Como alternativa a los colífagos se han propuesto otros grupos mejor definidos como los fagos RNA (7) o los fagos de *Bacteroides fragilis* (21). Los fagos RNA presentan los inconvenientes de su escasa cantidad en aguas contaminadas (7, 11, 15) o la posibilidad de multiplicación ambiental (4). Son por ello considerados marcadores de contaminación por aguas residuales, más que de contaminación fecal humana (4, 9). Los fagos de *B. fragilis* tampoco se encuentran en la cantidad apropiada en aguas contaminadas (2, 11), pero su origen se describe como exclusivamente humano y carecen de capacidad de multiplicación ambiental (21), por lo que pueden ser valiosos para discernir el origen de una contaminación (9).

Por estas razones, la investigación de fagos somáticos de *E. coli* y *B. fragilis* nos pareció inicialmente la más adecuada. Presentamos aquí los resultados obtenidos de recuentos de estos bacteriófagos y su relación con algunos indicadores bacterianos en aguas del Canal Imperial de Aragón y río Ebro a su paso por Zaragoza.

Materiales y métodos

Muestreo (Fig. 1). Se realizó a lo largo de los años 1990 y 1991.

- Punto 1 (90 muestras tomadas a intervalos de 1 semana). Canal Imperial de Aragón, a la entrada de la planta potabilizadora de la ciudad de Zaragoza y que trae agua del río Ebro recogida en Tudela (Navarra), sin recibir vertidos controlados a lo largo de 80 km.
- Puntos 2, 3 y 4 (33 muestras distribuidas regularmente a lo largo del estudio). En el río Ebro a su paso por Zaragoza y con características microbiológicas diferenciales (Tabla 3) (Marcen *et al.*, 1989. Res. V Simposio de Laboratorios e Institutos Municipales de Salud Pública).
    - Punto 2. A la altura del puente de la autopista, cuando aún no se han producido vertidos de la ciudad.
    - Punto 3. A la altura del puente de Santiago en el que se han producido vertidos urbanos de mediano caudal, además del efluente de una depuradora.

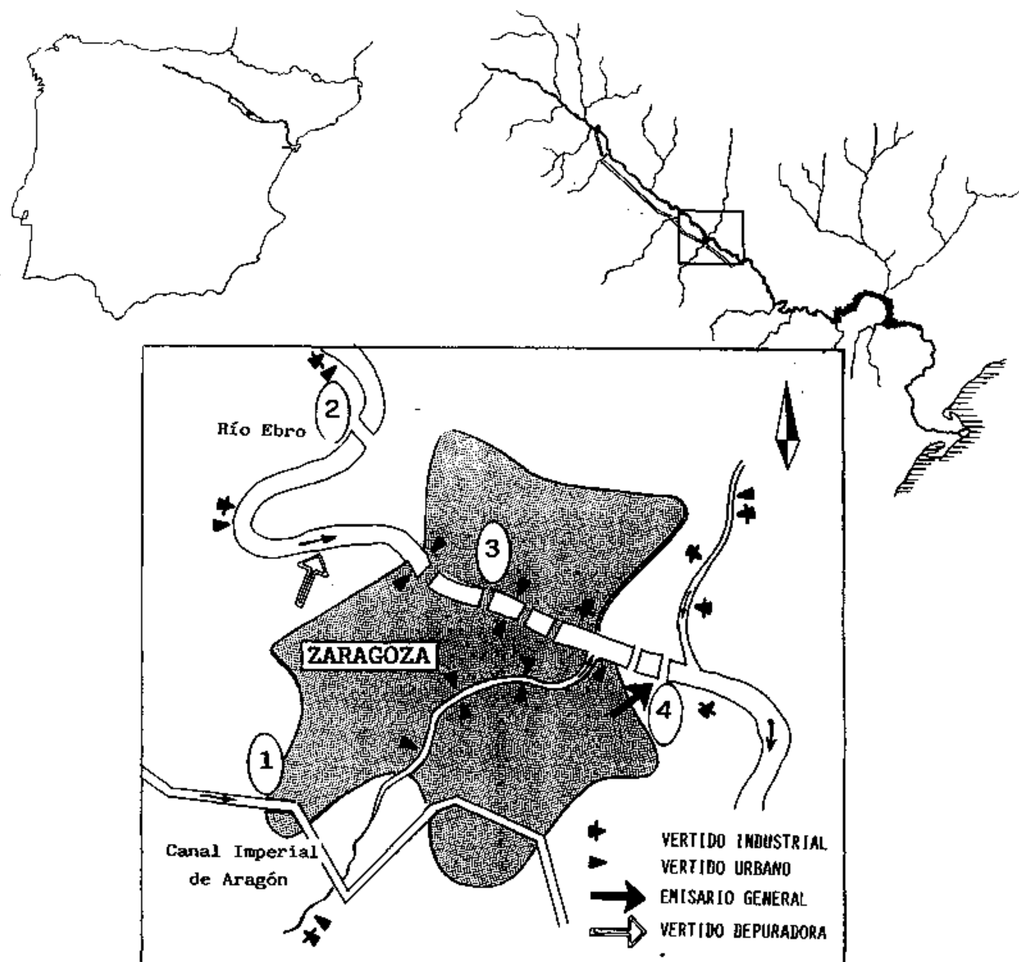

Fig. 1. Situación de los puntos de muestreo.

- Punto 4. En el puente del ferrocarril, en el que las aguas arrastran casi todos los vertidos domésticos de la ciudad, incluidas las aguas residuales que van al río Huerva, que desemboca antes de la toma. Quedan excluidos los vertidos que llegan con el río Gállego y algunos colectores que comprenden una pequeña parte de aguas residuales domésticas y una parte importante de las industriales.

Las muestras fueron recogidas en recipientes de 1 l, de plástico y estériles; por inmersión en la corriente a unos 50 cm de la superficie. Se guardaron en nevera hasta la realización del análisis (máximo 4 h para los recuentos bacterianos y 24 para los virales).

Recuento de colífagos. Técnica directa en agar monocapa de Grabow (6) con una variante nalidíxico resistente obtenida en el laboratorio a partir de la cepa CIP 5530 proporcionada por la Colección Española de Cultivos Tipo (CECT 543).

Recuento de fagos de B. fragilis *(sólo en las muestras del río Ebro en Zaragoza).* Modificación de la técnica de recuento directo de Tartera y Jofre (21) para analizar cada vez 10 ml de agua (Lafarga, M. A. *et al.*, 1989. Res. V Simposio de Laboratorios e Institutos Municipales de Salud Pública):

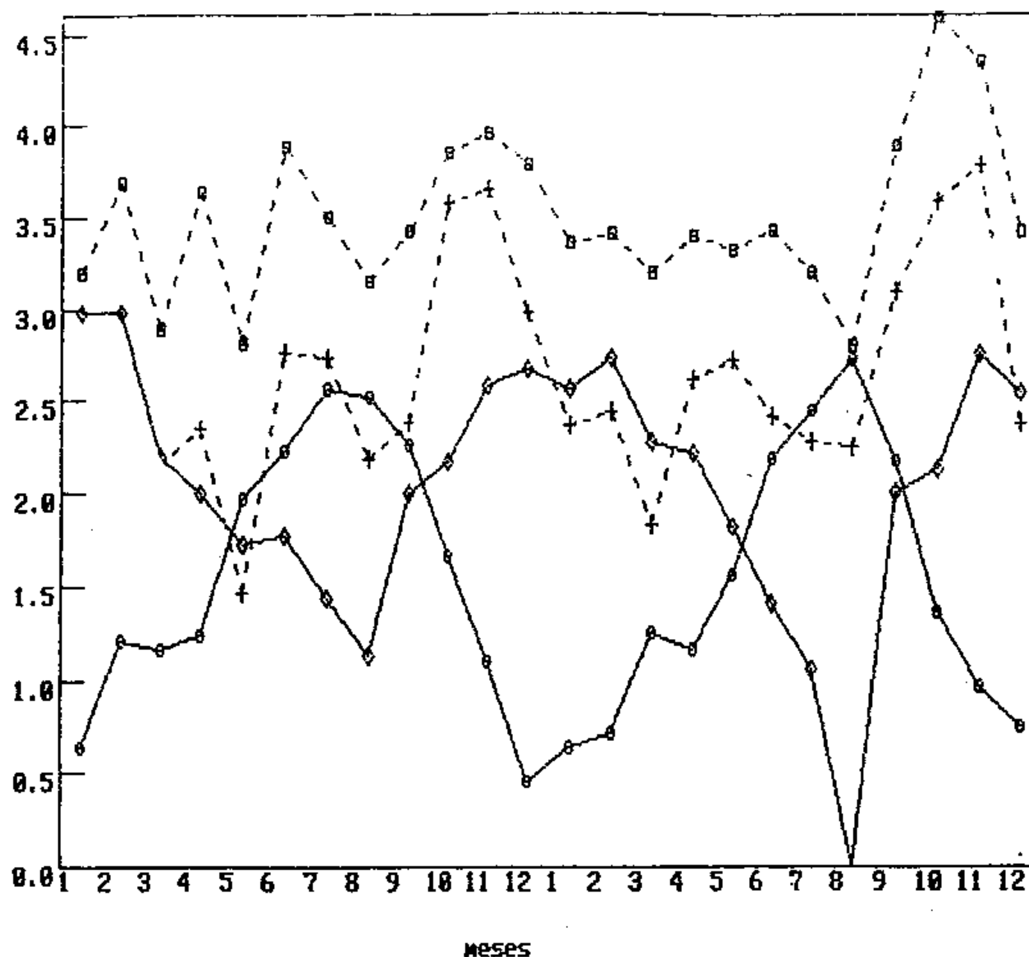

Fig. 2. Evolución mensual de colifagos, coliformes totales y fecales y temperatura ambiental (medidas geométricas). Canal de abastecimiento (p. 1), 1990-1991. –◇– Colifagos, ufp/100 ml, log 10. –□– Coliformes totales, ufc/100 ml, log 10. – + – Coliformes fecales, ufc/100 ml, log 10. –○– Temperatura ambiental, grados centígrados/10.

Se mezclan con suavidad en baño a 48° C, 10 ml de agua descontaminada con cloroformo (20 min 30% vol/vol) + 2,5 ml de crecimiento exponencial de la cepa hospedadora + 10 ml de medio MBB con agar (5 g/l). Se vierten sobre 6 placas con medio base. Se incuban en jarra de anaerobiosis, 37° C, 48 h. El límite teórico de detección es de 10 ufp/100 ml.

En las muestras negativas con el método anterior se utilizó el NMP con enriquecimiento propuesto por los mismos autores (21). La cepa receptora *B. fragilis* B40 fue cedida por el Departamento de Microbiología de la Universidad de Barcelona.

Recuentos bacterianos. Técnica normalizada de filtración (APHA) (1) con los siguientes medios y temperaturas de incubación: para los coliformes totales, caldo m-Endo MF (Difco) suplementado con 1,2% de agar e incubado a 36° C ± 1° C. Para los coliformes fecales el mismo medio incubando a 44° C ± 0,5° C, y para los estreptococos fecales medio de Slanetz (Oxoid) incubando a 44° C ± 0,5° C.

