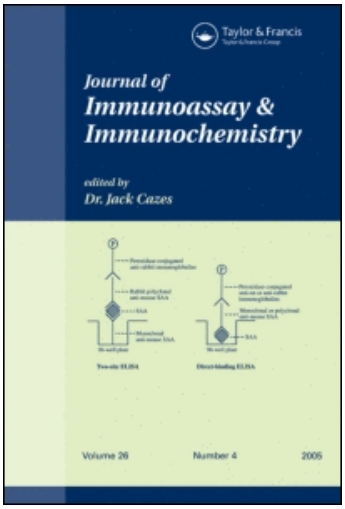

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ABSTRACT

Through a microscopical method, relying on the interaction between fluorescent antibodies and target antigen, it is possible to detect and enumerate *Escherichia coli* in seawaters. Various commercial monoclonal and polyclonal antisera have been tested in an indirect immunofluorescence (IIF) assay developed for microbiological monitoring of coastal waters. Prior to use, they have been titrated and screened for cross-reactions with a collection of clinical and environmental isolates. A comparison among counts obtained on field samples showed higher performance for microscopical than for plate methods, due to the ability of all antisera to label target cells specifically, regardless of their viability. Because of their different specificities, polyclonal antisera yielded better quantitative results than

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monoclonal antisera. The study further suggested the usefulness of the immunofluorescence assay as a rapid alternative analytical tool for the specific detection of bacterial pathogens in aquatic environments.

Key Words: Immunofluorescence; Immune sera; *Escherichia coli*; Seawaters

INTRODUCTION

In the framework of the basic measures established for the preservation of aquatic environments from pollution, monitoring of seawater quality plays a crucial role. The awareness of the limited availability of water resources, already greatly exploited by man, and of water-borne diseases and sanitary risks deriving from the use of seawaters contaminated by faecal wastes, makes any initiative for environmental conservation of global concern. Therefore, in recent years, an increasing interest by the scientific community has been addressed to searching new strategies for monitoring microbial indicators of faecal pollution discharged into aquatic environments. Major efforts have been devoted to the development of methodologies or advanced technologies[1–3] for the specific detection and enumeration of *Escherichia coli*, the most frequent species among faecal coliforms, which has recently been considered as the most effective indicator of faecal contamination.[4] Two reasons justify this operational choice: conventional methods for the determination of the extent of water pollution rely on the quantification of this microorganism by culture media, but the long analysis times required limit their feasible application in real-time environmental monitoring;[5] moreover, the same use of faecal coliforms as indicators has been controversial, due to their short survival times in waters and to their evolution in a viable but nonculturable form (VBNC).[6] Consequently, the search of new rapid methods as alternatives to standard techniques for *Escherichia coli* detection in waters has become more and more urgent.[7]

To this end, a promising approach is offered by the immunofluorescent method, based on the use of immune sera which react specifically with bacterial antigens; an indirect immunofluorescence (IIF) protocol has recently been applied at the Istituto Sperimentale Talassografico for the microbiological control of coastal areas heavily affected by urban discharges.[1,2,8] Immunological methods are widely used to detect pathogens in environmental samples, providing an exciting development in the field of rapid methods applied to monitoring the sanitary and microbiological quality of waters.

As a part of the research carried out during 2001 within the MIUR "Cluster 10-SAM" project for automatic seawater monitoring, six monoclonal and polyclonal antisera with different spectra of reactivity for the enteropathogenic (EPEC), enterotoxigenic (ETEC) and enteroinvasive (EIEC) serotypes of *E. coli*, were tested in an indirect immunofluorescent assay in order to evaluate their usefulness for the microbiological monitoring of contaminated areas. The aim of our experiments was to compare their performance and response in terms of microscopical detection; through the polyclonal sera assay which labelled a wider number of serotypes than those previously targeted, including serotypes other than EPEC,[8] we also evaluated the possibility to obtain accurate estimates of the abundance of the overall *E. coli* population.

EXPERIMENTAL

Principle of the Method

In indirect immunofluorescence (IIF), the primary antibody reacts specifically with the target antigen, yielding an antibody–antigen complex which may be visualised after labelling with a secondary antibody conjugated to a fluorochrome such as fluorescein isothiocyanate (FITC). So, the bright boundaries of labelled cells become visible under epifluorescence microscope when excited with a light of specific wavelengths for the particular fluorochrome used (i.e., FITC is excited at 490 nm and emits at 515–520 nm).

