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Molecular Typing of *Aeromonas* Isolates in Natural Mineral Waters

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A total of 103 isolates of *Aeromonas* spp. were obtained over a 3-year period from a natural mineral water and from surface streams located within the boundaries of the watershed of the natural mineral water wells and were typed by macrorestriction analysis of genomic DNA with *Xba*I and by pulsed-field gel electrophoresis. All *Aeromonas caviae* isolates from the natural mineral water belonged to the same clone, and an analogous clonal identity was found among *Aeromonas hydrophila* isolates. These two clones expressed no hemolytic or cytotoxic activities. *Aeromonas* isolates from surface waters showed high molecular heterogeneity and were not related to the clones found in the natural mineral water. The presence of aeromonads chronically found in the natural mineral water was a likely consequence of a localized development of a biofilm, with no exogenous contamination of the aquifer. Molecular fingerprinting of drinking water isolates is a useful tool in explaining possible reasons for bacterial occurrences.

The health consequences of the presence of motile species of the genus *Aeromonas* in drinking water are the subject of much debate (7, 11, 12, 20, 25). Recent studies have demonstrated that the presence of *Aeromonas* spp. in drinking water is a potential risk, since these microorganisms can produce a wide range of virulence factors (10, 13, 15, 18, 20, 26). The difficulty in assigning an unequivocal health hazard to motile aeromonads in drinking water stems from the facts that these microorganisms are heterogeneous and that only specific strains may be pathogenic (14, 16, 23).

There have been attempts in some countries, such as The Netherlands, Canada, and Italy, to introduce guideline standards for the presence of *Aeromonas* spp. in drinking water (4, 8, 9, 26). In Italy, provisional and cautionary limits were established in 1997 for natural mineral waters at their origin (10 CFU/100 ml) and after being bottled (100 CFU/100 ml) and maintained until the end of 1998.

These new health regulations aimed at the bottled-water industry compelled Italian public health professionals to make decisions about the safety of bottled mineral waters containing small quantities of *Aeromonas* spp., even after the cautionary limits were removed. In this paper we show how macrorestriction analysis of genomic DNAs of *Aeromonas* isolates with pulsed-field gel electrophoresis (PFGE) was useful in clarifying the reasons for the chronic occurrence of *Aeromonas* spp. in an Italian brand of bottled natural mineral water and the reasons these organisms are not presumed to be a hazard to human health.

The natural mineral water described in this study is a natural sparkling water obtained from wells drilled to depths between 60 and 100 m. Groundwater is first collected into stainless steel pipes leading to a single mixing tank and then pumped to four separate bottling lines. The average production volume of the plant is 2 million liters per day.

Microbiological analyses conducted during 1998 revealed none of the indicators set by the European health regulations for bottled natural mineral waters (total and fecal coliforms, fecal streptococci, sporulated sulfite-reducing clostridia, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) but did reveal the chronic presence of low numbers of *Aeromonas caviae*. This chronic occurrence prompted the establishment of a new protocol for the ensuing years 1999 to 2000, which includes (i) the seasonal sampling of the natural mineral water collected at different points of the production line of the plant, (ii) the seasonal microbiological analyses of samples collected from surface waters of the watershed, (iii) the macrorestriction analysis of genomic DNA of *Aeromonas* isolates with PFGE, and (iv) the determination of virulence properties of selected *Aeromonas* isolates.

Microbiological analysis. Mineral water samples were collected with seasonal frequency from the following sites: wells, the mixing tank, entrances of the bottling lines, and finished products after bottling.