Estudio estadístico. Se realizó mediante el programa Microsta (Microsoft). Análisis descriptivo, estudio del coeficiente de correlación de Pearson (r) de recuentos de colifagos con otros pa-

TABLA 1
RECUENTOS DE COLIFAGOS (ufp/100 ml), COLIFORMES FECALES Y COLIFORMES TOTALES (ufc/100 ml) EN EL CANAL DE ABASTECIMIENTO DE ZARAGOZA. PUNTO 1. 1990-1991

	N.° de muestras analizadas	Media*	Rango	Mediana
Colifagos	90	$2,44 \times 10^2$ ($3,68 \times 10^2$)	$0\text{-}2,10 \times 10^3$	$1,3 \times 10^2$
Coliformes totales	82	$3,01 \times 10^3$ ($2,90 \times 10^3$)	$2 \times 10^2\text{-}15 \times 10^3$	2×10^3
Coliformes fecales	73	$5,33 \times 10^2$ ($9,7 \times 10^2$)	$9\text{-}5,60 \times 10^3$	3×10^3

*Desviación típica.

rámetros, y chi-cuadrado para relacionar variables semicuantitativas. Al hacer los estudios de correlación se utilizó el log 10 de los recuentos.

Resultados

En el Canal Imperial, tal como aparece recogido en la Tabla 1, los recuentos de colifagos oscilaron entre 0 y 2.100 ufp/100 ml, con una media geométrica de 130. No se detectaron colifagos en 6 de las muestras, todas ellas con recuentos de coliformes fecales por debajo de 200 ufc/100 ml.

No encontramos correlación de los colifagos con los recuentos de coliformes totales ni con los de coliformes fecales (r = 0,0097 y 0,0498) (Tabla 2). Sin embargo, al comprobar la correlación con la temperatura ambiental, el índice fue r = –0,8050. La disminución en el número de fagos de *E. coli* conforme aumenta la temperatura se refleja también en la Fig. 2, en las que se representan las medidas por meses. Los niveles máximos de estos virus se producen de noviembre a febrero, o mejor dicho, con temperaturas medias de 5-10° C, comenzando el descenso en número alrededor de los 18° C y los picos más bajos a temperaturas de 30° C.

En el río Ebro a su paso por Zaragoza con unos recuentos medios de coliformes totales en los 3 puntos de muestreo de 24.200, 26.000 y 800.000 ufc/100 ml, los recuentos medios de fagos de *E. coli* fueron de 295, 380 y 8.000 ufp/100 ml (Tabla 3).

La Tabla 6 presenta las correlaciones de los colifagos con los otros parámetros estudiados que, al contrario que en las muestras del Canal Imperial, muestran una relación directa del número de colifagos con el de coliformes totales, fecales y estreptococos fecales y sin una apreciable influencia de la temperatura. La proporción media en log 10 de coliformes totales/colifagos fue de 1,67; coliformes fecales/colifagos, 1,40, y estreptococos fecales/colifagos, 1,00.

El estudio de los fagos de *B. fragilis* demostró un bajo porcentaje de muestras positivas y un bajo recuento en las mismas (Tablas 5 y 6).

Hubo 25 muestras con presencia de fagos de *B. fragilis* (5 positivas por recuento directo en

TABLA 2
CORRELACION DE LOS RECUENTOS DE COLIFAGOS (LOG 10) CON OTROS PARAMETROS. CANAL IMPERIAL (P. 1). 1990-1991

	Coliformes totales (log 10)	Coliformes fecales (log 10)	Temperatura* (° C)
r =	0,0097	0,0498	–0,8050

* Temperatura ambiental medida el día de la toma de la muestra. Centro Metereológico del Ebro.

TABLA 3
RECUENTOS DE FAGOS (ufp/100 ml) Y OTROS INDICADORES (ufc/100 ml)
EN EL RIO EBRO, 1990-1991

	Punto 2			
	N.° de muestras	Media*	Rango	Mediana
Colifagos	32	$4,34 \times 10^{2}$ ($4,58 \times 10^{2}$)	$35\text{-}2,36 \times 10^{3}$	$2,95 \times 10^{2}$
Coliformes totales	32	$4,40 \times 10^{4}$ ($6,01 \times 10^{4}$)	$4 \times 10^{2}\text{-}2,80 \times 10^{5}$	$2,42 \times 10^{4}$
Coliformes fecales	32	$6,04 \times 10^{3}$ ($7,18 \times 10^{3}$)	$2 \times 10^{2}\text{-}3,28 \times 10^{5}$	$3,15 \times 10^{3}$
Estreptococos fecales	32	$4,02 \times 10^{2}$ ($4,39 \times 10^{2}$)	$40\text{-}2,44 \times 10^{3}$	$2,70 \times 10^{2}$
Fagos de *B. fragilis*	33	0,22 (0,42)	0-1	0
	Punto 3			
	N.° de muestras	Media(*)	Rango	Mediana
Colifagos	31	$4,86 \times 10^{2}$ ($3,39 \times 10^{2}$)	$8 \times 10^{1}\text{-}1,47 \times 10^{3}$	$3,80 \times 10^{2}$
Coliformes totales	32	$3,49 \times 10^{4}$ ($2,69 \times 10^{4}$)	$1,40 \times 10^{3}\text{-}9,10 \times 10^{4}$	$2,60 \times 10^{4}$
Coliformes fecales	32	$7,19 \times 10^{3}$ ($8,06 \times 10^{3}$)	$4 \times 10^{2}\text{-}4 \times 10^{4}$	$4,60 \times 10^{3}$
Estreptococos fecales	32	$5,42 \times 10^{2}$ ($7,40 \times 10^{2}$)	$6 \times 10^{1}\text{-}3 \times 10^{3}$	$2,25 \times 10^{2}$
Fagos de *B. fragilis*	32	0,12 (0,33)	0-1	0
	Punto 4			
	N.° de muestras	Media(*)	Rango	Mediana
Colifagos	30	$1,05 \times 10^{5}$ ($3,51 \times 10^{5}$)	$3,60 \times 10^{2}\text{-}1,90 \times 10^{6}$	8×10^{3}
Coliformes totales	31	$3,85 \times 10^{6}$ ($8,83 \times 10^{6}$)	$6 \times 10^{4}\text{-}3,60 \times 10^{7}$	8×10^{5}
Coliformes fecales	31	$9,01 \times 10^{5}$ ($1,67 \times 10^{6}$)	$1 \times 10^{2}\text{-}8,40 \times 10^{6}$	$3,20 \times 10^{5}$
Estreptococos fecales	30	$1,44 \times 10^{4}$ ($1,53 \times 10^{4}$)	$1,20 \times 10^{3}\text{-}6,10 \times 10^{4}$	$8,50 \times 10^{3}$
Fagos de *B. fragilis*	31	6,06 (10,75)	0-50	2

* Desviación típica.

TABLA 4
CORRELACION DE RECUENTOS DE COLIFAGOS (LOG 10)
CON OTROS PARAMETROS. RIO EBRO (P. 2, 3 y 4). 1990-1991

	r
Coliformes totales (log 10)	0,7164
Coliformes fecales (log 10)	0,7773
Estreptococos fecales (log 10)	0,7380
Temperatura* (° C)	0,0255

* Temperatura ambiental medida el día de la toma de la muestra. Centro Metereológico del Ebro.

placa y 20 mediante enriquecimiento y NMP). Su número en relación con los otros parámetros aparece recogido en la Tabla 6. Hemos encontrado fagos para *B. fragilis* en aguas con recuentos en log 10 de colifagos por encima de 3, de coliformes totales por encima de 5 y de coliformes y estreptococos fecales por encima de 4 (chi-cuadrado, $p < 0{,}001$).

Discusión

Los recuentos obtenidos de colifagos (Tablas 1 y 3), en los diferentes puntos de muestreo, son inferiores a los descritos en la bibliografía para aguas de muy diverso grado de polución, en los que se mantiene a niveles semejantes o incluso superiores a los recuentos de coliformes fecales (2, 11, 20). Este hecho es seguramente debido al procedimiento utilizado. Para el recuento de colifagos, nosotros utilizamos la cepa *E. coli* CIP 5530, descrita como buena receptora de fagos. Posteriormente comparando los recuentos con la cepa C, universalmente utilizada con estos propósitos (6), comprobamos (datos no descritos) un rendimiento menor de la primera en la proporción 1:5, por lo que en la actualidad hemos pasado a utilizar de forma general la segunda. Hemos mantenido para el recuento la técnica de Grabow que en un estudio anterior demostró que nos permitía hacer el análisis de las muestras con una contaminación microbiana muy variable y era sencilla y reproducible (Lafarga, M. A. *et al.,* 1989. Res. V Simposio de Laboratorios e Institutos Municipales de Salud Pública).

En nuestro estudio aparecen marcadas diferencias en las características microbiológicas entre los puntos de muestreo 1, por un lado, y los 2, 3 y 4 por otro.

En las aguas del río Ebro a su paso por Zaragoza se observan altos niveles de contaminación fecal, según los índices bacterianos (Tabla 3). Los recuentos de colífagos aumentan progre-

TABLA 5
DISTRIBUCION DE FAGOS DE *B. FRAGILIS*
EN EL RIO EBRO (P. 2-4). 1990-1991

	N.°	%
Ausencia	61	70,93
Presencia:		
< 10 ufp/100 ml	19	22,09
≥ 10 ufp/100 ml	6	6,98
Total	86	

TABLA 6
CORRELACION DE RECUENTOS DE *B. FRAGILIS*** (LOG 10) CON OTROS PARAMETROS. RIO EBRO (P. 2, 3 y 4). 1990-1991

	r
Colífagos (log 10)	0,5360
Coliformes totales (log 10)	0,5471
Coliformes fecales (log 10)	0,6148
Estreptococos fecales (log 10)	0,7106
Temperatura* (° C)	–0,1302

* Temperatura ambiental medida el día de la toma de la muestra. Centro Metereológico del Ebro. ** Unicamente en muestras positivas.

sivamente a lo largo de los 3 puntos de muestreo, con unos niveles en el primer punto ligeramente superiores a los del agua del punto 1, mientras que en el último son semejantes o incluso superiores a los encontrados por nosotros y en un estudio anterior (Lafarga, M. A. *et al.,* 1989. Res. V Simposio de Laboratorios e Institutos Municipales de Salud Pública), y por otros autores (2, 8) en aguas residuales. Estos recuentos presentan, al igual que en numerosos trabajos en aguas de diferente origen (2, 5, 11, 12), una correlación muy alta con el resto de los indicadores de contaminación fecal, sobre todo con el grupo de coliformes fecales ($r = 0{,}7773$) (Tabla 4), sin apreciarse diferencias en esta relación entre los 3 puntos (datos no consignados).

En el caso del agua del canal de abastecimiento, los índices de contaminación bacteriana se mantienen en niveles mucho más bajos e independientes del número de colifagos (Tabla 1). Al estudiar los posibles factores que podían incidir en la evolución del número de colifagos, encontramos una marcada relación inversamente proporcional a la temperatura ambiental medida del día de la toma de la muestra (Tabla 2).

Aunque algunos autores han propuesto la utilización directa del número de colifagos como índice de contaminación fecal, por su gran correlación con el de coliformes fecales en cualquier tipo de agua (11, 23), numerosos trabajos han descrito también la falta de correspondencia en aguas muy diversas, pero con la característica común de su escasa contaminación. Así Haavelar *et al.* establecen que la correlación colifagos/coliformes fecales sólo puede esperarse en las cercanías de las fuentes de polución (9); O'Keege y Green en Escoia (12) y Rhodes *et al.* en Virginia (fagos RNA) (15) encuentran un alto grado de correlación en aguas de mar muy contaminadas y baja relación en aguas con menor polución. Cornax *et al.* describen ausencia de relación en aguas de playa poco contaminadas (2), al igual que Palmeteer *et al.* en ríos y lagos de Ontario escasamente contaminados (14).