Sampling and Microbiological Analyses

Water samples were drawn from three coastal sites, recognised as potentially subjected to pollution phenomena due to the presence of anthropic and industrial settlements. The areas of study were the Gulf of Palermo, the Gulf of Gioia Tauro, and the Straits of Messina; samplings were performed at 10 stations from the first two sites while, in the last zone, 12 stations were sampled. Samples collected were divided into sub-volumes of 100 mL and fixed in formalin (2% final concentration) within two hours of sampling. The density of faecal coliforms (FC) was estimated by filtration of aliquots equal to 10 and 100 mL aliquots, or less than 1 mL for highly polluted samples, through a 0.45 μm Millipore membrane and incubation at 44.5°C for 24 h on duplicate plates of the selective medium m-FC (Difco) + 2% agar.

For immunofluorescence counts, the following analytical protocol was used:[2] aliquots of waters equal to 100 mL or less, depending on the turbidity of the sample, were filtered through a 0.22 μm Nuclepore black membrane and the filter incubated, first for 30 min with a dilution of the primary serum, rinsed with phosphate buffered saline (PBS), and then incubated for the same time with the secondary antibody labelled with fluorescein isothiocyanate. In particular, for this step, we used goat anti mouse IgG (whole molecule) conjugated FITC (Sigma) for monoclonal antisera, and goat anti rabbit IgG (whole molecule) conjugated FITC (Sigma) for polyclonal ones. After mounting on a slide, the filter was observed under a Zeiss Axioplan epifluorescence microscope equipped with the filter set BP 490, FT 510, and LP 520. Cells binding the fluorescein conjugate appeared as fluorescent green rods under the microscope.

The *E. coli* counts were obtained from the mean value of cells calculated on 30 fields, using the formula: cells (mL) = (mean cellular value per field × area of the filter)/(volume of water filtered × 1.05) where 1.05 is a factor deriving from the fixation of the sample.

Characteristics of Specificity of the Sera

Polyclonal and monoclonal antisera used in the IF protocol were commercially distributed; they were specific for the following serotypes:

1. Monoclonal sera, both identifying serotypes O18, O44, O112, O125, were:
   (a) Chemicon Mouse Anti-*E. coli* monoclonal antibody (MAB 8381) and
   (b) ViroStat Monotope for *E. coli* (code 1011).
2. Polyclonal sera used had the spectrum of specificity reported below:
   (a) Behring antisera, a mix of three pools: A (O26, O55, O111, O128), B (O86, O119, O125, O126, O127), C (O114, O142, O158), which labelled a total of 12 serotypes,
   (b) Murex *E. coli* agglutinating sera, a mix of three pools: pool 2 (O26, O55, O111, O119, O126), pool 3 (O86, O114, O125, O127, O128), pool 4 (O44, O112, O124, O142), specific for a total of 14 serotypes, of which 12 EPEC and 2 (O112 and O124) EIEC,
   (c) Sifin Test sera "Anti-Coli" (I, II, and III), containing polyclonal antibodies towards O26, O44, O144, O125, O142, O158 serotypes (pool anti-Coli I); O55, O86, O111, O119,

O126, O127, O128 serotypes (pool anti-Coli II); O25, O78, O103, O118, O124, O145, O157, O164 (pool anti-Coli III); they label a total of 21 serotypes,

(d) Denka Seiken *E. coli* poly sera set 1(O-sera), a mix of eight polyvalent pools with the following reactivity spectrum: pool 1 (O1, O26, O86a, O111, O119, O127a, O128), pool 2 (O44, O55, O125, O126, O146, O166), pool 3 (O18, O114, O142, O151, O157, O158), pool 4 (O6, O27, O78, O148, O159, O168), pool 5 (O20, O25, O63, O153, O167), pool 6 (O8, O15, O115, O169), pool 7 (O28a, O112a, O124, O136, O144), pool 8 (O29, O143, O152, O164), for a total of 43 serotypes. In particular, pools 1–3 were specific for EPEC, pools 4–6 for ETEC and pools 7–8 for EIEC serotypes.