Water samples (10, 100, and 1,000 ml) were filtered through 0.45-μm-pore-size mixed-ester filters (Millipore). Membrane filters were placed directly on culture plates of ampicillin dextrin agar (BioLife Italiana) (6, 24). Plates were incubated aerobically for 48 h at 30°C. Colonies typical for *Aeromonas* spp. were counted, purified, and Gram stained, and their identification was confirmed on the basis of the results of the following tests: the oxidase test, the oxidation/fermentation test, a test to determine the resistance to vibriostatic agent O/129, and the API 20E test. Additional tests (to detect gas production from glucose, H_2S production from cysteine, esculin hydrolysis, and the suicide phenomenon) were performed for identification at the species level according to the criteria of Popoff (19), Namdari and Bottone (17), and Carnahan et al. (1).

CFU means were compared using the one-way analysis of variance and Student's *t* test.

Only one well tested positive for *Aeromonas* spp., with an

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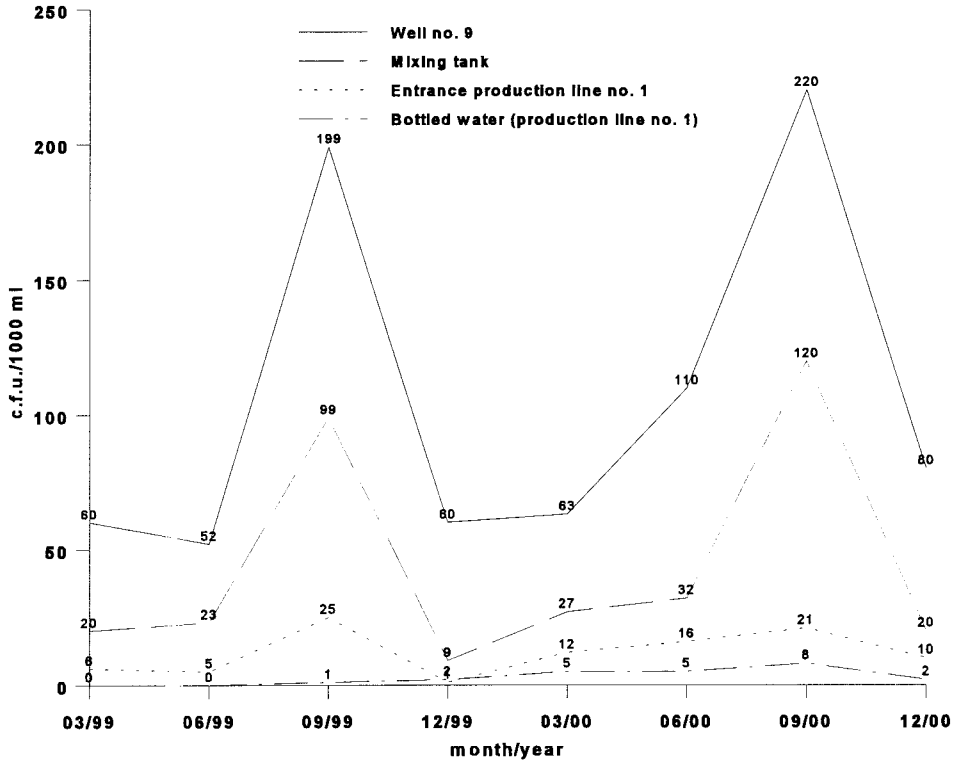

FIG. 1. Temporal trends of *Aeromonas* CFU counts at the sampling points chosen for the quality control program for natural mineral water. Only values for well no. 9 and for production line no. 1 are displayed. The other wells were always free of *Aeromonas* spp., whereas concentrations of aeromonads in the other three production lines were not different from those observed in production line no. 1.

average concentration of 105 CFU/1,000 ml (range, 52 to 220) (Fig. 1). Aeromonads were also found in the mixing tank (mean CFU/1,000 ml, 44) and at the entrances of production lines before (mean CFU/1,000 ml, 12) and after (mean CFU/1,000 ml, 2) the bottling process. The differences of *Aeromonas* concentrations observed among these sampling points were statistically significant ($P < 0.05$). By contrast, there were no significant differences in *Aeromonas* concentrations within the four production lines. A clear seasonal trend was observed, with highest CFU counts occurring in summer (Fig. 1). All samples positive for *Aeromonas* spp. during 1999 to 2000 contained *A. caviae*. As of December 1999, a multiple isolation of *A. caviae* and *Aeromonas hydrophila* occurred in 60% of the positive samples.

