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Rapid Determination of Members of the Family *Enterobacteriaceae* in Drinking Water by an Immunological Assay Using a Monoclonal Antibody against Enterobacterial Common Antigen

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An immunological method for the detection of members of the family *Enterobacteriaceae* in drinking water was developed. The method was based on a sandwich enzyme-linked immunosorbent assay (ELISA) with monoclonal antibody immunoglobulin G2a 898 against enterobacterial common antigen. The enterobacterial common antigen sandwich ELISA combined with selective preenrichment culture could be performed in only 24 h. Six hundred sixty-eight water samples from a variety of German public water supplies were screened to verify the effectiveness of the new method. Ninety-eight percent of the results obtained by the immunological method could be confirmed by conventional microbiological methods. The immunological method proved to be considerably faster and more specific and sensitive than the standard method specified by the German drinking water regulations.

Standard microbiological methods for testing drinking water are currently based on time-consuming culture techniques and biochemical analyses. These procedures are often inappropriate when rapid intervention is needed. In this report we describe the development and evaluation of a rapid and highly specific immunological method to detect members of the family *Enterobacteriaceae* in drinking water. A monoclonal antibody (MAb) (immunoglobulin G2a [IgG2a] 898) against the enterobacterial common antigen (ECA) was used in a sandwich enzyme-linked immunosorbent assay (ELISA) to detect *Enterobacteriaceae* in German drinking water as an alternative to the conventional identification of coliform bacteria. ECA, which was first described by Kunin et al. (8), is a stable family-specific antigen of all *Enterobacteriaceae*. *Erwinia chrysanthemii* is the only enterobacterial species without ECA, whereas *Plesiomonas shigelloides*, which was previously classified as being in the family *Vibrionaceae*, was found to produce ECA (9, 16). ECA is a glycophospholipid built up by a polymer of the trisaccharide repeating unit 4-3-β-MANNACUA-(1-4)-α-D-GLGNAO-(1-3)-α-FUC4NAC-(1-4) covalently linked to an L-glycophosphatidyl residue (7, 11, 13). ECA is located in the bacterial outer membrane (2). At present, ECA plays an important role in taxonomy (6) but is rarely used for analytical or diagnostic purposes (12).

The German drinking water regulations (1) require the absence of *Escherichia coli* and coliform bacteria in 100 ml of a water sample. To meet this limitation (i.e., to detect a single *E. coli* cell in 100 ml), filtration of the water sample and enrichment in a selective medium are needed before the sample is tested in the ELISA. The whole protocol, including the ELISA and overnight cultivation of the bacteria, requires only 24 h.

The new immunological method was compared with an extended modification of the microbiological standard method specified by the German drinking water regulations. Water samples with various degrees of contamination were taken from all steps of drinking water treatment and distribution systems and from groundwater, spring water, and private wells. Besides samples from the Mainz water supplies, samples were obtained from five other German public water supplies and from one public health authority. The samples were collected in sterile 250-ml water bottles containing 0.5 mg of sodium thiosulfate as a chlorine neutralizer, refrigerated, and transported to the laboratory in Mainz by road or rail as soon as possible (at least within 20 h). A 100-ml sample of water was filtered (0.2-μm-pore-size ME24/21 filter; Schleicher & Schuell, Dassel, Germany), and the filter was incubated in 10 ml of lactose-peptone broth (no. 10689; Merck, Darmstadt, Germany) at 37°C. An aliquot of 1 ml was taken after incubation for 20 h. Optimal release of ECA from the outer membrane of the bacteria was achieved by adjusting the pH of the aliquot to 11.0 and then boiling for 15 min in hot steam. The pH of the sample was then readjusted to 7.2 before the sample was tested in the sandwich ELISA.