Titration of the Sera

Before their use, sera were assayed for sensitivity and titrated by the same immunofluorescence technique using Teflon coated multiwell slides for immunofluorescence (Biomerieux). Briefly, a suspension of the control strain O125 and a mix of *E. coli* strains (O26, O125, and environmental isolates) were used in the titration of monoclonal and polyclonal sera, respectively; 10 μL of bacterial suspension were distributed over each wall of the slide, then fixed with cold acetone for 10 min and allowed to dry at room temperature. Separately, appropriate doubling dilutions (from 1 : 20 to 1 : 640) of both reagents (antisera and anti-IgG FITC-conjugated) were prepared in PBS prefiltered water, and in Evans Blue, respectively, in a 96-well microtiter plate. Different dilutions of antisera were added to each well of the slide and this latter was incubated in a moist chamber at 35°C for 35 min; after rinsing with PBS and being prefiltered (0.22 μm) distilled water, 10 μL of anti-IgG FITC conjugate were added to each well and incubated as described previously. The slide was then rinsed, mounted with FA mounting fluid (Difco), and observed by microscope.

From this assay, it was possible to test different combinations of antisera and anti-IgG and determine the optimal one to be used as working dilution.

Test of Sera Specificity

Once sera were titrated, they were assayed for their specificity against a collection of both clinical and environmental isolates and collection strains,

in order to determine the presence of cross-reactions with homologous and heterologous bacteria. For this aim, both enterobacterial and environmental strains were collected, which included strains of *Escherichia coli* as positive controls, clinical and marine isolates; they were maintained in the form of axenic cultures.

In the list below, the strains used for the test of specificity and their provenance are reported:

- *E. coli* O-125 serotype (Sclavo reference strain)
- *E. coli* environmental isolates: B1, B2, B4, B6, B7, B8, B9, B10, B11, B12, B13, B14, B15, B20, B21, B22 (sewage effluent of Messina, Italy)
- Clinical strain: *Pseudomonas aeruginosa*
- *Enterobacteriaceae: Enterobacter agglomerans**, *Klebsiella pneumoniae**, *Proteus mirabilis**, *Yersinia enterocolitica**, *Y. enterocolitica* O:3, *Y. enterocolitica* O:10, *Citrobacter freundii**, *Salmonella arizona* (Piccolo Sea, Taranto, Italy), *S. panama*, *S. gallinarum*, *S. bovis-morbificans*, *S. blockley*, *S. enteritidis*, *S. typhimurium*, *S. typhi*, *S. paratyphi* B, *Shigella flexneri*, *Escherichia vulneris**, *E. hermannii**, *E. coli**.
- Environmental autochthnous strains: *Pseudomonas* spp., *Vibrio* spp. (Adriatic Sea), *Vibrio metschnikovii*, *Aeromonas hydrophila* (Piccolo Sea, Taranto, Italy).

Statistical Analyses

Differences between counts, due to the kind of reagents and technique used, were tested using analysis of variance (ANOVA). Linear regression was used to examine correlation between direct (immunofluorescence) and indirect (culture) methods.

RESULTS

Titration of the Sera

The following dilutions of antisera yielding the maximum intensity of fluorescence by microscopic observation, namely 1 : 40 for Murex and Denka Seiken antisera, 1 : 20 for Sifin antisera, 1 : 10 for Behring antisera, 1 : 50 for monoclonal (Chemicon and ViroStat) antisera, were chosen as working dilutions. The fluorescent anti-IgG was always used at the dilution

1 : 80 and, in particular, the anti-mouse IgG and anti-rabbit IgG were used for monoclonal and polyclonal antisera, respectively.

Specificity Assay

The assay of specificity highlighted different characteristics of reactivity of the sera towards the homologous and heterologous strains of bacteria tested (Table 1). *Shigella flexneri* reacted positively with the majority of polyclonal antisera assayed. Cross-reactivity with some *Salmonella* strains, recorded for both Murex and Behring antisera, may interfere at the microscopical observation, yielding false positive results. These effects, probably ascribable to the presence of surface antigens common to *E. coli*, could be overcome through the adsorption of sera with a suspension of *E. coli*, in order to saturate all the binding sites of antibodies contained in the sera with the target antigen. Denka and Sifin sera displayed a lower number of cross-reactions. As expected, both monoclonal antisera exhibited the highest selectivity for *E. coli*. All the antisera used gave no cross-reactions with the strains of environmental bacteria assayed.