Hemos recogido escasas referencias de correlación de colifagos con temperatura. Tartera y Jofre (20) refieren un mayor número de colifagos en aguas del Delta del Ebro, en los meses de invierno, pero con elevaciones paralelas al número de coliformes totales. Rhodes *et al.* (15) en sedimentos de estuario demuestran una relación directa con la temperatura del agua y la salinidad, suponiendo una mejor multiplicación de fagos en dichas condiciones. La falta de relación en las aguas del canal de abastecimiento con los otros indicadores fecales y la relación inversa con la temperatura no parece debida a la multiplicación de los fagos, ya que faltaría una suficiente concentración de bacterias receptoras (24) y debería de tratarse de fagos con unas temperaturas de multiplicación óptimas entre 5-10° C, que corresponderían a los denominados fagos de baja temperatura (LT) por Seeley y Primrose (16), lo que estaría en contradicción con el hecho de que se han detectado con una técnica que utiliza una temperatura de incubación de 37° C. Parece más bien que en el agua con una cierta contaminación fecal de origen, una vez introducida en

un cauce artificial en el que difícilmente se producen procesos de sedimentación que favorecerían la multiplicación fágica (15), el nivel de bacterias puede ir decreciendo por procesos de autodepuración, y el de fagos depende de una inactivación directamente proporcional a los ascensos térmicos.

En cuanto a los fagos de *B. fragilis,* hemos limitado su estudio a las aguas del río Ebro al tener datos previos de su ausencia en aguas del canal (Lafarga, M. A. *et al.,* 1989. Res. V Simposio de Laboratorios e Institutos Municipales de Salud Pública). Nuestros resultados de bajos porcentajes de muestras positivas y bajos recuentos en comparación con otros indicadores, aunque con recuentos algo menores, no difieren grandemente de los descritos por Tartera y Jofre en el río Llobregat (20), utilizando la técnica propuesta en este trabajo.

Cornax *et al.,* en aguas de mar (2), aún introduciendo mejoras en el procedimiento para aumentar la sensibilidad (3), únicamente detectan, al igual que en nuestro estudio, fagos de *B. fragilis* de forma sistemática en aguas con recuentos de coliformes fecales por encima de 1×10^4 ufc/100 ml.

La correlación de los recuentos de *B. fragilis* en las muestras positivas con los otros indicadores sería la esperada en unas aguas que reciben desechos urbanos con importante, aunque no exclusiva, contaminación fecal de origen humano.

Estos resultados nos obligan a revisar tanto el procedimiento utilizado (22) para el recuento, como su valor indicador, puesto que con la técnica utilizada, que incluía la descontaminación con cloroformo y el cultivo de *B. fragilis* en MBBA, pensamos que aunque con una buena especificidad hemos obtenido un alto porcentaje de falsos negativos.

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Numerical analysis of fatty and mycolic acid profiles of *Corynebacterium urealyticum* and other related corynebacteria

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Summary

The fatty and mycolic acid profiles of 52 strains of clinical origin belonging to *Corynebacterium urealyticum* were subjected to numerical analysis along with those of representative members of *Corynebacterium ammoniagenes, Corynebacterium bovis, Corynebacterium glutamicum, Corynebacterium jeikeium, Corynebacterium minutissimum, Corynebacterium pseudodiphtheriticum, Corynebacterium pseudotuberculosis, Corynebacterium xerosis, Corynebacterium renale, Corynebacterium cystitidis, «Corynebacterium ulcerans»* and one strain of the *Corynebacterium* F1 group. Strains were divided into eight clusters at an amalgamation distance of 7.4. *Corynebacterium urealyticum* appeared as an homogeneous cluster clearly distant from others, that included several members of the genus *Corynebacterium,* and it was characterized by its content on unsaturated mycolic acids of mainly 28 (28:1) and 30 (30:3) carbon atoms. On the basis of these results the taxonomic «status» of *Corynebacterium urealyticum,* a new species within the genus *Corynebacterium «sensu stricto»,* is further justified.

Key words: Corybacterium urealyticum, *lipids, numerical analysis.*

Resumen

Se ha realizado un análisis numérico de la composición de ácidos grasos y ácidos micólicos de 52 aislados clínicos de *Corynebacterium urealyticum* y diversas cepas de *Corynebacterium ammoniagenes, Corynebacterium bovis, Corynebacterium glutamicum, Corynebacterium jeikeium, Corynebacterium minutissimum, Corynebacterium pseudodiphteriticum, Corynebacterium pseudotuberculosis, Corynebacterium xerosis, Corynebacterium renale, Corynebacterium cystitidis, «Corynebacterium ulcerans»* y una cepa del grupo F1 de *Corynebacterium.* Las cepas analizadas se separaron en 8 *clusters* a una distancia de amalgamación de 7,4. *Corynebacterium urealyticum* constituyó un *cluster* homogéneo, claramente diferenciado del resto de corinebacterias incluidas en el estudio, caracterizándose por su contenido en ácidos micólicos insaturados de 28 (28:1) y 30 (30:3) átomos de carbono. De acuerdo con los resultados obtenidos, queda justificada la consideración de *Corynebacterium urealyticum* como nueva especie dentro del género *Corynebacterium «sensu stricto».*

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Introduction

Corynebacterium urealyticum has been recently described (20) to accommodate the *Corynebacterium* group D2 (17). This gram-positive microorganism shows morphological, physiological, biochemical (22) and chemical (14) characteristics that are similar to those of the true corynebacteria. *Corynebacterium urealyticum* has been isolated from human urine samples and found to be mainly involved in urinary tract infections (21). Typically, it exhibits multiantibiotic resistance, negative oxidase and indole tests, positive urease and catalase tests and inability to produce acids from most carbohydrates (15).

The original description of the species (20) was based on genetic analyses (guanine plus cytosine and DNA-DNA hybridization) of several strains. The results confirmed its taxonomic «status» as a new member of the genus *Corynebacterium*, a conclusion that had been previously suspected by physiological (15) and chemical studies (14). The relatively poor metabolic activity of this microorganism has hindered so far the application of numerical approaches to its taxonomy. In a recent work (14) the fatty and mycolic acid compositions of a collection of strains of *C. urealyticum* were determined and compared with those of other species within the genus. Since numerical analysis of lipid composition has been successfully applied to group and/or speciate corynebacteria (5, 24), we have similarly applied this criterium to check the phenotypical homogeneity of *C. urealyticum*. Fatty and mycolic acid profiles, previously reported (14), have been subjected in this work to cluster analysis. Clusters integrated by more than one strain were also subjected to discriminant analysis to define the variables mainly involved in their separation.

Materials and methods

Strains studied and culture conditions

Fifty two strains of *Corynebacterium urealyticum* (kindly provided by Dr. F. Soriano, Departamento de Microbiología Médica, Fundación Jiménez Díaz, Madrid, Spain), 1 of *C. ammoniagenes*, 1 of *C. bovis*, 3 of *C. glutamicum*, 26 of *C. jeikeium* (also provided by Dr. F. Soriano), 1 of *C. minutissimum*, 3 of *C. pseudodiphtheriticum*, 3 of *C. pseudotuberculosis*, 1 of *C. renale*, 2 of *C. xerosis*, 1 of *«C. ulcerans»*, 1 of *C. cystitidis* and 1 of *Corynebacterium* F1 group were analyzed (Table 1). They were cultivated on blood agar for 48 h at 37° C.

Analysis of fatty and mycolic acids

As previously described (14), cells were subjected to acid methanolysis (19), the liberated methyl esters of fatty and mycolic acids were studied by combined thin-layer chromatography, gas-liquid chromatography, mass spectrometry and pyrolysis gas-liquid chromatography techniques.

Numerical analysis

The original computing data were the percentages of fatty and mycolic acids, provided by a Chromatopac CR1A (Shimadzu) integrator attached to a capillary gas chromatograph. The values were transformed according to the expression $y = ln\ (1 + x)$ (4), being y the introduced data

TABLE 1
DESIGNATION AND SOURCE OF THE STRAINS STUDIED

Laboratory number	Identification
	Strains assigned to cluster A
C1	*Corynebacterium pseudotuberculosis* NCTC 4655.
C96	*C. pseudotuberculosis* CNCTC 17/62 (= ATCC 19410^T).
C99	*C. pseudotuberculosis* CNCTC 14/61.
C95	*C. bovis* CNCTC 68/77 (= ATCC 7715^T).
	Strains assigned to cluster B
C4	*C. jeikeium;* F. Soriano FS 134-I-1.
C5	*C. jeikeium* FS 134-I-2.
C6	*C. jeikeium* FS 194-I.
C7	*C. jeikeium* FS 155-I.
C8	*C. jeikeium* FS 128-A.
C9	*C. jeikeium* FS 112-I.
C10	*C. jeikeium* FS 112-A.
C11	*C. jeikeium* FS 154-I.
C12	*C. jeikeium* FS 105-P.
C13	*C. jeikeium* FS 45-I.
C14	*C. jeikeium* FS 18-A.
C15	*C. jeikeium* FS 40-P.
C17	*C. jeikeium* FS 90-I.
C18	*C. jeikeium* FS 86-I.
C19	*C. jeikeium* FS 84-I.
C20	*C. jeikeium* FS, Newton.
C21	*C. jeikeium* FS, Lothers.
C22	*C. jeikeium* FS, Gee.
C23	*C. jeikeium* FS, Pearson.
C24	*C. jeikeium* Fs, Horn.
C25	*C. jeikeium* FS, Lewis.
C26	*C. jeikeium* FS, Hershan.
C27, 28, 29, 30	*C. jeikeium* FS, Baxter.
C31	*C. jeikeium* FS, Weeks.
C32	*C. jeikeium* FS, Durham.
C97	*C. pseudodiphtheriticum* CNCTC 5/78.
C107	«*C. ulcerans*» CNCTC 41/74 (= NCTC 7907).
C100	*C. xerosis* CECT 538 (= ATCC 373^T).
C101	*C. xerosis* CNCTC 32/70.
C16	*C. jeikeium* FS 57-I.
C102	*C. glutamicum* CECT 78 (= ATCC 13287).
C103	*C. glutamicum* CECT 79 (= ATCC 21253).
C104	*C. glutamicum* CECT 80 (= ATCC 21254).
C105	*C. minutissimum* CNCTC 67/77 (= ATCC 23348).
C98	*C. pseudodiphtheriticum* CNCTC 1/49.
C106	*C. renale* CNCTC 34/70 (= ATCC 19412^T).
	Strains assigned to cluster C
C33	*C. urealyticum* FS 126-I.
C34	*C. urealyticum* FS 111-A.
C35	*C. urealyticum* FS 133-I.
C36	*C. urealyticum* FS 118-I.
C37	*C. urealyticum* FS 117-I.
C38	*C. urealyticum* FS 110-I.
C39	*C. urealyticum* FS 136-I.
C40	*C. urealyticum* FS 162-I.

TABLE 1 (Continue)

Laboratory number	Identification
C41	*C. urealyticum* FS 156-I.
C42	*C. urealyticum* FS 122-A-I.
C43	*C. urealyticum* FS 133-P.
C44	*C. urealyticum* FS 122-I.
C45	*C. urealyticum* FS 167-P.
C46	*C. urealyticum* FS 128-I.
C47	*C. urealyticum* FS 85791.
C48	*C. urealyticum* FS 42909.
C49	*C. urealyticum* FS 85176.
C50	*C. urealyticum* FS 85176-B-2.
C51	*C. urealyticum* FS 85675.
C52	*C. urealyticum* FS 85675-50-3.
C53	*C. urealyticum* FS 82158.
C54	*C. urealyticum* FS 82153.
C55	*C. urealyticum* FS 82642.
C56	*C. urealyticum* FS 82642-B-2.
C57	*C. urealyticum* FS 70346.
C58	*C. urealyticum* FS 70293.
C59	*C. urealyticum* FS 68241.
C60	*C. urealyticum* FS 70261.
C61	*C. urealyticum* FS 43-457.
C62	*C. urealyticum* FS PT-2.
C63	*C. urealyticum* FS 41-1655.
C64	*C. urealyticum* FS 65245.
C65	*C. urealyticum* FS 82449-48-2.
C66	*C. urealyticum* FS 82449-48-1.
C67	*C. urealyticum* FS AB-23015.
C68	*C. urealyticum* ATCC 43042^T.
C69	*C. urealyticum* ATCC 43043.
C70	*C. urealyticum* ATCC 43044.
C71	*C. urealyticum* FS 64471.
C72	*C. urealyticum* FS, Sharpe.
C73	*C. urealyticum* FS, Cotmun.
C74	*C. urealyticum* FS, Bristol Marmount.
C75	*C. urealyticum* FS, PT-1.
C76, 77, 78, 79	*C. urealyticum* FS, 42-1682.
C80	*C. urealyticum* FS 161-P.
C81-C87	*C. urealyticum* FS 64-FS70.
C88	*C. urealyticum* FS 52-0.
C89	*C. urealyticum* FS C-120.
C90	*C. urealyticum* FS C-92.
C91	*C. urealyticum* FS C-81.
C92	*C. urealyticum* FS C-77.
C93	*C. urealyticum* FS C-31.
C2	*C. pseudodiphtheriticum* ATCC 10700^T.
C108	*Corynebacterium* group F1 FS 19-59724.
C3	*C. ammoniagenes* ATCC 6871^T.
C94	*C. cystitidis* ATCC 29593.