MAb IgG2a 898 (14) was purified by affinity chromatography on protein A-Sepharose (Pharmacia, Uppsala, Sweden) and used in the sandwich ELISA as a capture antibody and as a developing antibody. Since the trisaccharide repeating units of ECA function as epitopes, the same antibody can be used in the sandwich ELISA to capture and detect the antigen. U-shaped wells of nonflexible polystyrol plates (Greiner, Nürtingen, Germany) were coated with 20 μl of MAb IgG 2a 898 (10 μg of protein per ml) in buffer A (0.01 M potassium phosphate buffer made isotonic with saline [pH 7.5]) for 90 min at room temperature or overnight at 4°C. Plates were then washed twice with 200 μl of buffer A and incubated with 150 μl of buffer A containing 1% (wt/vol) bovine serum albumin (BSA; Sigma) for 30 min. After one wash with buffer A, 20-μl aliquots of the prepared samples were added and incubated for 1 h and 45 min. *E. coli* K-12 was used as a standard. Plates were then washed three times with buffer A, 20-μl aliquots of the biotinylated MAb IgG2a 898 (14) diluted 1:400 in buffer A-BSA were added, and the plates were incubated for 50 min. The MAb was biotinylated as previously described (4, 18). After three washes with

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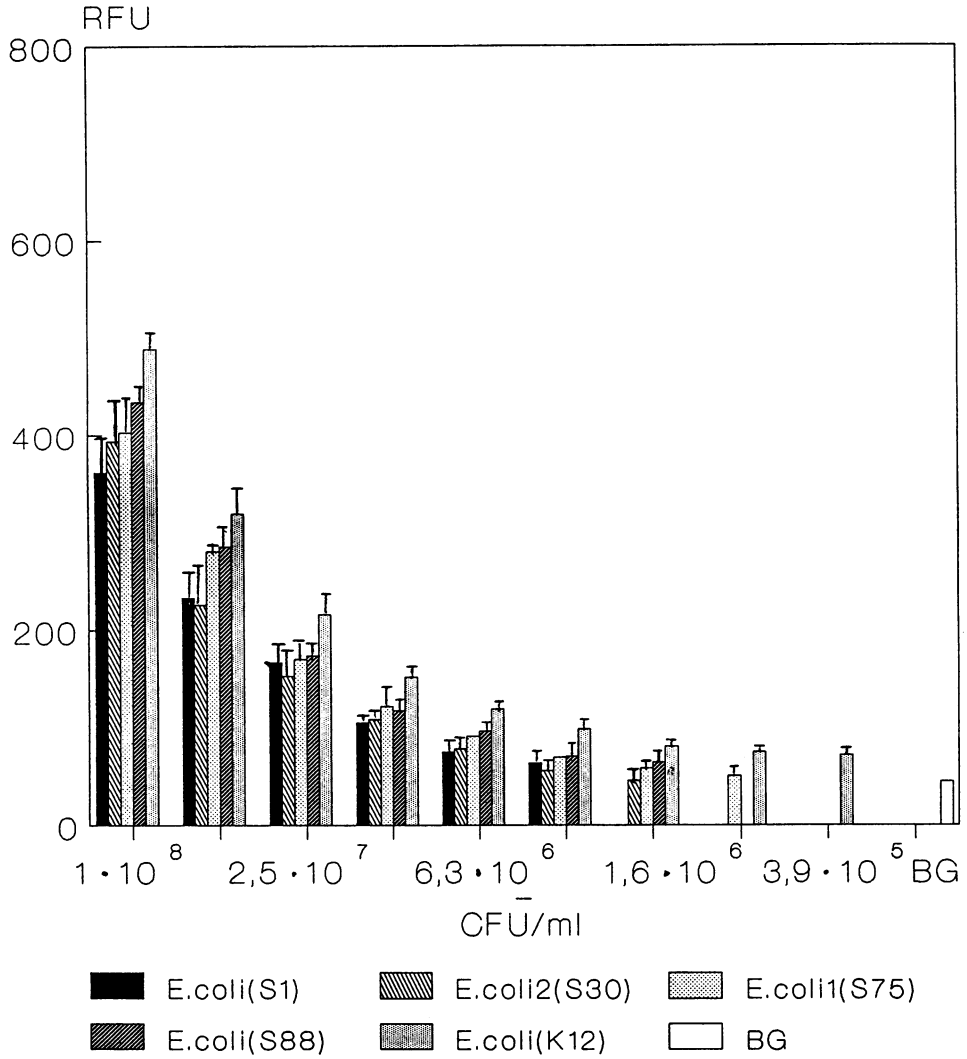