Modifications to the Analytical Protocol

During experimental trials, some modifications were made to the IIF labelling protocol in relation to problems arising from the type of serum used, with the aim of obtaining a better microscopical resolution of *E. coli* cells: for Sifin antisera, a preliminary filtration of the three mixed reagents through 0.22 μm Millex filter was included as additional step before their use, in order to avoid the precipitation of detrital particles observed in great number on the filter, thereby interfering with microscopical readings. By using sera Denka, the background fluorescence was reduced with a preliminary incubation of the filters with a 2% solution of gelatine hydrolyzed with hydrochloric acid as blocking agent. No additional treatments were required when using Murex and Behring sera.

Application to Field Samples and *E. coli* Counts

Values of bacterial densities obtained by immunofluorescence and plate techniques are summarised in Table 2. In terms of quantitative results, Murex sera displayed a good sensitivity, yielding, on average, counts in percentages of 74.3 and 72.8% higher than Behring and Sifin sera, respectively. Results

Table 1. Cross-Reaction Test of Antisera Used with Environmental and Clinical Strains (The Degree of Fluorescence Is Indicated by the Number of Symbols '+')

	Monoclonal Antisera		Polyclonal Antisera			
Strains	ViroStat 1 : 50	Chemicon 1 : 50	Murex 1 : 40	Denka 1 : 40	Sifin 1 : 20	Behring 1 : 10
E. coli O125	+++	+++	+++	+++	+++	+++
Pseud. spp.	−−−	−−−	−−−	−−−	−−−	−−−
Vibrio spp.	−−−	−−−	−−−	−−−	−−−	−−−
V. metschnikovii	−−−	−−−	−−−	−−−	−−−	−−−
Aerom. hydrophila	−−−	−−−	−−−	−−−	−−−	−−−
E. vulneris	−−−	−−−	−−−	−−−	−−−	−−−
E. hermannii	−−−	−−−	−−−	−−−	+−−	−−−
Sh. flexneri	−−−	−−−	++−	+−−	++−	−−−
Klebs. pneumoniae	−−−	−−−	−−−	−−−	−−−	−−−
Ent. agglomerans	−−−	−−−	−−−	−−−	−−−	+−−
Pr. mirabilis	−−−	−−−	−−−	−−−	−−−	−−−
Y. enterocolitica O:10	−−−	−−−	−−−	−−−	−−−	−−−
Y. enterocolitica O:3	−−−	−−−	+−−	−−−	−−−	−−−
Citrob. freundii	−−−	−−−	−−−	−−−	−−−	−−−
S. arizona	−−−	−−−	−−−	−−−	−−−	−−−
S. panama	−−−	−−−	−−−	−−−	−−−	+−−
S. blockley	−−−	−−−	++−	−−−	++−	++−
S. bovis morbificans	−−−	−−−	++−	−−−	−−−	−−−
S. gallinarum	−−−	−−−	++−	+−−	++−	−−−
S. typhimurium	−−−	−−−	++−	−−−	−−−	+−−
S. enteritidis	−−−	−−−	−−−	−−−	−−−	+−−
S. typhi	−−−	−−−	++−	−−−	−−−	+−−
S. paratyphi B	−−−	−−−	−−−	−−−	−−−	+−−

E. = *Escherichia*; *Pseud.* = *Pseudomonas*; *Aerom.* = *Aeromonas*; *Sh.* = *Shigella*; *Klebs.* = *Klebsiella*; *Ent.* = *Enterobacter*; *Pr.* = *Proteus*; *Y.* = *Yersinia*; *Citrob.* = *Citrobacter*; *S.* = *Salmonella*.

obtained using Denka sera were conflicting, reaching values sometimes one or two orders of magnitude higher than Murex sera, but they labelled, on average, a percentage of cells equal to 77.1% of those detected by Murex sera. Chemicon and ViroStat sera yielded comparable results, so that values reported in Table 2 refer to both sera. Counts recorded with monoclonal antibodies were always the lowest in magnitude; this finding was in agreement with their narrow range of reactivity, which was limited to 4 serotypes only, and consequently led to an underestimation of the bacterial concentration present in natural samples.

