Research Note

Behavior of *Aeromonas hydrophila* in Bottled Mineral Waters

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ABSTRACT

The growth and survival of *Aeromonas hydrophila* in three types of natural mineral waters were investigated. Mineral waters with different levels of mineral content (low, medium, and high) were experimentally contaminated with *A. hydrophila,* stored at different temperatures (10°C and 20°C), and analyzed at intervals over a 60-day period. Water samples that were not experimentally contaminated were investigated for indigenous *A. hydrophila*. The results confirmed that *A. hydrophila* may occur naturally in mineral waters and showed that the level of mineral content, temperature, length of storage, and, in some cases, the type of container used may favor the growth of *A. hydrophila*. The greatest proliferation was observed in water with a low mineral content stored in PET bottles at 10°C, in which *A. hydrophila* peaked at day 28 (4.47 ± 0.01 log CFU/100 ml). At 20°C, the same load was observed at day 60. The presence of high densities of *A. hydrophila* in bottled mineral water can constitute a risk for some groups of consumers, such as elderly and immunocompromised persons.

Aeromonas hydrophila is an opportunistic human pathogen that has been implicated as a cause of diarrhea in various countries *(2, 3, 5, 8)*. It is commonly associated with the aquatic environment and has been isolated from fresh water *(10, 17)* and natural mineral water *(6, 20)*, in addition to other food products such as poultry, raw meat, raw milk, and vegetables *(1, 11, 13, 14)*. The wide distribution of *Aeromonas* sp. is probably a consequence of its ability to adapt to different environments *(12)*.

Although the bacterial populations of mineral waters are diverse and brand-dependent, aeromonads constitute a minor part of the flora of most mineral waters *(16)*. Some studies have indicated that the bacteria in bottled mineral waters originate from the water source, where they are present in low number, later growing heterotrophically in the bottles *(18, 24)*. The behavior of *Aeromonas* sp. in water is affected by numerous extrinsic and intrinsic factors. Specifically, the survival of this bacterium may depend on various environmental factors, such as solar irradiation, availability of nutrients and direct or indirect antagonistic activity of existing microflora *(9, 23, 24)*.

The objective of the present study was to investigate the behavior of *A. hydrophila* in three natural mineral waters that differed in terms of mineral content. In accordance with current legislation (Gazzetta Ufficiale della Repubblica Italiana 17.2.92, D.L. n.105, carrying out of Directive of Council 80/777/CEE) content was classified as low (<500 mg/liter), medium (>500 and <1,500 mg/liter), and high (>1,500 mg/liter). The water samples were experimentally contaminated with *A. hy-*

drophila and maintained at different temperatures. The growth and survival of the microorganism were then investigated using viable counts.

MATERIALS AND METHODS

Water samples. Three types of Italian natural mineral waters were used: water with low mineral content (total dissolved solids at 180°C = 132.6 mg/liter), water with medium mineral content (total dissolved solids at 180°C = 597.0 mg/liter), and water with high mineral content (total dissolved solids at 180°C = 2,297.4 mg/liter). The ionic composition of each, as provided by producer firms, is reported in Table 1.

Each type of mineral water was bottled in both glass and PET (polyethylene) containers.

Bacterial strains. Two *A. hydrophila* strains were used in this study: *A. hydrophila* strain ATCC 7966 and an *A. hydrophila* strain isolated from mineral water in our laboratory. The strains were maintained on a slant of nutrient agar (Oxoid, Hampshire, UK) at 4°C and transferred every 15 days.

A. hydrophila was grown in nutrient broth (Oxoid) at 37°C for 24 h. The broth cultures were then washed three times by means of centrifugation (8,000 × *g* for 8 min) in 0.8% NaCl solution. The microbial suspensions were standardized by turbidimetry (40% transmittance at 540 nm—Bausch & Lomb Spectronic 20 turbidimeter). A parallel count of both *A. hydrophila* strains on plate count agar (Oxoid) yielded about 8 log CFU/ml.