FIG. 1. Sandwich ELISA specific for all *Enterobacteriaceae*. The reaction of the standard *E. coli* K-12 and four other strains of *E. coli* are shown. Cells were grown in lactose-peptone broth at 37°C for 20 h, prepared, and tested in the sandwich ELISA as described in the text. Mean values from four determinations are given. Signals exceeding the mean background plus three standard deviations are shown and considered positive. BG, mean background plus three standard deviations; RFU, relative fluorescence units.

buffer A, 20 μl of streptavidine–β-galactosidase conjugate (250 mU/ml diluted in buffer A-BSA; Boehringer Mannheim, Germany) was added for 25 min. The plates were washed three times with buffer A and once with 0.01 M sodium phosphate buffer (pH 6.9). The substrate solution (50 μl of 0.1 mM 4-methyl umbelliferyl-β-D-galactopyranoside [no. M 1633; Sigma) in 0.01 M sodium phosphate buffer [pH 6.9] per well) was added. After about 30 min, the fluorescent product was measured in a Fluoroskan II fluorometer (excitation wavelength, 355 nm; emission wavelength, 480 nm; Flow Laboratories, Meckenheim, Germany) as relative fluorescence units.

In the microbiological standard method specified by the German drinking water regulations, only primary cultures exhibiting the characteristics of lactose fermentation undergo further confirmatory tests. In the evaluation of the sandwich ELISA we extended the microbiological standard method in such a way that, regardless of any acid or gas formation, transfers from the standard enrichment culture (lactose-peptone broth) were streaked on Fluorocult Brolacin agar (Merck) containing 4-methylumbelliferyl-β-D-glucuronide as a substrate. Fluorescent colonies, indicating growth of *E. coli*, were confirmed by standard biochemical methods. If no fluorescence was observed, all colonies on the Brolacin agar were identified. In the absence of any *Enterobacteriaceae* on the Brolacin agar, additional subculture was done on Columbia blood agar (BioMérieux) and all visible colonies were differentiated.

A total of 668 water samples from the water supplies of five cities and from one public health authority were tested between October 1989 and November 1990 to compare the immunological method with the conventional microbiological method. To obtain a statistically satisfying distribution of negative and positive results, we used a larger number of more contaminated samples than would be expected from routine quality control samples. Ninety-eight percent of the samples yielded corresponding results with the immunological method and the conventional microbiological method;

