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Development of a fluorescent antibody method for the detection of *Enterococcus faecium* and its potential for coastal aquatic environment monitoring

Gabriella Caruso [a,*], L.S. Monticelli [a], R. Caruso [b], A. Bergamasco [a]

[a] *National Research Council, Institute for Coastal Marine Environment, Spianata S. Raineri, 98122 Messina, Italy*
[b] *University Hospital Agency "G. Martino", Via Consolare Valeria, 98121 Messina, Italy*

Abstract

A direct, microscopic fluorescent antibody method was developed to detect the occurrence of *Enterococcus faecium* in coastal aquatic environments and was compared with the conventional membrane filtering method. The "*in situ*" application of the antibody-based protocol in the analysis of water samples collected from coastal polyhaline habitats demonstrated good sensitivity and ease of implementation. Data obtained with the microscopic technique were in agreement with those obtained from culture counts. The fluorescent antibody method proved to be a rapid and reliable technique for the detection of *E. faecium*. The advantages and limitations intrinsic to the method are discussed, highlighting the potential of this new technique for monitoring coastal aquatic environments.

Keywords: Fluorescent antibody; Enterococci; *Enterococcus faecium*; Faecal pollution; Coastal aquatic environments

1. Introduction

Monitoring microbial pollution in aquatic environments is needed to maintain acceptable water quality standards and, consequently, to protect human health from sanitary hazards. Enterococci, members of the group D faecal streptococci, are common components of the normal flora of the intestinal tracts of all warm-blooded animals. They can enter streams or coastal waters through animal inputs, worsening bacteriological quality. The ability of these microorganisms to survive for long periods in aquatic environments has been demonstrated for both *Enterococcus faecalis* and *Enterococcus faecium* (Leclerc et al., 1996; Pinto et al., 1999; Lléo et al., 2001, 2005). For this reason, these microorganisms, in addition to faecal coliforms and *Escherichia coli*, are important indicators of faecal pollution in aquatic ecosystems. Based on the persistence of faecal streptococci in the environment, the ratio of faecal coliforms to faecal streptococci has been recognised as a means to calculate the date of the polluting event. According to Geldreich and Kenner (1969), values higher than 4 or lower than 1 indicate recent or medium-term pollution occurrences, respectively.

Knowledge of the distribution of enterococci in different matrices is important in assessing the value of this group as a faecal indicator. Among enterococci, the species *E. faecalis*, *E. faecium,* and *E. durans* are the most frequent in human faeces; *E. faecium* and *E. faecalis* are the most common enterococcal species found in environmental samples (Leclerc et al., 1996; Pinto et al., 1999). The density of enterococci in water correlates with the incidence of swimming-associated gastroenteritis (Kay et al., 1994; Toranzos and McFetters, 1997) and fever, stomach pain, and skin rash illness (Haile et al., 1999). Therefore, monitoring enterococcus distribution in aquatic environments is of primary importance for protecting human health. The quantitative determination of enterococci is included in the Italian Directive (Decree 152/99) and the European Union Water Framework Directive

* Corresponding author. Tel.: +39 090 669003; fax: +39 090 669007.
E-mail address: gabriella.caruso@iamc.cnr.it (G. Caruso).

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(WFD 2000/60/EC) within the standard guidelines for assessing the quality status of aquatic environments, with particular reference to bathing waters. The detection of enterococci has earned increasing interest in recent years, not only due to their widespread distribution and the diversity of the serogroups in clinical specimens (Teixeira et al., 1997), but also due to the isolation of some strains classified in epidemiological studies as emerging pathogens, etiological agents of severe diseases including urinary tract and intravascular infections, and endocarditis. Antibiotic resistance has been reported for many enterococci (Leclerq et al., 1988; Wegener et al., 1999), mainly towards vancomycin (vancomycin-resistant enterococci, indicated as VRE, see Uttley et al., 1988), underlying the importance of early identification of these species in clinical or environmental specimens to prevent the outbreak of disease and enact therapeutic measures.

