Behavior of Enteroaggregative *Escherichia coli* in Bottled Spring and Mineral Water†

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MS 02-66: Received 14 March 2002/Accepted 9 October 2002

**ABSTRACT**

The ability of enteroaggregative *Escherichia coli* (EAEC) to survive in bottled mineral and spring water at common storage temperatures was investigated. Filtered mineral and spring waters were inoculated with EAEC (ca. 10⁴ CFU/ml) and stored at 4, 10, and 23°C. Water samples were analyzed every 3 days for viable EAEC by plating on tryptic soy agar plates over 60 days of storage. EAEC survival levels were significantly higher (P < 0.01) at 23 and 10°C than at 4°C. Furthermore, EAEC survival levels were significantly higher (P < 0.01) in mineral water than in spring water at 4 and 10°C. The results of this study indicate that EAEC can survive in bottled mineral and spring waters for long periods of storage at 4, 10, and 23°C. The ability of EAEC to survive in bottled water indicates that the source water for bottling industries must be kept free of contamination. Furthermore, the refrigeration of bottled water is recommended to minimize the growth of EAEC in water.

Bottled water is any potable water intended for human consumption that is manufactured, distributed, or offered for sale in sealed food-grade bottles or other containers (28). Approved water sources can be springs, municipal systems, or other sources, and these sources are subjected to a number of treatments, such as distillation, carbonation, ozonation, and/or filtration (29). There has been a dramatic increase in the consumption of bottled natural mineral water over the last decade to a point at which consumption of this product exceeds that of carbonated soft drinks (11). This increase in demand reflects the increased concern of consumers based on their perceptions about the safety of municipal water supplies. Adequate knowledge about the fate of pathogens that may gain access to water sources is essential to assure public health surveillance agencies and consumers of the quality and safety of bottled water.

In general, waterborne disease outbreaks leading to gastroenteritis are a result of either poor protection of the source water or inadequate treatment (17). Outbreaks due to the ingestion of water containing *Escherichia coli* O157:H7 have occurred in many countries, including the United States (24). While many of these waterborne-disease outbreaks are related to the consumption of contaminated surface waters, there is now an increasing concern that the entry of pathogens into groundwater supplies may pose risks with regard to the consumption of bottled water (14). If bottled water is not produced under an approved hazard analysis critical control point plan, it could be a potential source of major food poisoning outbreaks (29).

Enteroaggregative *E. coli* (EAEC) is a group of pathogenic *E. coli* characterized by its ability to adhere to cultured cell monolayers in a characteristic “stacked brick” aggregative pattern (21). Epidemiological evidence suggests that strains of EAEC are a significant cause of protracted diarrhea in children and may cause traveler’s diarrhea in adults (15). During a diarrhea epidemic in a village in southern India, EAEC was identified in the stools of 11 of 20 persons with diarrhea. The intake of water from open wells was associated with diarrhea, even though EAEC was not identified from this source (22). Although outbreaks of EAEC in developing countries such as Brazil, Chile, Mexico, India, and Bangladesh have commonly been reported (15), some recent outbreaks have occurred in industrialized countries, including Japan and the United Kingdom. A massive outbreak of disease attributable to EAEC involving 2,697 school children in Japan was reported in 1993 (13). EAEC has also been described as a potential cause of traveler’s diarrhea in patients returning to the United Kingdom from a variety of locations (23). Gascon et al. (10) reported that EAEC strains were associated with diarrhea in Spaniards traveling to developing countries.

Although water has not been implicated as a source of infection in any EAEC outbreaks in industrialized countries, the potential role of water in the transmission of EAEC needs to be investigated. Hence, an understanding of the survival characteristics of EAEC in water is important. The objective of this study was therefore to determine the behavior of EAEC in bottled mineral and spring waters stored at 4, 10, and 23°C.
MATERIALS AND METHODS

Bacterial culture and media. EAEC cultures were obtained from the E. coli Reference Center, Penn State University, University Park, Pa. Strains 484, 485, and 486 (all human isolates) were used. Each strain was grown separately in 100 ml of tryptic soy broth (TSB; Difco Laboratories, Sparks, Md.) with agitation (150 rpm) at 37°C for 24 h. Following incubation, EAEC cells were harvested by centrifugation at 3,500 g for 15 min at 4°C. The bacterial cells were washed three times with 0.1% peptone buffer (Difco) and resuspended in the peptone buffer. Equal portions (50 ml) for each of the three strains were combined, and 5 ml of the mixture was used as the inoculum (10^6 CFU/ml). The bacterial count of the three-strain EAEC mixture was determined by plating 0.1-ml portions of appropriate dilutions on duplicate tryptic soy agar (TSA; Difco) plates and incubating the plates at 37°C for 24 h.