Table 2. Cellular Densities Estimated by Immunofluorescence (Cells $100\,mL^{-1}$) and Standard Plate (CFUs $100\,mL^{-1}$) Methods

Samples	FC CFU $100\,mL^{-1}$	IF Cells $100\,mL^{-1}$				
		Murex	Behring	Sifin	Denka	Chemicon
P1	5	2,141	1,512	1,638	252	839
P2	10	1,989	4,788	1,134	377	1,678
P3	381	163,795	61,198	160,195	25,199	22,679
P4	351	45,568	34,439	26,879	5,669	7,979
P5	1	882	3,150	1,359	1,385	420
P6	0	5,669	21,545	6,299	3,864	420
P7	3	5,669	3,780	1,134	4,536	839
P8	2	8,819	24,727	6,089	5,669	839
P9	0	12,389	43,199	3,359	2,489	839
P10	0	11,759	11,655	6,089	5,039	420
G1	3	1,134	2,646	1,134	1,889	839
G2	2,361	68,038	20,159	35,279	35,279	20,159
G3	9	3,919	1,631	1,512	839	1,678
G4	0	168	1,386	168	839	420
G5	2	1,176	1,061	1,134	1,512	420
G6	0	2,268	133	1,008	6,804	168
G7	0	2,519	133	1,008	37,295	168
G8	0	1,134	3,261	1,008	3,906	168
G9	0	1,008	2,835	1,008	46,871	420
G10	3	504	1,386	1,134	33,547	420
S1	3	1,386	3,683	1,134	145,316	839
S2	0	168	133	168	252	133
S3	104	4,199	3,276	6,089	7,279	1,678
S4	5,200	228,593	136,796	70,558	57,598	61,738
S5	4,780	135,446	39,058	35,279	62,788	36,538
S6	3,020	30,239	22,049	35,279	64,258	9,449
S7	73	840	24,947	6,089	29,609	1,678
S8	4,940	99,222	4,680	70,558	57,598	9,449
S9	44	2,099	3,024	3,024	839	420
S10	0	168	133	168	252	133
S11	1	504	1,512	1,134	839	168
S12	0	168	133	168	252	133
Mean	665	26,362	15,126	15,257	20,317	5,755
S.D.	1,556	54,818	26,989	32,555	31,285	13,034

Table 3. Results of ANOVA Test

FC vs:		Murex vs:		Behring vs:	
Murex	7.026*	FC	7.026*	Murex	1.082
Behring	9.156**	Behring	1.082	FC	9.156**
Sifin	6.414*	Sifin	0.971	Sifin	0.0003
Denka	12.595**	Denka	0.293	Denka	0.505
Chemicon	4.811*	Chemicon	4.279*	Chemicon	3.128
Sifin vs:		**Denka vs:**		**Chemicon vs:**	
Murex	0.971	Murex	0.293	Murex	4.279*
Behring	0.0003	Behring	0.505	Behring	3.128
FC	6.414*	Sifin	0.402	Sifin	2.349
Denka	0.402	FC	12.595**	Denka	5.907*
Chemicon	2.349	Chemicon	5.907*	FC	4.811*

*$p < 0.05$; **$p = 0.01$.

High standard deviation values were observed for direct and indirect counts; this suggested the high variability of bacterial concentrations among different samples (Table 2).

We used an ANOVA test to verify if our cell estimates were statistically comparable to each other, or whether they differed significantly; in other words, if they identified and were representative of two distinct populations of the same bacterial community, in the first hypothesis, or different subgroups belonging to different communities, in the second eventuality. While ANOVA values showed that there were no statistically significant differences among counts obtained with polyclonal antisera (Table 3), the significant F values obtained from plate (FC) counts, compared to immunofluorescence counts, suggested that culture and microscopical methods estimated two different populations. Quantitative differences between monoclonal and Denka ($F = 5.907$, $p < 0.05$, $n = 32$) or Murex ($F = 4.279$, $p < 0.05$, $n = 32$) sera were also statistically significant.