T: Type strain. CNCTC: Czechoslovak National Collection of Type Cultures. Prague. Czechoslovakia. ATCC: American Type Culture Collection, Rockville, Md., USA. CECT: Colección Española de Cultivos Tipo, Valencia, Spain. NCTC: National Collection of Type Cultures, London, UK.

in the cluster analysis, and x the percentage of the acid considered (values of $x < 1\%$ were excluded). The cluster analysis employed was P2M (12) contained in the BMDP program package (BMPD: Biomedical Computer Programs, University of California Press, Los Angeles, California, USA), available at Centro de Proceso de Datos, Universidad de Murcia, Murcia, Spain. We used Euclidean distance to estimate the separation between cases, with data standardized to z-scores before the computation of distances, and the single linkage algorithm to join clusters. Once the clusters were established, a discriminant analysis (P7M, from BMDP) (16) was applied to estimate the variables inducing the separation of clusters and also the statistical validity of cluster analysis.

Results

Cluster analysis

Three clusters were defined according to the fatty and mycolic acid compositions, at an amalgamation distance of 7.4, and were named A to C. A dendrogram showing the relationships between them is depicted in Figure 1. At the distance cited *C. bovis* (CNCTC 68/77^{T}), *C. pseudodiphtheriticum* (ATCC 10700^{T}), *Corynebacterium* F1 (FS 19-59724), *C. ammoniagenes* (ATCC 6871) and *C. cystitidis* (ATCC 29593^{T}) appeared isolated (Fig. 1).

Cluster A. This cluster contained the three strains of *Corynebacterium pseudotuberculosis* (NCTC 4655, CNCTC 17/62 and CNCTC 14/61). Major fatty acids were saturated and monounsaturated of 16 carbon atoms (16:1 and 16:0); tuberculostearic acid was absent (Table 2). Mycolic acids ranged from 28-34 carbon atoms; monounsaturated of 32 (32:1) and 34 (34:1) carbon atoms and diunsaturated of 34 (34:2) carbon atoms were predominant (14); however, 34:1 appeared as the characteristic one, as shown by discriminant analysis (Table 3).

Corynebacterium bovis (CNCTC 68/77) contained saturated, mono- and diunsaturated fatty acids of 18 carbon atoms, along with tuberculostearate (Table 2). Mycolic acids varied from 26 to 32 carbon atoms, being saturated, mono-, di- and triunsaturated. Monounsaturated of 26 and 28 carbon atoms were predominant (14).

Cluster B. All the strains of *Corynebacterium jeikeium, C. xerosis* (ATCC 373 and CNCTC 32/70), *C. pseudodiphtheriticum* (CNCTC 5/78 and CNCTC 1/49), «*C. ulcerans*» CNCTC 41/64, *C. glutamicum* (CECT 78, CECT 79 and CECT 80), *C. minutissimum* CNCTC 67/77 and *C. renale* CNCTC 34/70 fell into this cluster. Hexadecanoic (16:0), octadecenoic (18: 1) and octadecadienoic (18:2) fatty acids were predominant. Tuberculostearic acid was absent, except for *C. pseudodiphtheriticum* (CNCTC 5/78 and CNCTC 1/49), *C. glutamicum* (CECT 78, CECT 79 and CECT 80) and *C. minutissimum* CNCTC 67/77, where low amounts of this compound were detected (Table 2). Mycolic acids from 26 to 36 carbon atoms with 1 to 4 double bonds were found (14). According to the discriminant analyses 36:2, 34:1, 32:0 and 30:0 were the representative compounds (Table 3).

At an amalgamation distance of 3.3 all but one (C16) strains of *C. jeikeium* were recovered in an homogeneous subcluster (subcluster B1). Hexadecanoic (16:0), octadecenoic (18:1) and octadecadienoic (18:2) fatty acids were predominant; no tuberculostearic acid was found (Table 2). Mycolic acids varied from 28 to 36 carbon atoms (saturated, mono-, di-, tri- and tetraunsaturated) (14). These strains were mainly typified by its content on 36:2 and 34:1 mycolic acids (Table 3).

Cluster C. The strains belonging to the *Corynebacterium urealyticum* species were recovered in this cluster. The profiles of fatty acids consisted of 14:0, 16:1, 16:0, 18:2, 18:1, 18:0 and tuberculostearic. Hexadecanoic acid and the unsaturated forms of 18 C were the most abundant (Table 2). Mycolic acids from 26 to 36 carbon atoms were found, 28:2, 28:1, 30:3, 30:2, 32:3 and 32:2 mycolic acids being predominant, as defined previously (14). However, 30:3 and 28:1 were revealed as the most diagnostic for this cluster (Table 3).

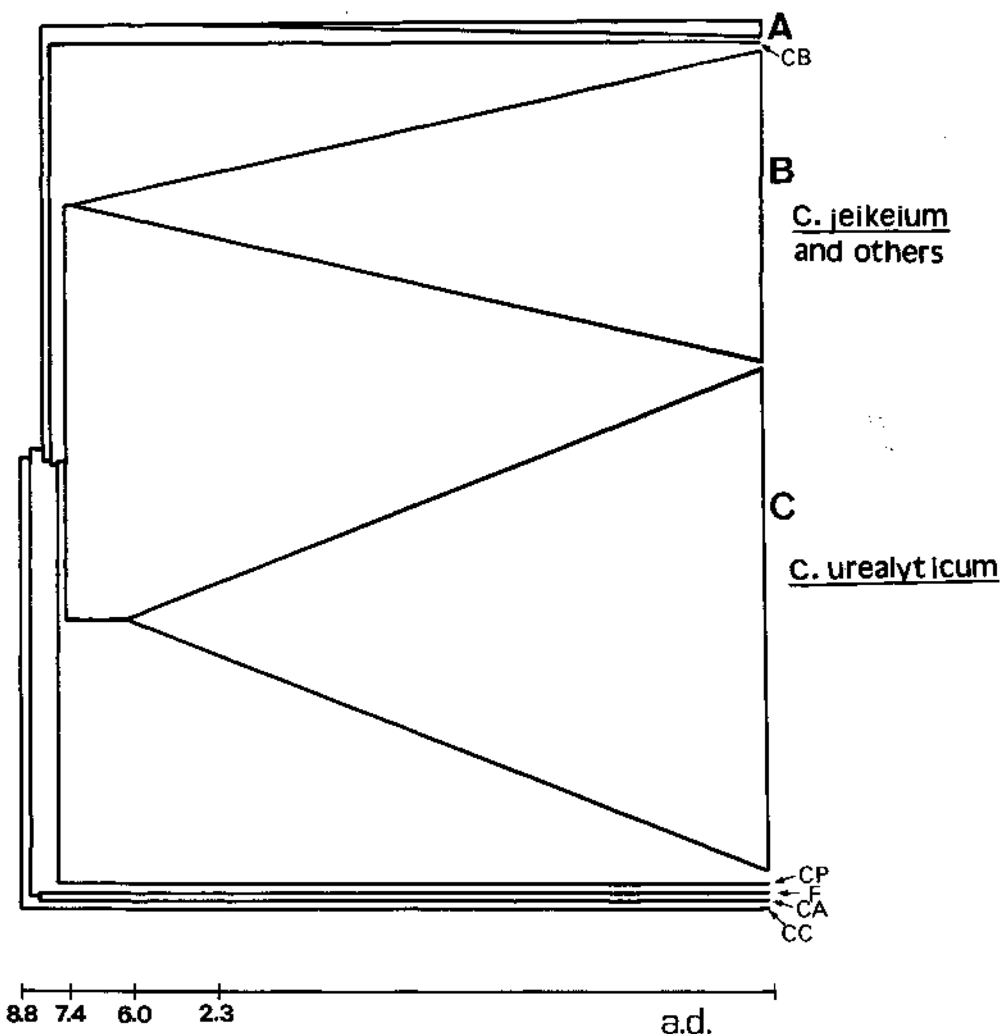

Fig. 1. Simplified dendrogram, based on fatty and mycolic acid profiles. Euclidean distance and single linkage algorithm, showing the relationship between *Corynebacterium urealyticum* and other corynebacteria. CB: *C. bovis* CNCTC 68/77; CP: *C. pseudodiphtheriticum* ATCC 10700. F: *Corynebacterium* sp FS 19-59724; CA: *C. ammoniagenes* ATCC 6871. CC: *C. cystitidis* ATCC 29593.

Corynebacterium pseudodiphtheriticum (ATCC 10700) contained mainly hexadecanoic acid (16:0); tuberculostearic acid was detected (Table 2). Mycolic acids were saturated, mono-, di- and triunsaturated, from 26 to 36 carbon atoms; 32:0 and 34:2 were predominant (14).

The strain of *Corynebacterium* F1 group (FS 19-59724) showed fatty acids from 16 to 18 C and were saturated (16:0 and 18:0) and unsaturated (18:2 and 18:1); it lacked 14:0 and tuberculostearic acids (Table 2). Mycolic acids varied from 32 to 36 C, 30:2, 36:3 and 30:1 being characteristics (14).

In *Corynebacterium ammoniagenes* (ATCC 6871) the profile of fatty acids consisted of saturated (16:0 and 18:0), unsaturated (18:1 and 18:2) and tuberculostearic (Table 2). Mycolic acids ranged from 32 to 36 carbon atoms; 36:2 and 36:3 were predominant (14).

The major fatty acids *Corynebacterium cystitidis* (ATCC 29593) were 16:0, 18:2 and 18:1, tu-

TABLE 2

FATTY ACID COMPOSITION (MEAN VALUE AND STANDARD DEVIATION) OF THE CLUSTERS OBTAINED

Clusters	Fatty acids*					
	14.0	16.1	16.0	18.1 + 18.2	18.0	TBS
A	1.5 (0.7)	33.2 (3.2)	47.9 (1.3)	12.3 (3.8)	4.9 (2.0)	—
C.b.**	1.0	1.4	26.1	32.0	29.9	9.3
B	3.0 (1.7)	6.2 (3.1)	40.8 (4.4)	35.8 (6.9)	13.0 (4.1)	1.1 (0.2)
B1**	2.0 (2.9)	4.6 (5.0)	34.0 (8.3)	40.5 (10.0)	18.3 (7.8)	—
C	0.8 (0.8)	1.5 (1.6)	29.4 (6.2)	43.3 (10.7)	17.0 (6.4)	7.6 (3.9)
C.p.**	—	0.6	62.3	27.2	9.0	0.9
F1**	—	—	28.5	28.1	43.2	—
C. a.**	—	—	25.9	47.3	23.3	3.4
C.c.**	16.0	4.0	26.0	37.0	14.0	3.0

* Identified by their number of carbon atoms and double bonds. TBS: Tuberculostearic acid. ** Included for comparison. C.b.: *Corynebacterium bovis* CNCTC 68/77. C.p.: *Corynebacterium pseudodiphtheriticum* ATCC 10700. F1: *Corynebacterium* F1 FS-19-59724. C.a.: *Corynebacterium ammoniagenes* ATCC 6871. C.c.: *Corynebacterium cystitidis* ATCC 29593. B1: Subcluster B1 (see results of cluster B). —: Not detected.

berculostearic acid was present (Table 2). Mycolic acids varied from 28 to 36 carbon atoms; unsaturated (32:2, 34:3 and 34:2) were predominant (14).