Inoculation of water samples. Locally purchased bottled spring and mineral waters were used for the study. Aliquots of 500 ml each of spring water and mineral water were filter sterilized with 0.2-μm cellulose nitrate filters (Nalgene, Rochester, N.Y.) as they were poured into sterile polystyrene bottles. The cell suspension was added to 500 ml of sterile water to obtain an inoculation level of approximately 10^4 CFU/ml. The inoculated water samples were incubated at 4, 10, and 23°C. Triplicate samples of each spring water and mineral water were included for sampling at each storage temperature. For both mineral and spring waters, three bottles each were kept as controls at each storage temperature, and samples from the controls were enriched in TSB and streaked on both TSA and sorbitol MacConkey agar (SMA; Difco) at regular intervals to ensure that the filtered water samples were free of any contamination.

Enumeration of EAEC. The levels of surviving EAEC in the inoculated water samples were determined on days 0, 1, and 3 and every 3 days thereafter until day 60 by plating 0.1-ml portions of samples directly or after serial dilutions (1:10 in 0.1% peptone) on duplicate TSA plates. The plates were incubated for 24 h at 37°C, and the colonies were counted. Representative colonies from the TSA plates were enriched in TSB at 37°C for 24 h and then streaked on SMA plates and incubated at 37°C for 24 h to confirm that the colonies being enumerated were EAEC colonies. On each specified sampling day, a volume of 1 ml of the inoculated water samples was also transferred to separate 250-ml Erlenmeyer flasks containing 100 ml of sterile TSB. The flasks were incubated at 37°C for 24 h. When growth was observed in TSB, the culture was streaked on TSA and SMA and the plates were incubated at 37°C for 24 h and observed for typical EAEC colonial morphology.

Statistical analysis. The EAEC populations in the water samples were analyzed with GLM subroutine of the Statistical Analysis Software (SAS Institute, Inc., Cary, N.C.). The model included types of water, temperatures, and days as the main effects. The least significant difference test was used to determine significant differences between the EAEC populations recovered from water samples stored at different temperatures.

RESULTS

The mean pH values of mineral water and spring water were 6.43 and 6.89, respectively. Uninoculated water samples (controls) showed no growth on TSA or SMA plates throughout the study, even after 48 h of incubation at 37°C. The average EAEC population recovered from the water samples on day 0 was 4.3 log CFU/ml. There was a significant difference (P < 0.01) between the ability of EAEC to survive in mineral water and their ability to survive in spring water at different storage temperatures. At 23°C, the numbers of EAEC in both types of water increased to approximately 6 log CFU/ml by day 3 and remained the same thereafter for the duration of the study (Fig. 1). At 10°C, the population of EAEC in mineral water increased to approximately 5.3 log CFU/ml on day 36 and remained the same until storage day 60 (Fig. 1). However, in spring water stored at 10°C, the EAEC counts increased to approximately 5.2 log CFU/ml on day 18 and then declined to the original inoculation level of 4.3 log CFU/ml by day 60.

At 4°C, the EAEC populations exhibited gradual de-
clines in both spring water and mineral water until they reached approximately 2 log CFU/ml, and thereafter they declined sharply to <1 log CFU/ml (Fig. 1). In spring water, the EAEC number declined by >3 log CFU/ml by day 30, and thereafter no bacteria were recovered by plating until the end of the study. However, viable EAEC cells could be recovered from spring water by enrichment in TSB until the end of the study. The survival level for EAEC at 4°C was significantly higher (P < 0.01) for mineral water, in which the bacterial population decreased to <1 log CFU/ml (detected by enrichment) only after 51 days of storage.