A total of 12 surface water samples were collected from three representative sites of two streams located within the boundaries of the watershed of the natural mineral water wells. Sampling was carried out every 3 months for a 1-year period in 1999. The main activities in the watershed area are rural, including farming and agriculture. All samples of surface water tested positive for *Aeromonas* spp. at concentrations ranging from 200 to 3,500 CFU/ml. *A. hydrophila* and *A. caviae* were recovered from 12 and 11 samples, respectively, whereas *Aeromonas sobria* was observed in only 3. All samples were positive also for indicators of fecal contamination, such as *Escherichia coli* (range, 750 to 73,000 most probable number/100 ml) and enterococci (43 to 11,000 most probable number/100 ml).

Molecular typing of *Aeromonas* isolates. A representative sample of 103 isolates of *Aeromonas* spp. were typed by using macrorestriction analysis of genomic DNAs with *Xba*I and PFGE, including 60 isolates of *A. caviae* recovered from the natural mineral water dating from 1998 to 2000, 23 isolates of *A. hydrophila* recovered from December 1999 through December 2000, and 20 isolates of *A. hydrophila* (10) and *A. caviae* (10) recovered from surface waters in 1999. The preparation of genomic DNAs of *Aeromonas* isolates for PFGE was performed as previously described (24). DNA restriction digestion was performed with *Xba*I (30 U/20-μl disk) according to the recommendations of the manufacturer (New England Biolabs). Electrophoresis was carried out in a CHEF-DR II apparatus (Bio-Rad) at 150 V at 10°C with pulse times of 20 s for 12 h and 5 to 15 s for 17 h.

On the basis of the assumption that a single base mutation in genomic DNA can introduce maximally a three-fragment difference in a strain's restriction pattern, variations in more than three fragments were assumed to represent major patterns (assigned capital letters), while one- to three-fragment differences were considered subtypes (assigned capital letters with numerical subcodes) (22). By these criteria, a major PFGE pattern (A) was displayed by all 60 isolates of *A. caviae* recovered from the natural mineral water samples, and two subtypes (A_1 and A_2) were represented. Subtype A_1 was detected in 59 of the 60 isolates (Fig. 2). Similarly, all *A. hydrophila* strains isolated from the natural mineral water showed a unique PFGE pattern (A) (Fig. 2). By contrast, the 10 isolates of *A. caviae* and the 10 isolates of *A. hydrophila* found in surface waters showed different PFGE patterns (B to K) (Fig. 3).