Previous studies performed in our laboratory (Caruso et al., 2000, 2002, 2003, 2006) demonstrated the suitability of a fluorescent antibody technique based on the use of immune sera for the detection of *Escherichia coli* as an indicator of faecal pollution. For the identification of group D streptococci, a direct staining procedure using specific fluorescent antibodies was proposed as a rapid test by Obiger (1966) and Pavlova et al. (1972). Cars et al. (1975) and Watson et al. (1975) demonstrated the effectiveness of this method for the specific characterization of streptococci to specific serological groups, and Levchak and Ellner (1982) proved that latex agglutination was suitable for group D streptococci identification. Fluorescent antibody techniques have also been used to quickly and specifically identify streptococci belonging to A (Freeburg, 1970; Ederer and Chapman, 1972), B (Smith, 1971), and E (Schueler et al., 1973) serogroups. However, apart from strictly diagnostic purposes, the use of serological methods has not been fully exploited to date and its application to environmental monitoring has rarely been investigated. Simple methods for the determination of enterococci are needed particularly for monitoring the microbiological quality of polyhaline aquatic habitats, wild ecosystems which frequently suffer faecal pollution from both point and non-point sources. Non-point sources, such as transcontinental and regional migratory birds that inhabit inland waters in great numbers, can spread bacterial pathogens such as faecal coliforms and streptococci (Chern and Olson, 2004; Kirschner et al., 2004) and are difficult to control. Thus, they are a major concern in programs devoted to coastal zone management and protection. For this reason, a rapid, fluorescent antibody-based method for the detection and enumeration of enterococci in waters was developed and compared with the standard culture method. The initiation of a multidisciplinary monitoring programme aimed at the ecological characterisation of coastal Sicilian aquatic environments provided a unique opportunity to verify the sensitivity and performance of this direct microscopic technique as a tool for assessing enterococcal abundance in the waters. Attention was paid, in particular, to *E. faecium*, as commercial antisera currently available are produced solely towards this species.

2. Materials and methods

2.1. Collection and treatment of water samples

From June 2005 to May 2006, surface (0.3 m depth) water samples were aseptically taken from shallow coastal polyhaline Sicilian habitats (Ganzirri, Marinello, Mergolo, Verde, Piccolo, Grande, Roveto) and stored in sterile polypropylene bottles (1 L) at +5 °C until analysis. For the fluorescent antibody (FA) method, 100 ml water samples were fixed with filtered formalin (2% final concentration). Culture counts were performed within 2 h of sampling.

2.2. Culture counts

The enumeration of enterococci was carried out according to the ISO 7899-2:2000 membrane filtering method (APAT/IRSA-CNR, 2003). Briefly, suitable sample volumes were filtered on a gridded Millipore membrane (0.45 μm porosity) and further incubated on Slanetz–Bartley plates (Oxoid, Hampshire, England), a selective medium for the recovery of faecal streptococci. Plates were incubated at 36 ± 1 °C for 44 ± 4 h; pink to brownish colonies growing on this medium were counted as presumptive enterococci (A.P.H.A., 1992). The membrane was further transferred to the surface of Bile Esculin agar (Oxoid) plates, a medium able to differentiate between group D (enterococci) and non-group D streptococci (according to Facklam and Moody, 1970); blackening of the colonies due to esculine hydrolysis after incubation at 44 °C for at least 2 h allowed the confirmation of suspected enterococci. Results were expressed as the number of CFU (Colony Forming Units) of confirmed enterococci per 100 ml of sample.

2.3. Microscopic counts

For microscopic counts, a direct fluorescent antibody (FA) procedure was applied. Sample volumes (60 to 100 ml) were filtered through a 0.22 μm polycarbonate black membrane (Whatman-Nuclepore, Maidstone, UK), which was allowed to stand for 30 min at room temperature with Omnitope *Enterococcus* sp. (ViroStat, Portland, Maine, USA) antiserum, prepared using whole cells of *E. faecium* as the immunogen and conjugated to fluorescein isothiocyanate (FITC). As a working solution, a 1:50 dilution in phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, USA) of the immune serum was used, according to the manufacturer's instructions, which indicated this dilution as the sensitivity threshold of the antiserum. After rinsing with PBS, the filter was mounted with buffered glycerin, pH 7.2 (FA mounting fluid, Difco), on a microscope slide and observed with an Axioplan 2 (Carl Zeiss Inc, Oberkochen, Germany) epifluorescence microscope