RAPID DETECTION OF *ESCHERICHIA COLI* IN COASTAL WATERS BY USE OF THE FLUOROGENIC SUBSTRATE 4-METHYLUMBELLIFERYL-β-D-GLUCURONIDE: PRELIMINARY RESULTS

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Abstract
The application of an enzymatic assay using the 4-methylumbelliferyl-β-D-glucuronide (MUG) substrate for the spectrofluorometric determination of fecal pollution in marine environments is evaluated. Results of the analysis carried out on a total of 80 samples collected from nearshore areas show the presence of significative correlations between plate (m-FC medium) and microscopical (direct immunofluorescence, IF) counts and the enzymatic methods (r=0.807 and r=0.747 respectively for MUG-FC and MUG-IF). The advantages and the quantitative and qualitative limits to the use of this biochemical assay are discussed.

Key-words: bacteria, pollution, monitoring

Introduction

Pollution of marine coastal waters is mainly caused by the discharge of sewage effluents containing high densities of human pathogens. The need for water quality monitoring is of great importance for public health protection and environmental safeguarding. *Escherichia coli* is the indicator organism universally accepted by the Italian directive (DPR 470/82) which defines the suitability of marine waters for bathing purposes: on the basis of the research and quantitative enumeration of this coliform it is possible to evaluate the degree of fecal contamination of seawaters. The inadequacy of the conventional standard methods based on the membrane filtration technique has already been demonstrated, because of their long analysis and incubation times which prevent the application of immediate remedial measures. The development and optimization of rapid methodologies, such as immunofluorescence, for the detection of *E. coli*, is becoming more and more urgent and it has been the first aim of the studies carried out in our laboratory over the last years (1). Considerable attention has recently been paid to the use of fluorogenic methylumbelliferyl substrates for the specific research of fecal and total coliforms in marine wastewaters, food and drinking waters (2).

We report here the results of a rapid method for the spectrofluorometric detection of *E. coli* in marine waters based on the 4-methylumbelliferyl-β-D-glucuronide (MUG). The analysis has been carried out in the general framework of the C.N.R. Strategical Project "Monitoring of the marine pollution in the South of Italy" aimed at developing new techniques for the assessment of bacteriological contamination levels in nearshore waters surrounding the city of Messina.

Material and methods

The use of the 4-methylumbelliferyl-β-D-glucuronide (MUG) compound in liquid medium was first proposed by Berg and Fiksdal (3) and then by Muller-Niklas and Herndl (4), Fiksdal *et al.* (5). The technique relies on the determination of the β-D-glucuronidase activity present in fecal coliforms, which causes the substrate hydrolysis with release of the fluorescing 4-methylumbelliferone (MU). The increase in fluorescence is measured after incubation of the sample with appropriate aliquots of the substrate.

From August 1996 to March 1997, a total of 80 seawater samples were collected monthly from coastal stations located along the Ionian coast of the Straits of Messina, heavily polluted by urban effluent discharges. In some cases the sampling was carried out during two successive days, in coincidence with opposing tidal patterns (called "montante" and "scendente") in order to evaluate the possible occurrence of variations in water quality in relation to the current regime.

For the biochemical assay, sample volumes ranging from 50 to 500 ml, according to their turbidity, were filtered through a Nuclepore membrane (0.22 μm pore size); the filter was then homogenized in 50 ml of PBS pH 7.2. Aliquots of a 0.5 mM stock solution of the MUG substrate (Sigma) dissolved in MethylCellosolve (Sigma) were added to 10 ml sub-samples in order to obtain final concentrations of 10, 25, 50 and 100 μM. A blank was prepared by boiling each sample. Fluorescence was measured at 0 time and after 3 hours of incubation in a water bath at 37°C in a Hitachi 2000 spectrofluorometer set at 365 nm (excitation) and 440 nm (emission); before measurements, pH was adjusted to >10, by adding NaOH 0.1 M. The fluorescence increase was transformed into mg of MU released based on a calibration curve obtained with MU standard (Sigma) and expressed in terms of Vmax of substrate hydrolysis, per hour and per litre (6).

In addition, the density in fecal coliforms (FC) was evaluated through filtration of 100 ml of sample on a Nuclepore membrane (0.45 μm) incubated on m-FC medium (Difco)+1.2% agar for 24h at 44.5°C. On the same samples the direct immunofluorescence count (IF) was carried out according to the procedure described by Zaccone *et al.* (1).

The three methods were compared through correlations by linear regression and Pearson analyses.

Results

As far as the optimization of the assay is concerned, on the basis of the results obtained from preliminary trials performed with a pure culture of *E. coli* O-26 collection strain, we decided to use a pH of 10 and an incubation temperature of 37°C. In these conditions, MU was highly fluorogenic and the early detection of fluorescence was possible rather than at 44.5°C. In our study, the use of the 25 min procedure proposed by Fiksdal *et al.* (5) was insufficient to appreciate significative differences between the initial and final measurements; therefore, the period of incubation was extended up to 3 hours, the same time as that used for other esoenzyme assays (7).

Samples analyzed were characterized by generally high contamination levels, in the order of at least 10^2 FC/100 ml of water. Bacterial plate counts ranged from $9.1x10^1$ to $2.48x10^6$ CFU/100 ml of water, whereas immunofluorescence counts varied between $5x10^2$ and $4.64x10^5$ cells/100 ml. Enzymatic values detected were quite variable, comprised between 0.0013 and 67.75 μg MU l^{-1} h^{-1}. Both the bacterial numbers and the levels of enzymatic activity increased corresponding to the sites subjected to higher input of urban effluents. With regard to the samplings repeated over two successive days (37 samples in total), the highest concentrations were generally observed during the "montante" current coming from the Ionian basin (data not shown).

A good linear correlation was found between β-glucuronidase and FC (r= 0.807) values and MUG-IF values (r=0.747) by pooling all data obtained, as well as during almost all sampling periods (Table 1 and Fig. 1 a,b).

Table 1. Pearson's correlation coefficients calculated between enzymatic activity (Vmax) and plate (FC) and microscopical (IF) counts.

		Vmax versus:	
	Pair no.	FC	IF
August	4	0.999(**)	0,865
September	8	0.999(**)	0,619
October	9	0.702(*)	0.875(**)
November	11	0.662(*)	0,23
December	12	0.644(**)	0.682(**)
January	12	0.891(**)	0.927(**)
February	12	0.837(**)	0.738(**)
March	12	0.881(**)	0.672(**)
All samplings	80	0.807(**)	0.747(**)

(*) significant value at P<0.05
(**) significant value at P<0.01

Discussion

In previous research carried out in our laboratory, we evaluated the accuracy and selectivity of a solid medium incorporating MUG; however, the long time of incubation required (24 h) did not allow to suggest the use of this method for rapid environmental monitoring (8). The method proposed here allows to overcome time limitation. The