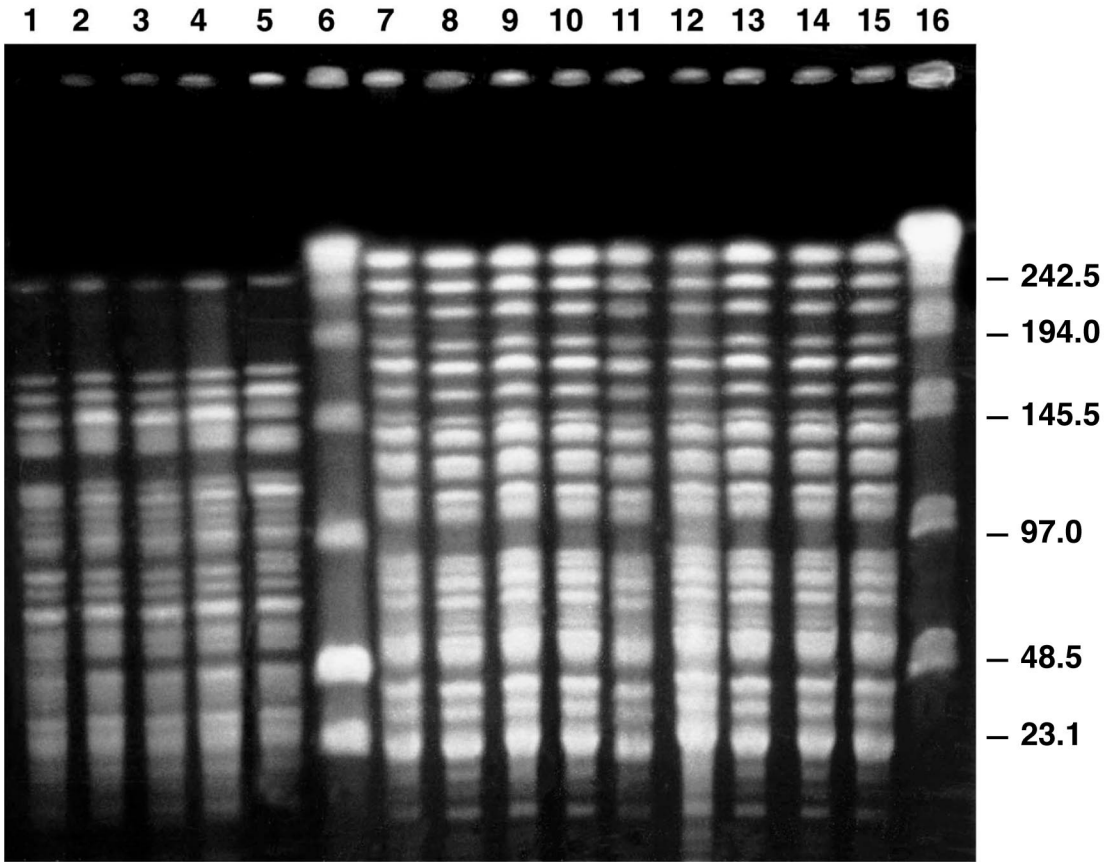

FIG. 2. *Xba*I PFGE patterns of *A. hydrophila* and *A. caviae* isolates from the sampling points chosen for the quality control program for the natural mineral water. Lanes 1 to 5 contain *A. caviae* strains with PFGE pattern A_1 isolated from well no. 9 (lane 1), the mixing tank (lane 2), the entrances of the bottling lines (lanes 3 and 4), and the bottled water (lane 5). Lanes 7 and 8 contain *A. hydrophila* strains with PFGE pattern A isolated from well no. 9, whereas lanes 9 to 15 contain *A. hydrophila* strains with the same PFGE pattern, A, isolated downstream (mixing tank, entrances of the bottling lines, and bottled water). Lanes 6 and 16 contain molecular size markers (lambda ladder; values to the right are kilobases).

Determination of virulence properties. A total of 22 isolates of *A. caviae* (11) and *A. hydrophila* (11) representing the major PFGE patterns were assayed for hemolytic and cytotoxic properties according to the methodology described by Kühn et al. (15). Detection of cytotoxin and hemolysin in sterile cell-free culture filtrates was assayed at 37°C with Vero cells and horse erythrocytes in agar plates, respectively. Isolates were considered cytotoxic if dilutions of one-eighth or more yielded destruction of at least 50% of the Vero cells.

No hemolytic or cytotoxic activities were detected in the two clones recovered from the natural mineral water. By contrast, three *A. caviae* (PFGE patterns B, D, and K) and seven *A. hydrophila* (PFGE patterns B, C, E, G, H, J, and K) strains isolated from surface water showed both hemolytic and cytotoxic properties. Hemolysis production and cytotoxin production were strongly correlated, since only one *A. hydrophila* strain isolated from surface water (PFGE pattern D) was hemolytic without showing evidence of cytotoxic activity.