Discriminant analysis

This analysis defined several discriminant functions based only on mycolic acids, showing a correct classification for the clusters considered (A, B, C) with a high statistical significance:

TABLE 3

RANGE OF VARIATION (PERCENTAGES) OF THE FIRST EIGHT DISCRIMINANT VARIABLES (MYCOLIC ACIDS) IN THE CLUSTERS SUBJECTED TO DISCRIMINANT ANALYSIS (A, B AND C)

Cluster	Variables (mycolic acids)*							
	30:3	36:2	34:1	32:0	28:1	28:0	30:0	36:3
A	—	t	18-38	—	—	1-4	—	t
C.b.**	—	—	—	—	37	—	—	—
B	nd-2	nd-25	t-25	nd-32	nd-4	nd-10	t-20	nd-15
B1**	—	6-25	6-13	1-5	—	1-10	1-7	2-6
D	13-18	—	—	t-2	5-21	t-11	t-5	t-1
C.p.**	—	14	10	3	t	1	2	8
F1**	—	—	—	—	—	—	—	20
C. a.**	—	26	4	t	—	—	—	50
C.c.**	1	—	t	—	—	—	t	3

* Identified by their number of carbon atoms and double bonds. ** Included for comparison. C.b.: *Corynebacterium bovis* CNCTC 68/77. C.p.: *Corynebacterium pseudodiphtheriticum* ATCC 10700. F1: *Corynebacterium* F1 FS-19-59724. C.a.: *Corynebacterium ammoniagenes* ATCC 6871. C.c.: *Corynebacterium cystitidis* ATCC 29593. B1: Subcluster B1 (see results of cluster B). —, nd: Not detected. t: Traces (< 1 %).

$F (34/178) = 2066.68$ ($p < 0.0001$); $F_{AB} (17/89) = 461.8$ ($p < 0.0001$); $F_{AC} (17/89) = 1373.64$ ($p < 0.0001$); $F_{BC} (17/89) = 8884.63$ ($p < 0.0001$). The range of variation (as percentages) of the first eight variables in the clusters defined is given in Table 3.

Reproductibility of the results

Strains C27 *(Corynebacterium jeikeium)* and C76 *(C. urealyticum)* were studied in quadruplicate. The qualitative lipid profiles obtained in the four analyses were consistent with those of their respective species, but there were quantitative variations, mainly related to some mycolic acid percentages. In the case of *C. jeikeium* C27, the major variation was found for the compound 36:2 (16 to 25 %), and in the case of *C. urealyticum* C76 the most relevant were those of 28:1 (4 to 17 %), 30:2 (10 to 20 %), 26:1 (0 to 4 %), 26:0 (0 to 5 %) and 30:0 (0 to 5 %). However, strain *C. jeikeium* C27 always fell into cluster B at an amalgamation distance of 3.0, and strain *C. urealyticum* C78 in cluster C at an amalgamation distance of 5.0.

Discussion

Corynebacterium urealyticum appeared in the present study as an homogeneous cluster, clearly distant from other related corynebacteria, some of them also presenting urea-splitting activity (10). Other studies have emphasized its morphological and physiological peculiarities (15), but until recently its taxonomic position remained unclear (20). Cell wall chemotype IV, «*sensu*» Lechevalier (18), and presence of tuberculostearic acid and mycolic acids from 26 to 36 carbon atoms have been found to be the most significant chemical features relating *C. urealyticum* and the true corynebacteria (14).

As previously established (5), lipid profiles can be numerically evaluated to group coryneform bacteria, and a variety of statistics can be applied to the analysis of fatty acids with different degree of success. Although Euclidean distance did not seem to be very convenient in this approach (5), it was employed to assist in the identification of gram-positive rods (24), grouping *Corynebacterium urealyticum* (*Corynebacterium* group D2) together with *C. bovis* and other coryneforms, *C. jeikeium* included, and separating them from *C. renale, C. xerosis, C. minutissimum, C. pseudodiphtheriticum, «C. ulcerans», C. diphtheriae* and *C. pseudotuberculosis.* In this study both fatty and mycolic acids were taken into consideration and, to solve a possible distortion of the data by Euclidean distance, we used logarithm (4) and z-score standardization. Such an approach has proved to be successful, because the clusters obtained were well delineated. The discriminant analysis, however, revealed the null value of fatty acids, contrary to mycolic acids, in the process of classification. Nevertheless, it is clear that fatty acid profiles are very similar between the strains analyzed (Table 2) and that mycolic acids are quantitatively and qualitatively more dissimilar (Table 3). The classification obtained was, then, exclusively dependant on mycolic acid composition. The delimitation of the different taxa is improved by the inclusion of these components in the numerical analysis, as compared with the results obtained when only fatty acids were considered (5, 24).

Corynebacterium urealyticum was, according to discriminant analysis, characteristically typified by its content on 30:3 and 28:1 mycolic acids. However, in a recent study on the structure of mycolic acids of three reference strains of this species (11), the presence of triunsaturated mycolic acids was not reported, although the overall chain length in major compounds was similar to that previously published (14). This discrepancy could be attributed to the different culture conditions employed in both studies, mainly because the former authors supplemented the medium

with Tween 80, a compound that induces variations in double bonds of corynemycolates (6). Triunsaturated C30 mycolic acids, one of the most abundant in *C. urealyticum* (14), could derive from diunsaturated C16 and monounsaturated C14 acids, as revealed by pyrolysis-gas liquid chromatography (14). Given that the strains studied grew under the same conditions, the relevance of the characteristic mycolates of *C. urealyticum* is warranted, and mainly that of the 30:3, a mycolic acid scarcely distributed in other species (1, 8, 13, 14) and, therefore, considered an important marker for *C. urealyticum.*

Other discrepancies, also attributed to the culture medium, were noted with reported composition of mycolates of *C. jeikeium, C. ammoniagenes, C. pseudotuberculosis, C. pseudodiphtheriticum, C. renale, C. minutissimum* and *«C. ulcerans»* (1, 6, 7, 8, 11, 25). In general, this is due to the degree of unsaturation and, to some extent, the higher chain length of corynemycolates in our study (14).

That culture conditions influence lipid profiles of corynebacteria is also exemplified by the fatty acid composition of *C. urealyticum* reported by several groups (3, 11, 14, 24). Except for Couderc *et al.* (11), octadecadienoic acid is always present in this species. On the other hand, with a more sophisticated analytical technique, these authors (11) were able to establish the presence of several new compounds in *C. urealyticum,* and notably that of a 10-methylen octadecanoic acid, a precursor of tuberculostearate, not previously detected in corynebacteria. However, fatty acids of *C. urealyticum* are predominantly straight-chain type, like *Corynebacterium «sensu» stricto* (2, 9, 23), with significant amounts of tuberculostearic acid, a compound occasionaly present in the genus (10).

According to several cell wall and membrane components (14), *Corynebacterium urealyticum* appeared as chemically related to the true corynebacteria; a numerical evaluation of its fatty and mycolic acid composition has shown that it constitutes an homogeneous group, clearly separated from other corynebacteria. On the basis of these data we can further support the current taxonomic status of this microorganism as a new species within *Corynebacterium «sensu stricto»,* as recently demonstrated (20).

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Phenotypic and phylogenetic evidence for a close relationship between *Lactococcus garvieae* and *Enterococcus seriolicida*

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Summary

Cultural, biochemical and protein profiling studies were performed on *L. garvieae* strains isolated from diseased rainbow trout and on the fish patoghen *Enterococcus seriolicida* ATCC 49156. The results, confirmed by 16 rRNA sequence analyses, indicate that *E. seriolicida* ATCC 49156 should be reclassified in the genus *Lactococcus.* Contrary to previous reports, both *L. garvieae* and *E. seriolicida* were found to be β-haemolytic.

Key words: Taxonomy, fish disease, Lactococcus garvieae, Enterococcus seriolicida.

Resumen

Se han estudiado las características de cultivo, el perfil bioquímico y la composición proteínica en cepas de *Lactococcus garvieae* aisladas de truchas arcoiris enfermas y en *Enterococcus seriolicida* ATCC 49156, especie descrita como patógena en peces. Los resultados, confirmados por análisis de la secuencia de 16S rRNA, indican que *E. seriolicida* debería reclasificarse en el género *Lactococcus.* En contraposición a lo descrito previamente, tanto *L. gariveae* como *E. seriolicida* mostraron actividad β-hemolítica.

Introduction

During 1991 we investigated an infectious disease in farmed rainbow trout *(Oncorhynchus mykiss)* in Central Spain. Typical symptoms exhibited by the diseased fish were bilateral exophtalmous, marked darkening of the skin and haemorrhages in the eyes and on the pectoral fins. Swimming activity was depressed; many fish became moribund. Internally there was congestion in the intestine, liver, spleen and kidney. The disease occurred throughout the year but the symptoms were mo-

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re severe and the mortality rate higher during the summer months. The symptoms were virtually identical to those reported by Kusuda *et al.* (6) in fish infected with *Enterococcus seriolicida.* Bacteriological investigation, however, indicated that the etiological agent of the Spanish outbreak was phenotypically very similar to *Lactococcus garvieae* (9). This bacterium, isolated originally from bovine mastitis (1, 4) and more recently, from human clinical sources (3, 10) has to our knowledge not been incriminated previously as a fish pathogen.

In this paper we report the results of comparative cultural, biochemical and protein profile on our fish isolates and the type strains of *Lactococcus garvieae* and *E. seriolicida.* Strains of the Gram-positive bacteria *Lactococcus piscium* and *Carnobacterium piscicola,* also isolated from fish, were included. To elucidate the phylogenetic relationships of our fish isolates and *E. seriolicida,* 16S rRNA sequencing analysis was performed.

Methods

Strains

The test strains are listed in Table 1 and Figure. 2.

Cultural characteristics

Growth at various temperatures was tested on Brain Heart Infusion Agar (BHIA, Oxoid) and Blood Agar (BA). Haemolysis was tested on both BHIA and Columbia Agar (CA, Oxoid) supplemented with 5 % (v/v) defibrinated whole sheep blood with an equal volume of saline solution (0.85 % NaCl).

Biochemical tests

The tests were done using the API 50 CH and API 20 Strep systems (Biomerieux, Lyon, France) according to the manufacturer's instructions. Tests were incubated at 37° C, 24° C in the case of *Lactococcus piscium,* and readings made after 24 and 48 h and 7 days.

Preparation of membrane protein samples, electrophoresis and staining

The test strains were grown overnight in 20 ml BHI and harvested by centrifugation. The resulting cell pellet was resuspended in 0.5 ml PBS buffer and disrupted by sonication. After centrifugation to remove cell debris, sample loading buffer (SDS reducing buffer, Mini Protean II system, Bio-Rad Manual) was added to 100 μl of the supernatant SDS-PAGE. Analysis of the proteins was done as described in the Mini-Protean II system (Bio-Rad Manual). The method is based on the discontinuous buffer system of Laemmli (7). A 12 % acrylamide separating gel was used. The gel was then stained with 0.5 % Coomassie Blue in 40 % methanol, 10 % acetic acid solution to visualize the protein bands.