Sample contamination. Two hundred fifty-five bottles of each mineral water originating from the same lot (153 in glass bottles containing 1 liter of water and 102 in PET bottles containing 1.5 liters of water) were delivered to our laboratory within 24 h of bottling. The bottles were divided into three groups, each consisting of 51 glass bottles and 34 PET bottles. Two groups were experimentally contaminated with *A. hydrophila* (one strain per group) with an appropriate dilution in 0.8% NaCl solution of

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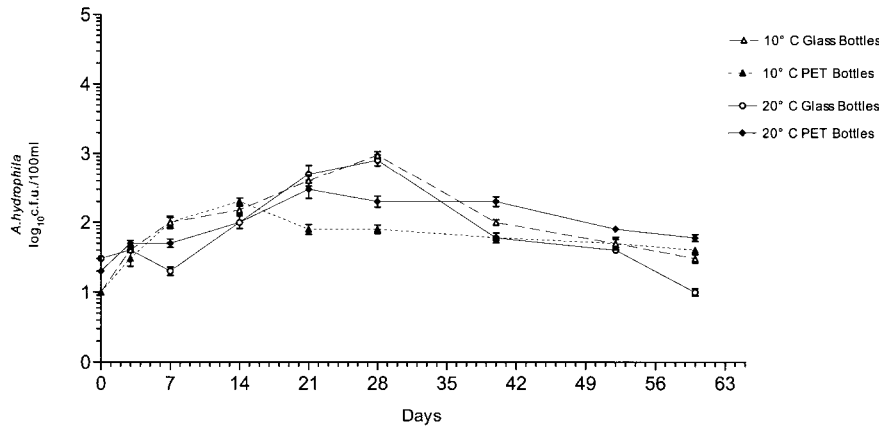* Author for correspondence. Tel +39(06)49902477; Fax +39(06)49387101; E-mail: luciana.croci@iss.it.

FIGURE 1. A. hydrophila *count in noncontaminated water of low mineral content by type of container and storage temperature. Each point is the mean of three experiments (error bars are given).*

A. hydrophila broth culture, obtained as previously described, to obtain an initial concentration of approximately 1 to 2 log CFU *A. hydrophila* per 100 ml water. Bottles were sealed immediately after inoculation. The third group remained noncontaminated for use as controls.

Both contaminated and control samples were divided into two groups: one was stored at 10 °C and the other was stored at 20 °C in temperature-controlled rooms.

For all analyses, each sample consisted of 3 liters of water (i.e., three 1-liter glass containers and two 1.5-liter PET containers) as required by Italian law (Gazzetta Ufficiale della Repubblica Italiana n. 14, D.L. 13.1.93).

At day 0 (after contamination), three samples of water for each type of container were analyzed (one for *A. hydrophila* ATCC 7966 water, one for the other strain, and one for noncontaminated water). At days 3, 7, 14, 21, 28, 40, 52, and 60, three samples of water were analyzed as above for each of the two storage temperatures. Noncontaminated samples were tested to determine both total bacterial count and count of naturally present *A. hydrophila*. Experimentally contaminated samples were tested for *A. hydrophila* count only. Each experiment (250 bottles per type of mineral water) was conducted in triplicate.

Preparation of samples. The contents of the bottles constituting a single sample (three 1-liter glass containers and two 1.5-liter PET containers) were combined in a single container under sterile conditions and used for the total bacterial count and *A. hydrophila* count.

Total bacterial count. The total bacterial count (noncontaminated samples only) was determined by pour plate method, inoculating four plate count agar dishes with 1 ml of each sample. Two dishes were incubated at $20 \pm 1°C$ for 72 ± 2 h, and the

other two dishes were incubated at $37 \pm 1°C$ for 24 ± 2 h in accordance with Italian law (Gazzetta Ufficiale della Repubblica Italiana n.14, D.M. 13.1.93). Results are expressed as log CFU/ml.