The chronic occurrence of microorganisms belonging to the genus *Aeromonas* in natural mineral waters may be the result of the exogenous contamination of the groundwater source or may be a consequence of biofilm blooms. The health hazard due to the exogenous contamination of the aquifer is high, since it is possible that some strains of *Aeromonas* deriving from external sources are pathogenic and may induce disease in humans. Moreover, an exogenous contamination actually signifies that the aquifer is vulnerable also to other more pathogenic bacteria (e.g., salmonellae) or viruses. By contrast, the occurrence of a biofilm bloom has different public health implications, as biofilm colonization may derive from strains of the normal autochthonous microbial flora of the mineral water which have no pathogenic potential (7). For diagnostic purposes, a molecular heterogeneity of *Aeromonas* isolates is expected in the former case whereas a clonal relationship of the isolates may be observed in the latter (2).

Recently, digestion of DNA with rare cutting endonucleases and separation of the fragments by PFGE has proved to be a very useful tool in the epidemiological analysis of *Aeromonas* isolates (5, 21, 23). A previous study from our laboratory demonstrated a high level of genetic heterogeneity of *Aeromonas* isolates from ready-to-eat foods and that consumers are regularly exposed to many genetically distinct strains of *Aeromonas* (23). In this study, the molecular typing of *Aeromonas* isolates through PFGE showed that (i) all *A. caviae* strains isolated from the natural mineral water collected from 1998 to 2000 belonged to the same clone, (ii) all *A. hydrophila* strains iso-