Analysis of 16S rRNA

DNA was extracted from cells in estimated mid-late logarithmic growth phase and purified by the method of Lawson *et al.* (8). 16S rRNA fragments were generated by PCR amplification

TABLE 1

ACID PRODUCTION FROM CARBOHYDRATES USING API 50 CH SYSTEM. READING WERE MADE AT 24 HOURS

	*L. garvieae**	CP1 CP2	*E. seriolicida* ATCC 49156	*L. piscium* NCFB 2778	*C. piscicola* C 462
Glycerol	0	–	–	–	+
Erythritol	0	–	–	–	–
D-Arabinose	0	–	–	+	–
L-Arabinose	0	–	–	+	–
Ribose	100	+	+	+	+
D-Xylose	0	–	–	–	–
L-Xylose	0	–	–	–	–
Adonitol	0	–	–	–	–
β-Methylxylose	0	–	–	–	–
Galactose	100	+	+	+	+
D-Glucose	100	+	+	+	+
D-Fructose	100	+	+	+	+
D-Mannose	100	+	+	+	+
L-Sorbose	0	–	–	–	–
Rhamnose	0	–	–	–	–
Dulcitol	0	–	–	–	–
Inositol	0	–	–	–	+
Mannitol	75	+	+	+	+
Sorbitol	0	–	–	–	+
α-Methyl-D-Mannoside	0	–	–	-	+
α-Methyl-D-Glucoside	0	–	–	+	+
N-Acetyl-glucosamine	100	+	+	+	+
Amygdalin	100	+	+	+	+
Arbutin	100	+	+	+	+
Aesculin	100	+	+	+	+
Salicin	100	+	+	+	+
Cellobiose	100	+	+	+	+
Maltose	75	+	+	+	+
Lactose	100	+**	+**	+	+
Melibiose	0	–	–	+	+
Sucrose	50	+	–	+	+
Trehalose	100	+	+	+	+
Inulin	0	–	–	–	+
Melizitose	0	–	–	+	+
D-Raffinose	0	–	–	+	+
Starch	0	–	–	+	+
Glycogen	0	–	–	–	–
Xylitol	0	–	–	–	–
β-Gentibiose	100	+	+	+	+
D-Turanose	0	–	–	+	+
D-Lyxose	0	–	–	–	–
D-Tagatose	50	+	–	+	–
D-Fucose	0	–	–	–	–
L-Fucose	0	–	–	–	Weak
D-Arabitol	0	–	–	–	–
L-Arabitol	0	–	–	–	–
Gluconate	50	+	+	+	+
2-Keto-gluconate	0	–	–	–	–
5-Keto-gluconate	0	–	–	–	Weak

*The results corresponding to *L. garvieae* are the percentage of value from four collection strains (NCDO 2155, 2156, 2157 and 2159). Data obtained by Collins *et al.* (1). **Weakly positive results after 24 hours and positive after 48 hours.

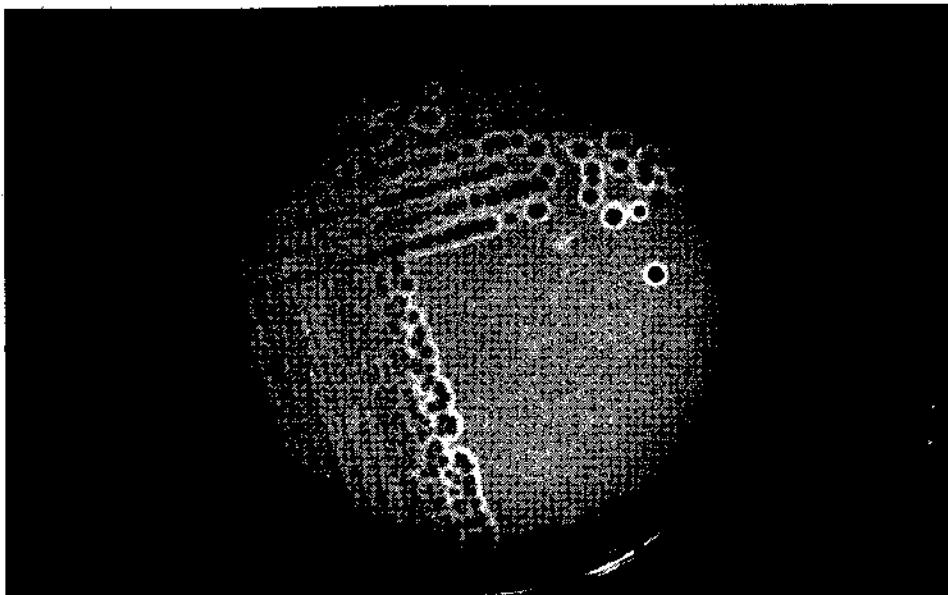

Fig. 1. Zones of β-haemolysis displayed by *Lactococcus garvieae* NCDO 2155 on Blood Agar after incubation at 37° C for 24 hours. Strains CP1, CP2 of *Lactococcus garvieae, Enterococcus seriolicida* ATCC 49156 and *Lactococcus piscium* NCFB 2778 showed a similar haemolysis.

using Taq polymerase as previously described (5). The amplified products were purified using a Gene-clean II kit (Bio 101 Inc., USA) and directly sequenced using (^{35}S)-dATP and sequenase Version 2.0 sequencing kit (USB).

Results and discussion

With the exception of *L. piscium,* which did not grow at 37° C, all strains tested grew at 4, 10, 24 and 37° C.

Lactococcus garvieae strains (NCDO 2155, CP1 and CP2), *E. seriolicida* and *L. piscium* displayed clear zones of β-haemolysis on BHIA and CA supplemented with either whole blood or SBRC after incubation at 37° C for 24 h; 24° C in the case of *L. piscium* (see Fig. 1). The haemolysis was enhanced after storage at 4° C. These results are not in agreement with the description of *L. garvieae* as non-haemolytic (1) nor with that of *E. seriolicida* as α-haemolytic (6). To our knowledge, there is no published information on the haemolytic activity of *L. piscium.*

The results of the biochemical pattern are presented in Table 1 and Table 2. *Lactococcus piscium* and *C. piscicola* were readily distinguished from each other and from all the other strains tested. All the *L. garvieae* strains (NCDO 2155, CP1 and CP2) exhibited almost identical biochemical profiles that correlated with the characteristics of *L. garvieae* as described by Collins *et al.* (1) and confirmed the previous preliminary studies of Prieta *et al.* (9) on the same strains. The biochemical profile of *E. seriolicida* ATCC 49156 differed from that of the *L. garvieae* strains only in that no acid was produced from sucrose and tagatose (Table 1).

The protein profile patterns of all the strains studied plus *Enterococcus faecium* ATCC 19434 (included for comparison with *E. seriolicida*) are shown in Figure 2. *L. garvieae* (NCDO 2155,

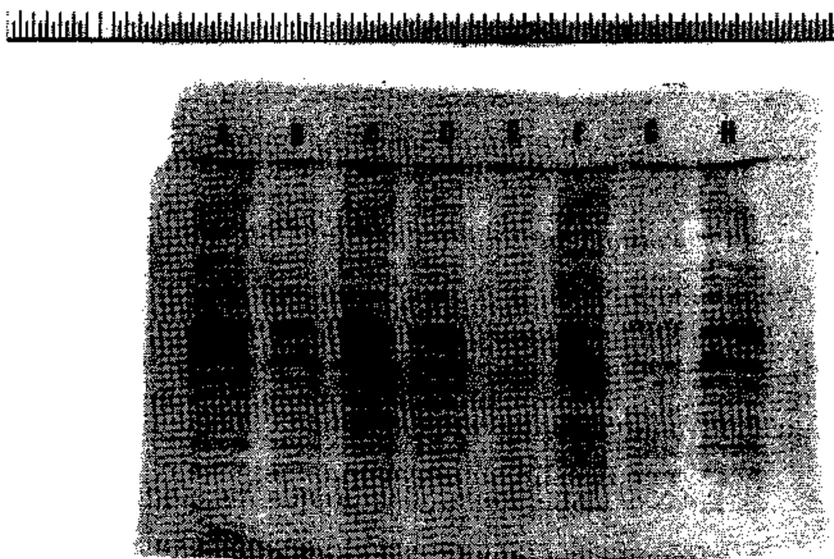

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of membrane proteins of all the strains studied. Lanes: A: FS1; B: FS2; C: *Lactococcus garvieae* NCDO 2155; D: *Enterococcus seriolicida* ATCC 49156; E: *Lactococcus piscium* NCFB 2778; F: *Enterococcus faecium* ATCC 19434; G: *Carnobacterium piscicola* C 462; H: *Enterococcus seriolicida* ATCC 49156 (double concentration).

CP1 and CP2) and *E. seriolicida* exhibited identical patterns, clearly distinguishable from *E. faecium, L. piscium* and *C. piscicola.*

Confirmation of this surprisingly close relationship between the *L. garvieae* strains and *E. seriolicida* was provided by 16S rRNA gene sequence analysis. The almost complete 16S rRNA gene sequence of the type strain of *E. seriolicida* was determined (approx. positions 50 to 1500, *Escherichia coli* numbering system) and found to be indistinguishable from the previously published 16S rRNA sequence of *L. garvieae* (2). A short fragment (approx. position 50 to 500) which includes the diagnostic variable regions V1-V3 of the 16S rRNAs of the two fish isolates was also sequenced and found to be identical to *L. garvieae,* thus confirming the genealogical identity of the strains.

The species *Enterococcus seriolicida* was described by Kusuda *et al.* (1991) for bacteria iso-

TABLE 2
RESULTS OF ENZYME ACTIVITY DETERMINED USING API 20 STREP SYSTEM. READINGS WERE MADE AT 4 AND 24 HOURS

	*L. garvieae**	CP1 CP2	*E. seriolicida* ATCC 49156	*L. piscium* NCFB 2778
Arginine dehydrolase	100	+	+	–
α-Galactosidase	0	–	–	–
β-Galactosidase	0	–	–	+
Pyrrolidonyl arylamidase	100	+	+	–
Leucine arylamidase	100	+	+	–

*The results corresponding to *L. garvieae* are the percentage of value from four collection strains (NCDO 2155, 2156, 2157 and 2159). Data obtained by Collins *et al.* (1).

lated from diseased yellowtail *(Seriola quinqueradiata)* and eels *(Anguilla japonica)* in Japan. The bacteria were classified in the genus *Enterococcus* on the basis of phenotypic similarity and DNA-DNA homology studies. The data of Kusada *et al.* (6), however, indicate only low levels of DNA homology (> 25 %) between *E. seriolicida* and representative strains of twelve other species of the genus *Enterococcus.* Furthermore, no DNA homology studies were done with representatives of any other phylogenetically related Gram-positive bacteria.

On the basis of the results presented here, *E. seriolicida* ATCC 49156 is clearly a member of the genus *Lactococcus.* Further, the presence of an identical 16S rRNA sequence indicates *E. seriolicida* and *L. garvieae* possess a close genealogical affinity, and probably represent a single species. Chromosomal DNA-DNA pairing studies are however necessary to determine with confidence the genospecific relatedness of these taxa.

Acknowledgments

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MICROBIOLOGIA SEM 9 (1993), 69-71 **NOTA**

Comparación de tres medios de enriquecimiento para el estudio de *Listeria monocytogenes* en los alimentos

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Summary

Analysis organised by the BCR (Bureau Communautaire de Référence) using material in form of capsules containing a strain of *Listeria monocytogenes* of known concentrations in various types of foods were carried out within the interlaboratory trials. In this work, it is demonstrated that the LEB (Listeria enrichment broth) medium is, of the three media assayed, the most adequate to investigate this organism in foods.