A. hydrophila count. For each sample, 200 ml of water were collected and subdivided into two 100-ml aliquots. Each aliquot was filtered through a Millipore membrane (0.45 μm). The membranes were then placed in petri dishes containing 15 ml of Aeromonas agar base with ampicillin supplement (5 mg/liter, Oxoid). The petri dishes were incubated for 24 h at $28 \pm 2°C$. After incubation, suspect colonies, which appeared dark green in color surrounded by a yellow-green area or totally dark green, were counted. To identify the suspect colonies in the control samples, a proportion of these colonies (5%) was subjected to the following procedures.

Identification. Suspect colonies were transferred onto tryptone soya agar (Oxoid) slants and allowed to grow for 24 h at $37 \pm 1°C$. They were then identified by means of API 20E and API 20NE galleries. Furthermore, to distinguish *A. hydrophila* from other species of aeromonads, the following tests, described elsewhere *(22),* were carried out: oxidase reaction, production of gas from glucose, production of gas from cysteine, β-hemolysis, and suicide phenomenon. Results are expressed as log CFU/100 ml.

Statistical analysis. The results of three sets of experiments were analyzed by analysis of variance with Statview 4.02 (Abacus Concepts, Inc., Cary, N.C.) on an Apple Macintosh.

RESULTS AND DISCUSSION

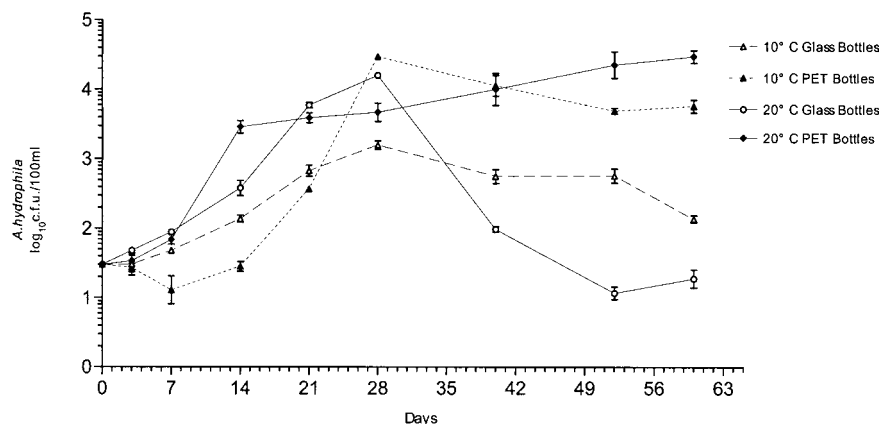The total microbial load for all of the noncontaminated water samples, irrespective of the type of container, re-

FIGURE 2. A. hydrophila *ATCC 7966 count in experimentally contaminated water with low mineral content by type of container and storage temperature. Each point is the mean of three experiments (error bars are given).*