The calculation of the coefficients of variation, as an index of data dispersion, compared to the arithmetic mean (C.V. = standard deviation/arithmetic mean*100, Fig. 1), gives some information on the variability in cell distribution among repeated measurements within each sample, performed both on filter (30 microscopical fields) or on plate (two replicates). As suggested,[9] there was usually an inverse relationship between the total variance contributed by replicates, as estimated by C.V. values, and the number of bacteria per field. For Murex, Behring, and Sifin sera, a higher variability, as shown by higher CVs, was associated to microscopical counts

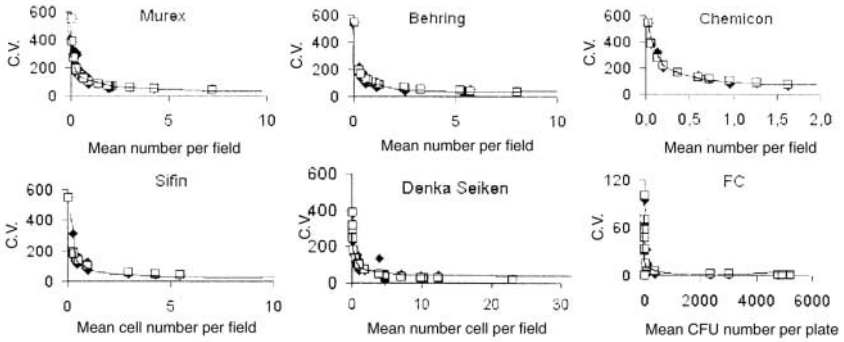

Figure 1. Variability in cell distribution within each sample by microscopical (IF) and culture (FC) methods. Coefficients of variation (C.V. = standard deviation/arithmetic mean*100) calculated with respect to cell numbers per microscopical field or plate; theoretical C.V. ($=1/\sqrt{x}$) from the Poisson distribution.

lower than 2 cells per field. A similar variation range was observed using the monoclonal Chemicon sera but, in presence of a minimum number of cells per field. Using polyclonal Denka sera, an increase in the number of cells counted per field resulted in a lower variability (C.V. values lower than 400). Due to subjectivity of the microscopic observation, IF estimates led to enhanced variability, whereas reduced values of C.V.s were found with culture method, suggesting a lower variability of plate counts and, consequently, higher data reproducibility within one sample.

On average, *E. coli* counts by immunofluorescence exceeded faecal coliform counts estimated by standard plate method by two or one orders of magnitude with respect to polyclonal or monoclonal antisera, respectively (Table 2). This discrepancy provides additional evidence for the presence of viable nonculturable cells. Although *E. coli* is the main component of the faecal coliform group, and one may expect the reverse result, this was not surprising, since the ability of the microscopical technique to quantify cells which are stressed and unable to grow on media, but still have an intact surface antigenic structure, makes the immunofluorescence counts not directly comparable with plate counts. The statistical analysis of logarithmic transformed data through linear regression, however, revealed the positive correlation between the microscopical and culture methods, as shown by the high values of the regression coefficients and by the low dispersion of data with respect to the theoretical regression line (Fig. 2).

Error rates ($E\% = (\text{IF} - \text{FC})/\text{FC}$) between the two methods, calculated for the different sera separately, increased above 100% for low

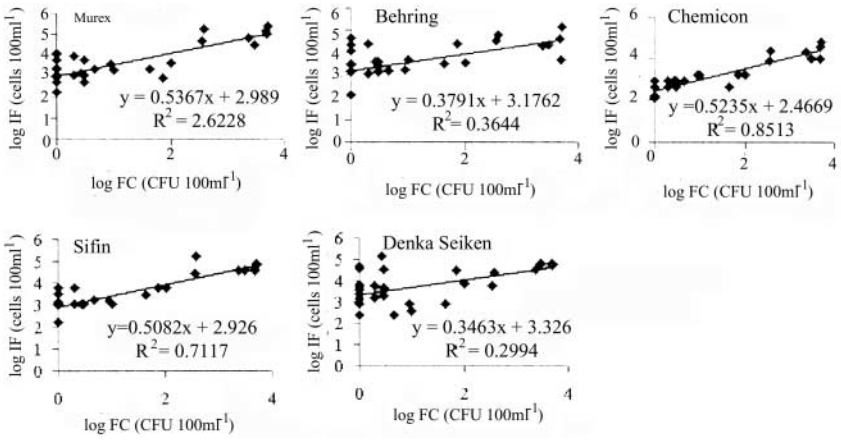

Figure 2. Linear regression analysis of microscopical vs. plate counts calculated for each serum separately.

contaminated samples, with cell densities lower than 10 CFU $100\,mL^{-1}$; lower error rates were found for Chemicon sera (Fig. 3).