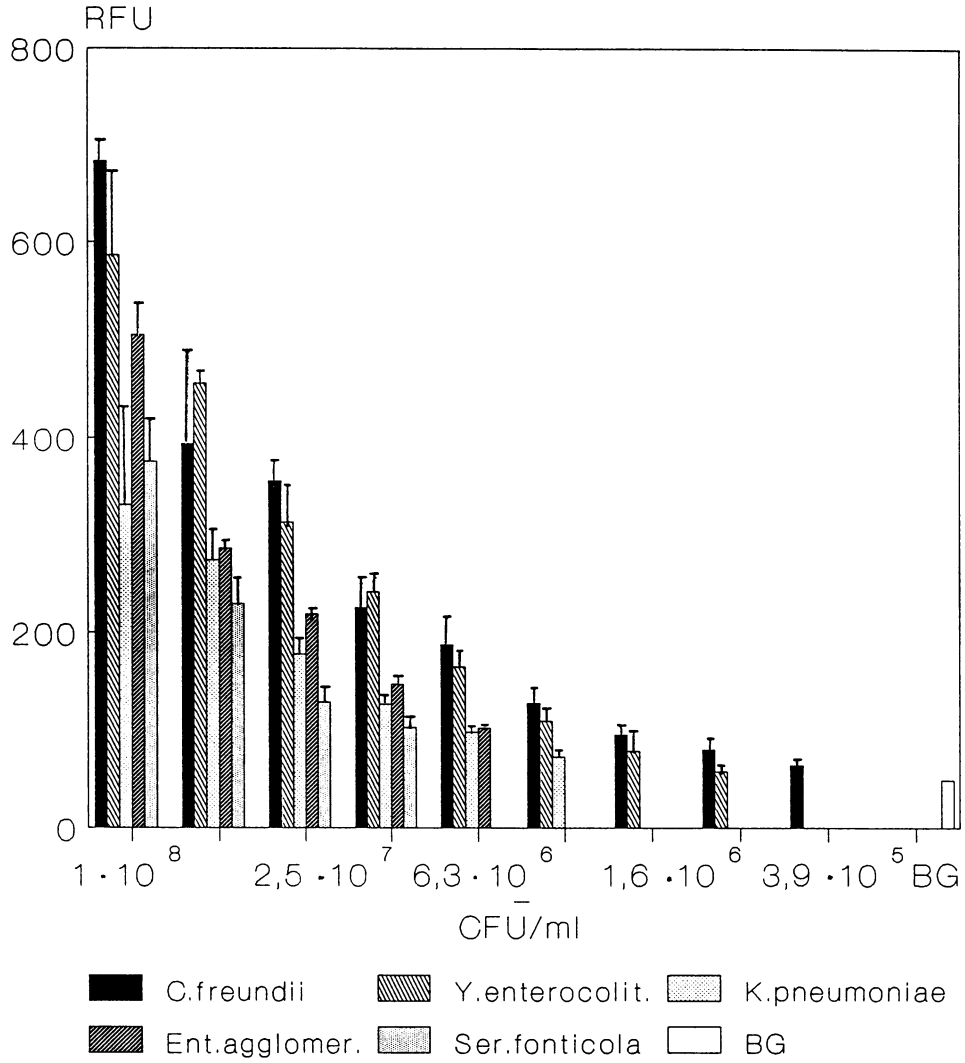

FIG. 2. ECA expression of different *Enterobacteriaceae* isolated from water samples in the sandwich ELISA. Bacteria were grown in lactose-peptone broth at 37°C for 20 h, prepared, and tested as described in the text. Mean values of four determinations are given. Abbreviations are as in Fig. 1.

with both methods, 39.2% of these samples were positive and 58.8% were negative. For 0.8% of the samples (all with bacterial growth), the ELISA yielded a positive result but no *Enterobacteriaceae* could be cultured. None of the other bacteria isolated from these samples showed ECA expression when recultured and tested in the ELISA. It is possible that the *Enterobacteriaceae* in those samples were not culturable anymore and therefore could not be isolated. Interference and antagonistic effects between coliforms and heterotrophic bacteria were previously described (10, 15). In 8 (1.2%) of the samples, *Enterobacteriaceae* were detected but a positive signal in the ELISA was not obtained within 24 h of culture. However, for 7 of these 8 samples the ELISA yielded a positive result after 44 to 48 h of culture. The species isolated from this collection of samples were *Enterobacter agglomerans* (three times), *Serratia fonticola* (twice), *Escherichia vulneris* (once), and one species that could not be definitively identified. One *E. agglomerans* strain, although weakly positive for ECA when tested after isolation on agar, did not produce a detectable ECA concentration in lactose-peptone broth even after 72 h of incubation. When recultured in peptone-lactose broth, these strains grew very slowly.

We also examined the ECA expression of these strains and compared them with those of other water isolates (see below). The sensitivity of the sandwich ELISA for a standard *E. coli* K-12 isolate was about 3.9×10^5 CFU/ml. The amount of ECA detected in another five *E. coli* strains isolated from water samples did not differ very much (Fig. 1). Depending on the ECA content, the detection limit varied for other species (Fig. 2). The values ranged from 1.3×10^7 to 3.9×10^5 CFU/ml. The level of ECA expression of a strain of *Citrobacter freundii* was higher than those of the *E. coli* isolates tested. Representative isolates of *E. agglomerans* and *S. fonticola* expressed only low levels of ECA. Differences in the ECA contents of different species and even different strains of one species were described previously (3, 13).