Key words: Listeria monocytogenes, *reference material, foods.*

Resumen

En el marco de los ensayos interlaboratorios organizados por el BCR (Bureau Communautaire de Référence) se han realizado unos análisis con material de referencia en forma de cápsulas conteniendo una cepa de *Listeria monocytogenes* a concentraciones conocidas en diversos tipos de alimentos. Se ha puesto de manifiesto que de entre los 3 medios estudiados, el más eficaz para la investigación de este microorganismo en los alimentos es el LEB (Listeria enrichment medium).

En el marco de los ensayos interlaboratorios organizados por la Oficina Comunitaria de Referencia (Trials BCR/FOOD) de la Comunidad Económica Europea con *Listeria monocytogenes,* se propuso llevar a cabo una prueba adicional como consecuencia de las discrepancias observadas entre las publicaciones relativas a los resultados de las técnicas USDA (2) y LEB (1) y los resultados obtenidos en el ensayo IV en el que se había añadido por primera vez una flora de competición a las cápsulas de *Listeria monocytogenes.* El objetivo de este ensayo era evaluar el comportamiento de las listerias del material de referencia (cepa Scott A, serotipo 4B) en contacto con diferentes alimentos y en nuestro caso la eficacia de 3 técnicas distintas.

El protocolo de un ensayo de esta naturaleza es, en resumen, el siguiente: envío por correo de 2 viales conteniendo cápsulas de leche descremada contaminada con el microorganismo a ensayar en una concentración conocida en 2 paquetes distintos con una semana de intervalo a fin de conocer el efecto del transporte sobre las cápsulas. Cada laboratorio investiga la presencia, en este caso, de listerias según una técnica estándar (con todo el material facilitado por el laborato-

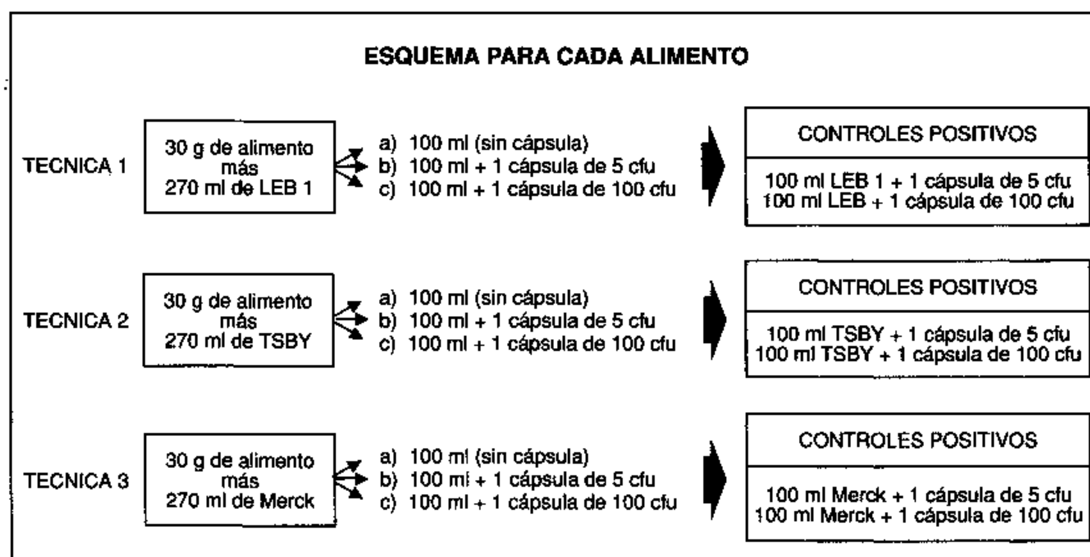

Fig. 1. Esquema utilizado para el análisis de los alimentos.

TABLA 1
RESULTADOS GLOBALES DEL ENSAYO

	5 cfu/12 m	100 cfu/12 m	Control+/4 5 cfu	Control+/4 100 cfu	Sin cápsula/12
Técnica 1					
LEB 1: 24 horas	5	6	0	0	3*
LEB 1: 48 horas	7	8	2*	4*	3
LEB 1: 7 días	9*	12*	2	4	3
LEB 2: 24 horas	6	9	1	1	3
LEB 2: 48 horas	6	8	1	3	3
LEB 2: 7 días	6	9	1	3	3
Técnica 2					
TSBY: 24 horas	7*	8	4*	4*	3*
LEB 2: 24 horas	6	7	4	4	2
LEB 2: 48 horas	7	11*	4	4	2
LEB 2: 7 días	7	11	4	4	3
MERCK: 2 24 horas	5	3	0	0	2
MERCK: 2 48 horas	4	4	1	1	2
MERCK: 2 7 días	4	6	1	0	1
Técnica 3					
MERCK 1: 24 horas	3	4	0	0	2*
MERCK 1: 48 horas	3	6*	0	2	2
MERCK 1: 7 días	5*	6	0	4*	2
MERCK 2: 24 horas	1	2	0	0	2
MERCK 2: 48 horas	3	5	0	0	2
MERCK 2: 7 días	3	4	0	0	2

* Resultados más significativos.

rio organizador) y la técnica propia del laboratorio. De aquí se puede deducir principalmente la calidad de las cepas de referencia, pero también la respuesta de las diferentes técnicas y la calidad del trabajo de cada laboratorio.

Se han empleado 3 técnicas, 2 concentraciones diferentes de *Listeria monocytogenes,* 3 controles y 2 tipos de alimentos. Las técnicas empleadas fueron: LEB (Listeria enrichment broth) Lovett *et al.*, 1987, y TSBY (non selective buffered pre-enrichment broth) RIVM y MERCK.

Los controles que se utilizaron fueron: sin alimento con una cápsula de 5 cfu, sin alimento con una cápsula de 100 cfu y alimento sin cápsula.

Las clases de alimentos fueron productos lácteos (2 quesos frescos, 2 quesos mantecosos, 2 quesos curados) y productos cárnicos (3 hamburguesas de ave crudas, 3 productos cocidos de charcutería). La metodología empleada se refleja esquemáticamente en la Figura 1.

Los resultados consignados en la Tabla 1 ponen en evidencia la superioridad de la técnica 1 para la recuperación de *Listeria monocytogenes* en alimentos. Tenemos 9 positivos de los 12 alimentos ensayados con la concentración inferior (5 cfu de L.m. por cápsula). Los 3 negativos son quesos. Con la concentración de 100 cfu, el 100% es positivo. Para obtener estos resultados hace falta llegar al séptimo día de incubación del medio de enriquecimiento. También se pone de manifiesto la ineficacia del segundo enriquecimiento.

En la técnica 2 los resultados positivos se consiguen ya a las 24 horas de incubación para los controles positivos con 5 cfu. Para los de 100 cfu tenemos el máximo de positivos a las 48 horas del subcultivo. Los negativos siguen siendo los quesos. En cuanto al caldo selectivo de Merck se demostró su alto poder selectivo en el ensayo III, pero en nuestro caso es la técnica que da los mejores resultados con la concentración de 5 cfu en quesos: 4/6 positivos cuando sólo hay 3/6 con LEB y 2/6 para el TSBY.

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MICROBIOLOGIA SEM 9 (1993), 72-76

Escherichia coli enterotoxigénicos K99$^+$ del serotipo 08:K25 producen el factor necrosante citotóxico CNF1 y α-hemolisina

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Summary

A total of 54 K99$^+$ and/or F41$^+$ *Escherichia coli* strains isolated of calves and piglets with diarrhoea in different countries were investigated for production of heat-labile (LT) and heat-stable (STa) enterotoxins, verotoxins (VT1 and VT2), cytotoxic necrotizing factors (CNF1 and CNF2), α-haemolysin (Hly) and enterohaemolysin (EntHly). Fifty-one (94%) strains were STa$^+$, two (4%) were STa$^+$CNF1$^+$Hly$^+$ and one (2%) was not toxigenic. The two STa$^+$CNF1$^+$Hly$^+$ *E. coli* strains expressed the K99 intestinal colonization factor and belonged to serotype O8:K25. The majority of STa-producing *E. coli* were K99$^+$ and F41$^+$ and belonged to serotypes O9:K(A)35, O101:K(A)28 and O101:K(A)30. All enterotoxigenic strains assayed in this study were negative for expression of the Att25, Vir and B23 adhesins recently detected in *E. coli* that cause infections in calves.

Key words: K99 antigen, colibacillosis, E. coli, *enterotoxins, toxins.*

Resumen

Un total de 54 cepas de *Escherichia coli* K99$^+$ y/o F41$^+$ aisladas de terneros y lechones con diarrea en diferentes países fueron investigadas para la producción de la enterotoxina termolábil (LT) y termoestable (STa), verotoxinas (VT1 y VT2), factores necrosantes citotóxicos (CNF1 y CNF2), α-hemolisina (Hly) y enterohemolisina (EntHly). Cincuenta y una (94%) cepas fueron STa$^+$, dos (4%) STa$^+$CNF1$^+$Hly$^+$ y una (2%) resultó ser no toxigénica. Las 2 cepas STa$^+$CNF1$^+$Hly$^+$ pertenecieron al serotipo O8:K25 y expresaron únicamente el antígeno de colonización intestinal K99. Entre los *E. coli* que produjeron solamente la enterotoxina STa predominaron las cepas K99$^+$ y F41$^+$ de los serotipos O101:K(A)35, O101:K(A)28 y O101:K(A)30. Ninguna de las 54 cepas examinadas expresó las adhesinas Att25, Vir y B23 recientemente detectadas en *E. coli* que causan infecciones en terneros.

(*) A quien debe dirigirse la correspondencia.

Escherichia coli puede producir al menos 4 categorías de toxinas: a) enterotoxinas termolábiles (LT) y termoestables (STa); b) verotoxinas (VT1 y VT2); c) factores necrosantes citotóxicos (CNF1 y CNF2), y d) hemolisinas (α-hemolisina —Hly— y enterohemolisina —EntHly—) (3, 6). Los estudios realizados en los años setenta pusieron de manifiesto que los *E. coli* que causan diarrea en terneros lactantes producían la enterotoxina termoestable STa y expresaban en su superficie el antígeno de colonización intestinal K99 (F5) (8, 12). Posteriormente, se comprobaría que la mayor parte de las cepas enterotoxigénicas K99$^+$ bovinas poseían un segundo factor de adhesión conocido como F41 (9, 10). Los *E. coli* enterotoxigénicos (ECET) K99$^+$ y/o F41$^+$ también son capaces de producir diarrea en lechones (11). No obstante, la mayoría de las colibacilosis sufridas por el ganado porcino son debidas a ECET que presentan los antígenos de colonización K88 ó P987 (3). Recientemente se ha comprobado que muchas cepas de *E. coli* de origen bovino producen verotoxinas (VT1 y VT2) o sintetizan el factor necrosante citotóxico CNF2 y expresan nuevas adhesinas (Att25, Vir y B23) que se han relacionado con la virulencia (5, 6, 7, 14, 15). El objetivo de este trabajo era examinar la producción de verotoxinas y factores necrosantes citotóxicos en las cepas de *E. coli* K99$^+$ y/o F41$^+$, causantes de diarrea en terneros y lechones, así como estudiar la expresión de los nuevos antígenos adhesivos recientemente descubiertos en los *E. coli* causantes de infecciones en ganado bovino.