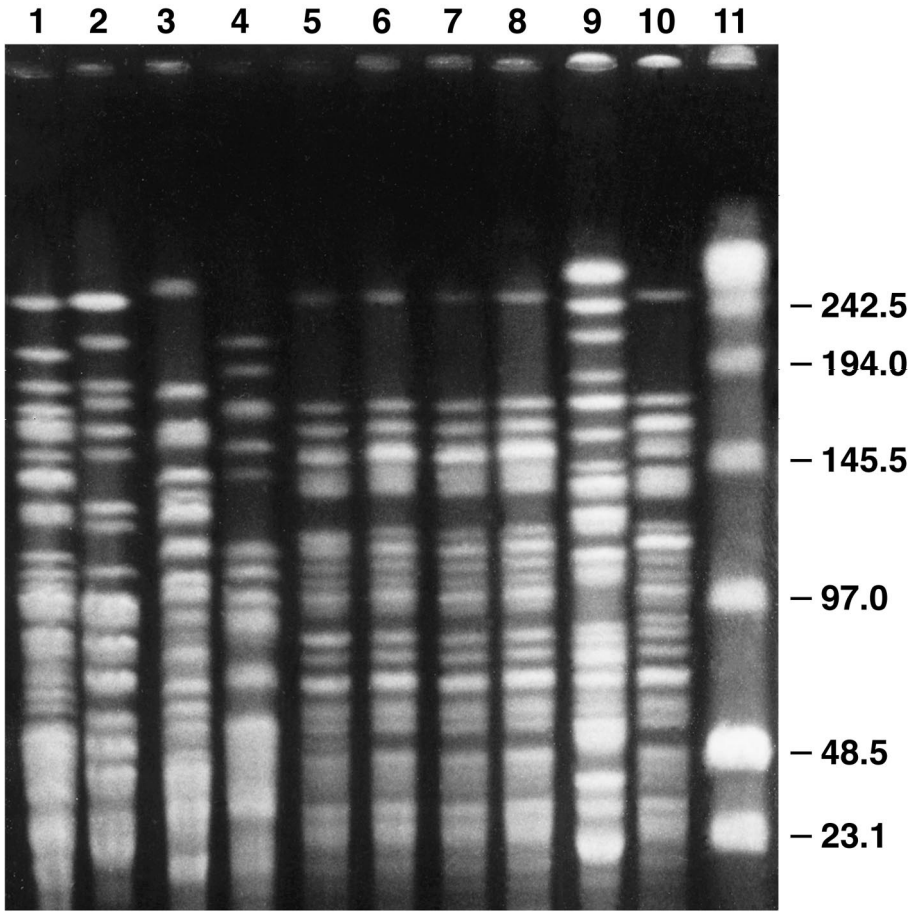

FIG. 3. *Xba*I PFGE patterns of *A. hydrophila* and *A. caviae* isolates from natural mineral water (lanes 5 to 10) and the surface waters of the watershed (lanes 1 to 4). Lanes 1 to 4 contain *A. hydrophila* strains with PFGE patterns B and F (lanes 1 and 2) and *A. caviae* strains with PFGE patterns C and G (lanes 3 and 4). Lanes 5 to 8 and 10 contain *A. caviae* strains with PFGE pattern A_1, whereas lane 9 contains an *A. hydrophila* strain with PFGE pattern A. Lane 11 contains molecular size markers (lambda ladder; values to the right are kilobases).

lated from the same mineral water in the period between December 1999 and December 2000 had an analogous clonal identity, and (iii) *A. caviae* and *A. hydrophila* isolates of the surface streams located within the watershed boundaries showed a very high level of molecular heterogeneity and displayed no relationship to the clones discovered in the natural mineral water.

Combined, these results indicate that the chronically present aeromonads in the natural mineral water were a likely consequence of a localized development of a biofilm somewhere in the water intake structures of well no. 9. The seasonal trend of *Aeromonas* CFU counts corroborates this conclusion, since it is well known that biofilms are most active in the warmer months (3). The biofilm was colonized first by *A. caviae* and then by *A. caviae* and *A. hydrophila* together. The origins of these two clones are obscure, but the normal autochthonous microbial flora of the natural mineral water remains the probable source. The decrease of *Aeromonas* concentrations observed between well no. 9 and the mixing tank was due to the effects of dilution, since at the mixing tank the waters of different wells converge and all these other wells proved to be free of aeromonads. The further decrease in *Aeromonas* CFU counts noticed downstream before and after the bottling process may have been caused by the addition of carbon dioxide at the entrances to the production lines. In any case, these decreases seem to indicate the absence of other biofilms along the production process of the natural mineral water.

Other researchers have performed biochemical or molecular typing of *Aeromonas* isolates in drinking water. Talon et al. (21) used PFGE to characterize *A. hydrophila* isolated from a cluster of hospital infections and from the water supply, concluding that the drinking water was not the source of the infections. Kühn et al. (15) performed a biochemical fingerprinting of *Aeromonas* isolates from 13 Swedish drinking water distribution systems, showing that, although raw surface water may contain many diverse *Aeromonas* populations, only a few persist and multiply within the distribution system. However, to our knowledge, our study is the first to use PFGE to demonstrate that the chronic presence of aeromonads in a natural mineral water was the result of a biofilm bloom and that exogenous contamination was not a factor.

Since relatively little is known about the pathogenic mechanisms of *Aeromonas* species, it is not yet possible to identify virulent strains definitively. However, the two clones of *A. caviae* and *A. hydrophila* found in the mineral water did not express the virulence properties considered in this study. The

labels of the majority of bottled mineral waters produced in Italy offer a free customer service telephone number to allow consumers to report any kind of problems. No cases of gastrointestinal complaints were registered during the time frame of the study. The fact that consumers of the bottled mineral water were exposed to two distinct clones of *Aeromonas* spp. and reported no signs of malaise further demonstrates the absence of any pathogenic potential of these clones.

Whereas the *Aeromonas* occurrences detailed in this study may not raise public health concerns, these occurrences should not be ignored, since they suggest the existence of a habitat that could be used by pathogens. As a matter of fact, a careful maintenance and disinfection program of pipelines downstream from the water intake structures of the wells was instituted and no evidence of other biofilms was observed within the production lines. More generally, the results of this study indicate that molecular fingerprinting of drinking water isolates is a useful tool for explaining possible reasons for bacterial occurrences.

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