Serial dilutions of a bacterial suspension of *E. coli* having a density of 7.28×10^8cells mL^{-1}, as estimated by DAPI counts, were performed in order to establish the sensitivity threshold of the IF technique. With all the polyclonal sera, a comparable threshold value can be achieved, since a minimum of 15 CFU, as estimated by plate count, can be detected (Fig. 4).

Concerning an overall judgement on the performance of the sera tested, in comparison with Behring sera, the only one used in previous studies,[8] Murex sera yielded higher counts and showed good performance in the immunofluorescence protocol, allowing a clear detection of *E. coli* cells without needing any further treatment. Contrary to what was expected on the basis of their reactivity range, counts obtained with Denka sera were frequently lower than Murex counts; since Denka sera were directed towards a greater number of serotypes, a possible explanation of this result may be the limited occurrence in the aquatic environment of the serotypes targeted by Denka sera.

DISCUSSION

The need for new rapid and accurate methods for the determination of microbial indicators is one of the specific goals of research devoted to the control and preservation of water quality. The methodological problems

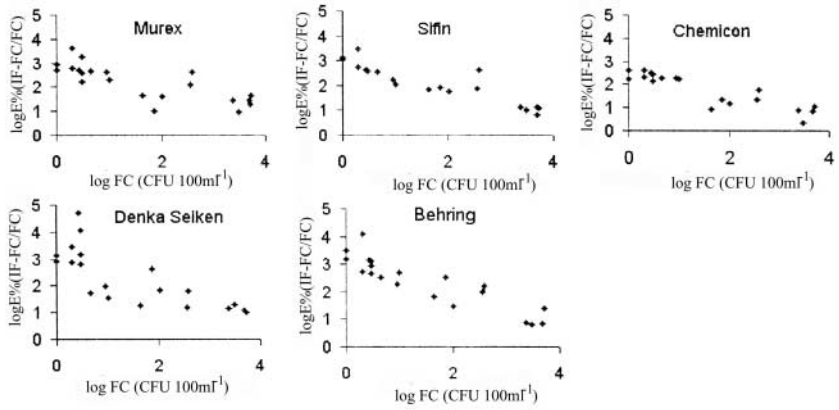

Figure 3. Error rates between immunofluorescence (IF) and faecal coliform (FC) counts obtained as $E\% = ((\mathrm{IF} - \mathrm{FC})/\mathrm{FC})$ for each serum tested.

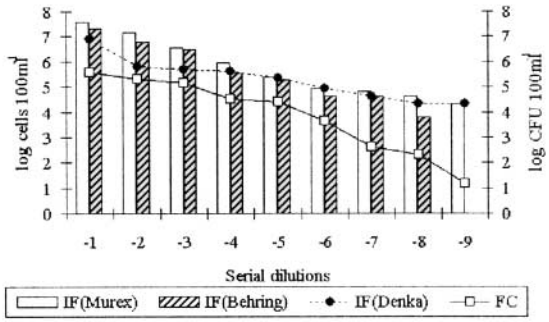

Figure 4. Assay of sensitivity performed on serial dilutions of a bacterial suspension of *E. coli* (initial density $= 7.28 \times 10^8$ cells mL^{-1}).

linked to the detection and enumeration of *E. coli* are still not completely resolved and, to date, there is no method which is considered completely reliable.[7] In the field of seawater analysis, the immunofluorescence approach has been successfully applied to detect pathogens[8,10–12] or bacterial species involved in biogeochemical cycles.[13] At the moment, however, this method is used at an experimental level only and it has not been included in the routine microbiological practices by Italian regulation.

Results obtained in our study further suggest that immunofluorescence data are not significantly different from indirect plate counts

and, therefore, this approach could represent a reliable and efficient alternative to culture techniques. Immunofluorescence results are also in accordance with the values of β-glucuronidase obtained with a rapid enzymatic fluorogenic assay with 4-methylumbellyferil-β-d-glucuronide (MUG) which has been experimented in our laboratory for the study of microbial pollution.[14] Other than specificity, advantages offered by the use of immunofluorescence in environmental monitoring consist in the reduced times required for analysis and response, and in the simplicity of the analytical protocol. The use of fixed samples allows postponing the execution of analyses; the filters obtained may be kept at -20°C for several months, allowing repeated microscopical observations over time. The technique is not very expensive, as the costs depend on the reagents used; and they have a reduced weight when calculated on the basis of a single determination. In any case, costs are well balanced by advantages: through the IF technique, quantitative direct estimates of the abundance of *E. coli* are obtained; results are available in near real-time, within 2 h of sampling, and this encourages the possible inclusion of this method for early warning of microbial pollution of seawaters. This possibility is also supported by the high number of samples (50–60) that may potentially be processed and analysed per day.