**DISCUSSION**

Contaminated food and water are commonly implicated in traveler’s diarrhea (3, 6, 9). Although EAEC has been associated with cases of traveler’s diarrhea (10, 27), the potential of water as a vehicle for EAEC has not been investigated. Therefore, this study was undertaken to determine the viability of EAEC in bottled spring and mineral waters at storage temperatures commonly used for bottled water. Previous studies have shown that the survival times for *E. coli* strains in water can vary greatly, depending on the type of water and the storage temperature. Burge and Hunter (5) found that at 23 to 25°C, *E. coli* was able to persist in bottled mineral water for about 42 days at an initial inoculation level of 10^4 CFU/ml. Tsai and Yu (25) reported the growth of *E. coli* in autoclaved mineral water from an initial count of 10^5 CFU/ml to a maximum of 10^8 CFU/ml. However, in another study investigating the survival of *E. coli* cells in bottled water stored at 22°C at inoculation levels of 10^3 and 10^5 CFU/ml, the organism did not survive for more than 1 and 4 days, respectively (8). Moreira et al. (20) found that *E. coli* barely survived for 20 days in mineral water in polyvinyl chloride and glass bottles, both in the presence of autochthonous bacteria and in sterile water. In contrast, Warburton et al. (29) reported that *E. coli* O157:H7 could survive in bottled spring water for >200 days.

In the present study, it was observed that EAEC survived in both mineral and spring waters for 60 days at all three storage temperatures, with significantly larger populations (P < 0.01) being observed at 23 and 10°C. EAEC grew in the water samples by approximately 2 and 1 log CFU/ml at 23 and 10°C, respectively. Furthermore, the EAEC population surviving in mineral water was significantly (P < 0.01) larger than that surviving in spring water at 4 and 10°C. Although water represents a nutrient-limiting medium, the growth of bacteria such as *E. coli* (25), *Pseudomonas aeruginosa* (16), and *Aeromonas* species (4, 7) in bottled mineral water has been reported. Furthermore, a substantial increase in the inherent bacterial count in bottled water kept at room temperature for 30 days was observed in a 1998 Health Canada study (12). Tsai and Yu (25) reported that the autochthonous bacteria in bottled mineral water are able to multiply even with a very limited supply of nutrients. A variety of agents present in water have been reported to support the survival and growth of bacteria. Kerr et al. (14) opined that trace amounts of nutrients from bacterial growth medium may allow the extended survival of the cells in water. Ducluzeau et al. (8) reported that the presence of even minute amounts of organic matter in water can extend the survival of *E. coli*. The availability of assimilable organic carbon (26) and phosphorous (19) is important in supporting bacterial growth in drinking water. The low levels of nutrients from soap used to clean the glass bottles or leaching from the polyethylene matrix have been reported to be sufficient to support bacterial growth (29). Furthermore, many enteric bacteria are able to carry on a maintenance level of metabolism in water by hydrolyzing endogenous macromolecular reserves (17). In the present study, trace amounts of nutrients from the peptone buffer in which the cells were suspended (0.001% peptone) could have partly contributed to EAEC growth. A similar result was observed by Warburton et al. (29), who reported that unwashed tryptic phosphate broth-cultured *E. coli* O157:H7 cells grew by 1 to 2 log CFU/ml in sterile distilled and mineral waters compared with cells washed in water prior to inoculation.

The ability of EAEC to survive better in mineral water than in spring water could be attributable to the phenomenon of natural bacterial genetic competence. Metal ions such as Ca^{2+} and Mg^{2+} have been reported to be key elements for competence induction in *E. coli* in low-nutrient environments (2). Since mineral water contains a higher concentration of minerals like Ca^{2+} and Mg^{2+} than spring water does (1, 18), genetic competence induced by these minerals in EAEC would explain the better survival of the organism in mineral water.

The results of this study indicate that EAEC can survive in bottled mineral and spring water for extended periods, especially in the presence of traces of nutrients. The survival of EAEC in both mineral and spring waters was significantly (P < 0.01) lower at 4°C than at 10 and 23°C. Hence, refrigeration of bottled water is recommended for the minimization of the growth of EAEC in water. Moreover, source water for the bottling industries should be devoid of EAEC, and effective disinfection of the distribution lines in water-bottling plants must be implemented to ensure the production of safe bottled water. Regular coliform screening and cultural tests for generic *E. coli* will not specifically identify EAEC. Furthermore, there are no specific cultural tests available for the confirmation of EAEC. Therefore, molecular methods such as polymerase chain reaction or DNA probe methods should be employed for the specific detection of EAEC in water.

**REFERENCES**