FIGURE 3. A. hydrophila *count in non-contaminated water with medium mineral content by type of container and storage temperature. Each point is the mean of three experiments (error bars are given).*
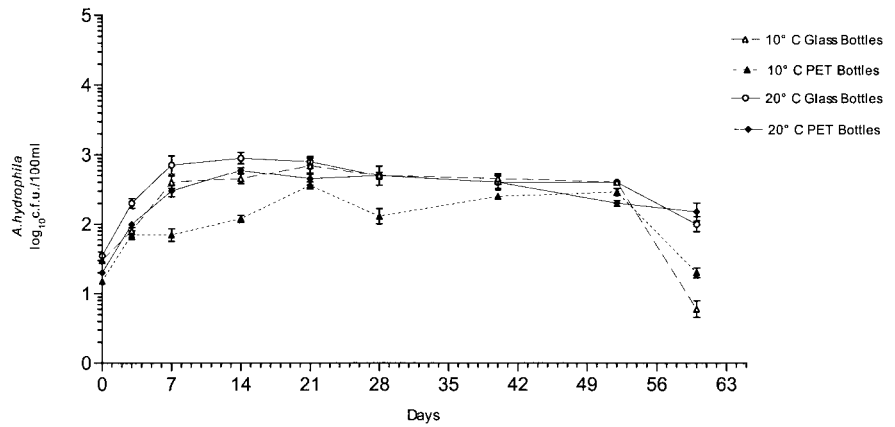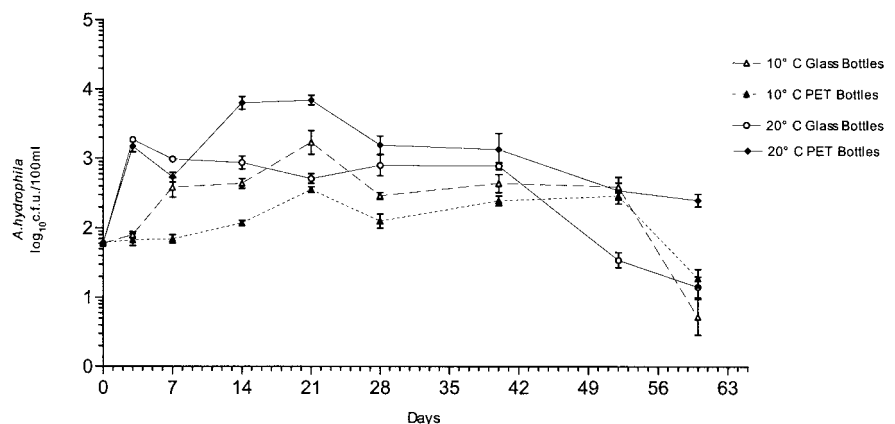

mained consistently low, peaking at 3 log CFU/ml at day 15. A small quantity of *A. hydrophila* (1 to 1.301 log CFU/100 ml) was detected in some of these samples for water with a low or medium mineral content. *A. hydrophila* tended to multiply over the study period at both storage temperatures (Figs. 1 and 3), and proliferation was more evident in water with low mineral content, especially in glass bottles, with a peak count of approximately 3 log CFU/100 ml for both temperatures at day 28 (Fig. 1). In water with the highest mineral content, *A. hydrophila* was not detected.

This behavior was confirmed by the results obtained from tests carried out on experimentally contaminated samples. Because the results for the two *A. hydrophila* strains were very similar, only those for *A. hydrophila* ATCC 7966 are presented. The analysis of variance indicated that the results of the three sets of experiments were not significantly different ($P > 0.05$). The greatest proliferation was observed in water with low mineral content, compared to water with medium and high mineral content. In particular, at 20°C, a rather high load (3.46 ± 0.12 log CFU/100 ml) was observed in PET bottles as early as day 14. The highest load (4.47 ± 0.04 log CFU/100 ml) was observed for PET bottles at day 60 (Fig. 2). At 10°C, a peak of the same magnitude was observed at day 28, followed by a slight reduction.

For water with a medium mineral content, proliferation was greatest for PET bottles stored at 20°C (peak of 3.80 ± 0.09 log CFU/100 ml at day 14) and lowest for PET bottles at 10°C (peak of 2.55 ± 0.04 log CFU/100 ml at day 21) (Fig. 4).

In water with a high mineral content at both temperatures, samples contained in PET bottles showed constant *A. hydrophila* levels for some time, followed by drastic decreases (microorganism not detected at day 40 for samples stored at 20°C or at day 60 for samples stored at 10°C). In samples stored in glass bottles at 20°C, growth peaked between days 14 and 21, followed by a progressive reduction. In glass bottles at 10°C, growth peaked at day 21 and then decreased drastically (Fig. 5).

The results of this study confirm that *A. hydrophila* may occur in natural mineral waters, albeit in low quantities, and can multiply at low substrate concentrations.

In experimentally contaminated waters, the growth of the microorganism was highest in water with low mineral content. It was also high in water with medium mineral content, especially when stored in PET containers at 20°C.