The sandwich ELISA was highly specific for *Enterobacteriaceae*. No cross-reactions with nontarget species were

TABLE 1. Species of *Enterobacteriaceae* isolated from the preenrichment cultures of the immunological test[a]

Species	No. of samples containing the species
Escherichia coli	170
Citrobacter freundii	30
Enterobacter cloacae	17
Ent. agglomerans	14
Klebsiella pneumoniae	10
Hafnia alvei	5
Enterobacter amnigenus	5
Enterobacter sp.	5
Enterobacter adecarboxilata	4
Citrobacter amalonaticus	4
Citrobacter sp.	3
Escherichia vulneris	2
Serratia fonticola	2
Enterobacter aerogenes	1
Serratia liquefaciens	1
Serratia plymthia	1
Yersinia enterocolitica	1
Unidentified species of *Enterobacteriaceae*	8

[a] A total of 668 water samples were tested. Few samples contained more than one species, and 398 samples were negative.

observed. Two samples (0.3%) contained *Staphylococcus aureus* but no *Enterobacteriaceae*. Possible nonspecific reactions caused by binding of the MAb IgG2a 898 to *S. aureus* protein A were not observed under the test conditions. However, when these *S. aureus* strains were isolated, grown in monoculture to a high cell density, and then tested in the ELISA, bovine gamma globulin (5 mg/ml; Sigma) had to be added in excess to the detector antibody solution to eliminate nonspecific binding. This measure was not taken routinely because *S. aureus* occurred very seldom and native water samples with *S. aureus* in monoculture were not monitored.

The use of lactose-containing media particularly favors the isolation of lactose-degrading species of *Enterobacteriaceae* and therefore meets the legal requirements of the drinking water regulations for the detection of coliform bacteria. Most samples with a positive ECA result contained species belonging to the genera commonly referred to as total coliforms: *Escherichia*, *Citrobacter*, *Klebsiella*, and *Enterobacter*. *E. coli* was the most common species isolated, followed by *C. freundii* (Table 1). The high number of *E. coli* isolates might reflect the identification procedure used (as described above), which favors the detection of *E. coli* only although other *Enterobacteriaceae* might additionally be present. Slow-growing species, e.g., *E. agglomerans*, often did not produce acid or gas within 44 to 48 h of incubation time and would not be detected with the standard methods specified by the German drinking water regulations, which are based mainly on lactose degradation (5). In most cases, the slow-growing species produced a positive signal in the ELISA within the same incubation period and so demonstrate the higher sensitivity of this method. If routine detection of slow-growing species is desirable, prolonging the preenrichment culture could be considered. Most of the non-*Enterobacteriaceae* isolated were species of *Pseudomonas*, *Bacillus*, and *Aeromonas* and gram-positive cocci (data not shown).

The ECA ELISA allows a general determination of all *Enterobacteriaceae* species. This immunological method could therefore be extended to other groups of the family, especially to pathogenic *Salmonella* or *Shigella* species, provided that special selective preenrichment media are applied. On the other hand, if the ELISA is combined with an unselective medium it should be possible to detect all family members. Wiedenmann et al. (17) used the presence of any member of the family *Enterobacteriaceae*, instead of *E. coli* or coliform bacteria only, as an indicator of unsatisfactory drinking water quality.

When used for routine quality control of the laboratory of the Stadtwerke Mainz AG, the immunological method was more specific than the standard method. One sample that had been subjected to conditioning and chlorination produced acid and gas in the standard analysis but showed a negative ELISA result. As a consequence, no supplementary sanitation procedures were applied. The ELISA result was confirmed by the subsequent isolation of a lactose-degrading *Bacillus* sp.

The immunological method is easy to perform and suitable for large numbers of samples during routine drinking water surveys. In combination with growth in the preenrichment medium, the test is particularly designed to detect coliform bacteria. Analysis of drinking water by the standard method requires a minimum of 2 days and a maximum of 5 days. In contrast to the standard method, signs of lactose degradation in samples and time-consuming confirmatory tests are not needed with the immunological method. With this test it is possible to analyze drinking water for the presence of *Enterobacteriaceae* in only 24 h. Our results with the test in the routine quality control of drinking water at the laboratory of the Stadtwerke Mainz AG indicate the advantages of the new immunological method over the standard method.

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