Se investigaron 54 cepas de *E. coli* K99$^+$ y/o F41$^+$ aisladas de terneros y lechones con diarrea en diferentes países. La mayoría de las cepas examinadas eran de origen bovino y fueron aisladas en Estados Unidos, Canadá, Holanda, Suecia y España. Tras sembrar las cepas en 5 ml de caldo triptona-soja (Oxoid) contenidos en matraces de 50 ml e incubar (37° C/20 h/200 rpm), se obtuvieron los fluidos extracelulares y los extractos con mitomicina C tal y como describimos previamente (4, 5). La detección de la enterotoxina STa se realizó en ratones lactantes empleando fluidos extracelulares (5), mientras que la detección de las toxinas LT, VT1, CNF1 y CNF2 se llevó a cabo en monocapas de células Vero y HeLa usando los extractos con mitomicina C (4). El estudio de hemolisinas fue efectuado en agar sangre base (Merk) con un 5% (v/v) de sangre de carnero y en agar sangre base con un 5% (v/v) de eritrocitos de carnero lavados 2 veces. Para potenciar la expresión de adhesinas, las cepas se inocularon en caldo Mueller-Hinton (Difco) (37° C/5 días/estático) y posteriormente, partiendo de la película-anillo superficial, se sembraron en agar Minca con Isovitale X (9). La detección de los antígenos adhesivos K99, F41, Att25, Vir y B23 fue realizada por coaglutinación estafilocócica usando antisueros obtenidos y absorbidos en nuestro laboratorio como previamente describimos (Blanco, M., 1991. Tesis Doctoral en microficha número 191 del Servicio de Publicacións e Intercambio Científico de la Universidade de Santiago de Compostela). La determinación de los antígenos somáticos O y capsulares K de las cepas fue realizada siguiendo el método descrito por Guinée *et al.* (9), empleando antisueros obtenidos en el National Institute of Public Health and Environmental Protection, Bilthoven, Holanda.

Los resultados obtenidos con las 54 cepas de *E. coli* examinadas en este estudio se indican en la Tabla 1. Los fluidos extracelulares de 53 (98%) cepas provocaron deshidratación al ser inyectados oralmente a ratones lactantes, induciendo coeficientes IMT $\geq$ 0,100, indicativos de la producción de la enterotoxina STa. En contraste, solamente 2 cepas sintetizaron CNF1 y la α-hemolisina, y ninguna fue positiva para la producción de la enterotoxina LT, el factor necrosante CNF2 y para la enterohemolisina EntHly. Treinta y cuatro (63%) de las 54 cepas investigadas expresaron conjuntamente los antígenos de colonización intestinal K99 y F41, mientras que 13 (24%) presentaron únicamente el antígeno K99 y 7 (13%), solamente la adhesina F41. Los nuevos antígenos adhesivos Att25, Vir y B23 no fueron detectados en ninguno de los *E. coli* K99$^+$ y/o F41$^+$ examinados. La mayoría de las cepas de *E. coli* K99$^+$ y/o F41$^+$ pertenecía a un número bastante reducido de serogrupos O, siendo los serotipos más frecuentemente encontrados el O101:K(A)30 (11 cepas), el O101:K(A)28 (8 cepas) y el O9:K(A)35 (8 cepas). Las 2 cepas

TABLA 1

SEROTIPOS, TOXINAS Y ADHESINAS DE LOS *E. COLI* K99+ Y/O F41+ CAUSANTES DE DIARREA EN GANADO PORCINO Y BOVINO

Cepa	Serotipo	Toxinas: Ratones lactantes*	Toxinas: Células Vero y HeLa**	Hemolisinas	Antígenos de colonización***
H1751/77	O8:K25	0,120	CNF1	Hly	K99
H1929/75	O8:K25	0,112	CNF1	Hly	K99
H472/82	O8:K85	0,114	—	—	K99
H1946/75	O8:K85	0,140	—	—	K99
WS10	O8:K208	0,131	—	—	K99
H1726/78	O9:K(A)30	0,132	—	—	K99 F41
H44/82	O9:K(A)30	0,177	—	—	K99 F41
483	O9:K(A)35	0,137	—	—	K99 F41
B30a	O9:K(A)35	0,150	—	—	K99 F41
H1927/75	O9:K(A)35	0,130	—	—	K99
H1763/77	O9:K(A)35	0,160	—	—	K99 F41
H140/84	O9:K(A)35	0,165	—	—	K99 F41
H110/82	O9:K(A)35	0,128	—	—	K99 F41
H211/82	O9:K(A)35	0,137	—	—	K99 F41
H215/82	O9:K(A)35	0,141	—	—	K99 F41
H275/81	O9:K(A)37	0,122	—	—	K99 F41
H1916/75	O20:K?	0,121	—	—	K99 F41
637	O64	0,169	—	—	K99 F41
Bd3433/76IV	O64:K-:H-	0,124	—	—	K99
Bd600/76VI	O64:H-	0,117	—	—	K99
H1303/78	O101:K-	0,110	—	—	K99 F41
B41mc	O101:K-	0,135	—	—	F41
H107/81	O101:K-	0,144	—	—	F41
H726/81	O101:K-	0,160	—	—	F41
H416/75	O101:K(A)?	0,133	—	—	K99 F41
VC/1751	O101:K(A)27	0,137	—	—	F41
505	O101:K(A)28	0,129	—	—	K99 F41
H1548/78	O101:K(A)28	0,161	—	—	K99 F41
H1550/78	O101:K(A)28	0,152	—	—	K99 F41
H155/84	O101:K(A)28	0,182	—	—	K99 F41
H113/82	O101:K(A)28	0,176	—	—	K99 F41
H152/82	O101:K(A)28	0,143	—	—	K99 F41
H183/82	O101:K(A)28	0,178	—	—	K99 F41
H448/84	O101:K(A)28	0,119	—	—	K99 F41
431	O101:K(A)30	0,137	—	—	K99 F41
12Ae	O101:K(A)30	0,142	—	—	K99 F41
VAC/1676	O101:K(A)30	0,126	—	—	F41
KATI/1706	O101:K(A)30	0,148	—	—	F41
Bd2068/75	O101:K(A)30	0,109	—	—	K99 F41
490	O101:K(A)30	0,109	—	—	K99 F41
H352/82	O101:K(A)30	0,124	—	—	K99 F41
H138/84	O101:K(A)30	0,166	—	—	K99 F41
H148/84	O101:K(A)30	0,179	—	—	K99 F41
H154/84	O101:K(A)30	0,145	—	—	K99 F41
H108/81	O101:K(A)30	0,158	—	—	F41
H11/79	O101:K(A)32	0,120	—	—	K99 F41
H1298/78	O101:K(A)32	0,122	—	—	K99 F41
H185/84	O101:K(A)32	0,101	—	—	K99 F41
H235/84	O101:K(A)103	0,145	—	—	K99 F41
BD82b	O101	0,064	—	—	K99
BC98c	O8, O141	0,171	—	—	K99
BC99a	O8, O141	0,173	—	—	K99
BC100b	O8, O141	0,123	—	—	K99
BD48a	O141	0,133	—	—	K99

* Coeficientes de enterotoxigenicidad. Valores superiores a 0,100 son indicativos de producción de la enterotoxina STa. ** En las células Vero y HeLa se pueden detectar las toxinas LT, VT1, VT2, CNF1 y CNF2. *** Todas las cepas fueron negativas para los antígenos Att25, Vir y B23.

STa$^+$CNF1$^+$Hly$^+$ pertenecieron al serotipo O8:K25 y expresaron únicamente el antígeno de colonización K99.

En nuestro estudio, 53 (98%) de las 54 cepas de *E. coli* K99$^+$ y/o F41$^+$ produjeron la enterotoxina STa, corroborando la correlación existente entre la producción de STa y la expresión de los antígenos K99 y F41 (8, 10, 12). En este trabajo es la primera vez que se detecta la producción de CNF1 por *E. coli* enterotoxigénicos bovinos K99$^+$. Nosotros (1) hemos examinado en los últimos años miles de cepas de origen humano y animal para la producción de los factores necrosantes citotóxicos CNF1 y CNF2, lo que nos ha permitido concluir que la síntesis de CNF1 es característica de *E. coli* causantes de infecciones extraintestinales en seres humanos, mientras que la producción de CNF2 es típica de cepas de *E. coli* bovinas. Así, 266 (99%) de 269 *E. coli* necrosantes humanos aislados en España entre 1979 y 1991 resultaron ser CNF1$^+$. En contraste, 123 (97%) de las 127 cepas bovinas necrosantes produjeron CNF2 (1). Por tanto, CNF1 es un factor de virulencia de *E. coli* causantes de infecciones urinarias y sepsis en seres humanos (2), y solamente de forma esporádica se ha detectado hasta la fecha en cepas aisladas de terneros con diarrea. Los *E. coli* bovinos CNF1$^+$ previamente detectados no eran enterotoxigénicos (LT$^-$STa$^-$) ni expresaban el antígeno K99 y pertenecían a los serogrupos O6, O8 y O153 (1) (Blanco, M., 1991. Tesis Doctoral). Nosotros creemos que la producción de CNF1 y Hly puede incrementar la virulencia de las cepas K99$^+$ del serotipo O8:K25. La α-hemolisina contribuye a la virulencia al facilitar el crecimiento del microorganismo en los tejidos del huésped, gracias al suministro de iones Fe^+ mediante la lisis de los eritrocitos (2). Aunque se sabe que CNF1 posee actividad necrosante y letal, y se cree que es un factor de virulencia que incrementa la patogenicidad, se desconoce el papel exacto que desempeña en la patogénesis de las infecciones causadas por *E. coli* (7).

En los últimos años se han descubierto 3 nuevas adhesinas (Att25, Vir y B23) en las cepas *E. coli* de origen bovino (6, 14). El antígeno Att25 también se conoce como FY y F17 (14). No se ha encontrado una correlación entre la presencia de Att25 y la producción de enterotoxinas, aunque sí se ha encontrado una asociación entre las cepas Att25$^+$ y diarrea y septicemia en terneros (14). Los estudios de Pohl *et al.* (14) en Bélgica indican que aproximadamente el 17% (27/157) de las cepas de Att25$^+$ son STa$^+$K99$^+$ y que un 13% (18/139) de las cepas STa$^+$K99$^+$ poseen la adhesina Att25. No obstante, las 171 cepas Att25$^+$ aisladas en Japón por Shimizu *et al.* (15) resultaron ser todas no productoras de enterotoxinas. Nuestros resultados corroboran los encuentros de Shimizu *et al.* (15), ya que previamente habíamos encontrado la adhesina Att25 en 119 (14%) de 853 *E. coli* bovinos STa$^-$ aislados en Galicia entre 1980 y 1991 (Blanco, M., 1991. Tesis Doctoral) y ahora ninguna de las 53 cepas bovinas enterotoxigénicas STa$^+$ expresó Att25. Los antígenos Vir y B23 se encontraron asociados fundamentalmente con la síntesis del factor necrosante CNF2 (6, 13). Oswald *et al.* (13) comprobaron que un mismo plásmido llevaba la información para la producción de la toxina CNF2 y la adhesina Vir. Por otra parte, nosotros encontramos los antígenos superficiales Vir y B23 en el 48% (59/123) y 22% (27/123) de las cepas bovinas CNF2$^+$, respectivamente. En las cepas STa$^-$ no productoras de CNF2 el antígeno Vir lo detectamos en el 16% (114/730) ($p < 0{,}001$) y el B23 en solamente el 2% (17/730) ($p < 0{,}001$) (Blanco, M., 1991. Tesis Doctoral). En el estudio actual, ninguna de las 53 cepas enterotoxigénicas STa$^+$ expresó estos 2 antígenos.

En conclusión, nuestros resultados confirman la correlación existente entre la expresión de los antígenos K99 y F41 y la síntesis de la enterotoxina termoestable STa, e indican que los *E. coli* K99$^+$STa$^+$ del serotipo O8:K25 también producen el factor necrosante citotóxico CNF1 y la α-hemolisina. Además, nuestros encuentros sugieren que los *E. coli* enterotoxigénicos K99$^+$ y/o F41$^+$ no suelen expresar los antígenos adhesivos Att25, Vir y B23 ni producir verotoxinas.

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