Particular care must be devoted to the interpretation of readings: observations must be performed by experienced personnel in order to avoid subjectivity of the counts and to distinguish target cells from detritus. This is particularly important in seawater samples, which are usually rich in organic and inorganic debris onto which cells may attach and, therefore, detritus may mask their identification. Therefore, a preliminary strong homogenisation and/or sonication of the sample is recommended in order to obtain the dispersion of organic aggregates that may interfere with microscopical detection.

The quality of the immunological assay is related to the characteristics of antibody preparation;[15] polyclonal antibodies recognise several antigens, while monoclonal antibodies are specific to single epitopes, resulting in high levels of specificity.

One of the most evident limitations of the immunofluorescence method is the scarce availability of specific immune sera and, in particular, by the narrow range of specificity of the antisera used for *E. coli* detection. In fact, the majority of those commercially distributed include most of the agglutinins recognised in human infections; in particular they are directed towards the enteropathogenic (EPEC) serotypes of *E. coli* only, responsible for gastroenteritis and diarrhea in children. On the other hand, the specificity of a polyclonal serum is not extensive enough to detect all *E. coli* serotypes potentially occurring in a clinical or environmental sample.

An attempt to extend the spectrum of reactivity of the sera to a wider number of serotypes, other than EPEC, has been carried out in our study; this goal has only partly been reached, as the Denka sera expected to label in absolute the great range of *E. coli* serotypes (42 among EPEC, EIEC, and ETEC) sometimes failed to give the highest counts. However, with respect to Behring sera previously used,[8] Murex sera, tested in our investigation, also label EIEC other than EPEC serotypes and, therefore, may provide an estimate of the overall *E. coli* population more accurately than was formerly possible.

Using polyclonal antibodies, problems may arise from non-specific bindings to bacterial antigens other than target, but the adsorption of sera with strains known from the specificity test to cross-react, may exclude the occurrence of false positive results.[12]

Detection of pathogens in environmental samples is limited by the low sensitivity of the method which requires a bacterial concentration high enough ($10–10^2$ cells $100\,mL^{-1}$) for detection; this threshold value may be overcome through the concentration (i.e., by preliminary filtration or centrifugation) of large volumes of seawater. The sensitivity threshold of the method depends on the affinity of the antibodies contained in the serum used.

An interesting perspective of the research is related to checking the viability conditions of the cells labelled. Unlike the culture methods, detection by immunofluorescence does not provide information concerning this aspect, because all the target bacteria, dead, viable and culturable or viable but nonculturable, are labelled, provided that binding sites are not degraded; therefore from IF results it is impossible to verify if positive reactions truly indicate the presence of infectious pathogens and to determine their health significance. The viability concept is very important for interpreting the detection of pathogenic bacteria in relation to public health issues.[6,16] Besides, dead cells, which may bind antibodies not specifically, can make the assessment of sanitary risks related to the use of contaminated waters very difficult. It must not be forgotten, however, that the recovery of gastrointestinal pathogens in a sample in itself demonstrates that sewage contamination has taken place. In particular, the detection of *E. coli* cells which are still alive and replicate on culture media, is indicative of a recent pollution episode, since 2 or 3 weeks after introduction in oligotrophic environments and exposure to sunlight, this microorganism undergoes sublethal injury and enters the viable but nonculturable state.[17] It has been demonstrated, however, that *E. coli* strains may retain their pathogenicity under adverse conditions.[18] Furthermore, future developments in the immunoassay technology include the combination of the fluorescent antibody staining with fluorochromes or substrates (i.e., fluorescein diacetate or CTC,

5-cyano-2,3-ditolyl tetrazolium chloride) able to give indication about cell viability at the same time. This double staining procedure could specifically identify the active metabolic cells only, allowing discarding non-viable cells from immunofluorescence analysis. This modification to the treatment of sample could expand the potential of the microscopical method and broaden the range of future applications, providing a more precise evaluation of the microbiological quality of seawaters.

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