The level of growth was much lower in water with high mineral content; nevertheless, the microorganism remained vital up to day 60 (last observation) at both temperatures.

The level of mineral content, the temperature and length of storage, and in some cases, the type of container used can influence the development of *A. hydrophila* naturally present in water, constituting a risk for some groups of consumers, such as elderly and immunocompromised persons *(3, 7, 10, 15)*. Unfortunately, very few data are available on the concentration of aeromonads present in drinking water implicated in cases of *Aeromonas*-associated diarrhea. Cases have been reported in association with concentrations as low as 1 to 50 CFU/100 ml *(4)*; a greater number of cases have been associated with concentrations

FIGURE 4. A. hydrophila *ATCC 7966 count in experimentally contaminated water with medium mineral content by type of container and storage temperature. Each point is the mean of three experiments (error bars are given).*
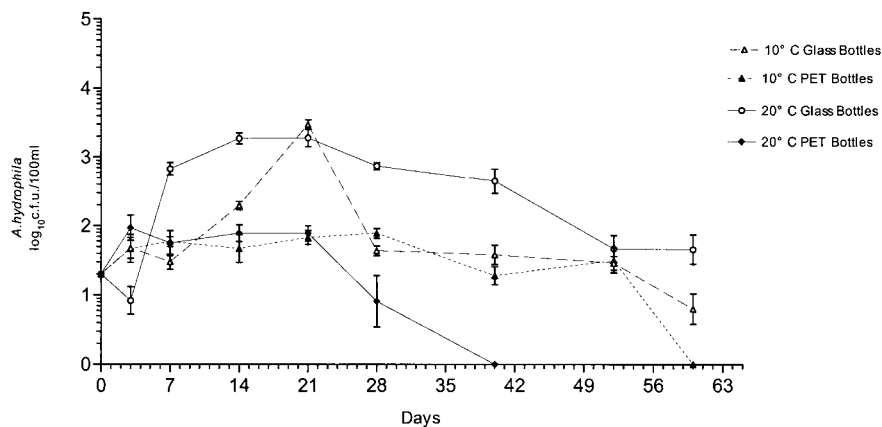
FIGURE 5. A. hydrophila *ATCC 7966 count in experimentally contaminated water with high mineral content by type of container and storage temperature. Each point is the mean of three experiments (error bars are given).*

of *Aeromonas* ranging from 50 to 150 CFU/100 ml *(21)*. Further studies are needed to determine the relationship between cases and the concentrations of these microorganisms in food and in drinking water; however, it is advisable to prevent exposure of the consumers to high concentrations of aeromonads. Recently, the Scientific Commission for Foods of the European Commission (CS/FMH/NMW/2-1998 *(19)*), which does not consider *A. hydrophila* and *A. caviae* as appropriate microbiologic criteria for the acceptability of spring water because of a lack of epidemiologic evidence, has recognized the possibility of growth of *Aeromonas* in bottled water. The Commission has suggested possible solutions, such as informing consumers, which is particularly important when considering that bottled water is often given to young children and used for therapeutic purposes.

ACKNOWLEDGMENT

TABLE 1. *Ionic composition of natural mineral waters*

	Water mineral content (mg/liter)		
Ionic residue	Low	Medium	High
Ca^{2+}	30	124	304
Mg^{2+}	6.1	41	75.5
Na^+	6.3	6.4	252
HCO_3^{2-}	103.1	305	1,926.4
SO_4^{2-}	19.3	235	268.8
Cl^-	7.3	5.6	48.6
NO_3^-	2.8	5.5	0.2
K^+	1.0	—	247
F^-	—	0.4	1.9
SiO_2	8.0	8.3	138.0
Sr^{2+}	0.2	—	4
Mn^{2+}	—	—	1.5
Li^+	—	—	0.3
NH_2^+	—	—	0.8
BO_3^{2-}	—	—	9.5
PO_4^{2-}	—	—	0.5
Total dissolved solids at 180°C	132.6	597.0	2,297.4

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