Bottled Water: How Safe Is It?

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ABSTRACT: Sales of bottled water have increased dramatically in recent years, with worldwide sales of more than $35 billion, largely because of the public perception of purity and safety and public concern about the quality of tap water. Presently, there are no Food and Drug Administration (Washington, D.C.) recommendations regarding temperature and duration of storage for bottled water once it is opened and used. The objectives of this study were to examine the effects of time and storage temperature on bacterial growth and characterize the types of microorganisms contaminating bottled water after drinking once from the bottle. Bottled and tap water were tested using standard microbiology culture techniques. The bacterial count in bottled water increased dramatically, from less than 1 colony per milliliter (col/mL) to 38,000 col/mL over 48 hours of storage at 37°C. Bacterial growth was markedly reduced at cold temperatures (refrigeration) compared with room temperature, with 50% fewer bacterial colonies in 24 hours and 84% fewer colonies in 48 hours. Interestingly, tap water resulted in only minimal growth, especially at cold temperatures (<100 col/mL at 48 hours). These findings may be useful to increase public awareness and development of guidelines on storage temperature and expiration time for bottled water once it is opened and used. Water Environ. Res., 77, 3013 (2005).


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Introduction

Bottled water has become increasingly popular, with a current U.S. market of more than $5.7 billion and a worldwide industry of approximately $35 billion (Bottled Water Web, 2002). The market is growing at a rate of approximately 10% per year, and industry experts anticipate that bottled water will become second only to soft drinks as America’s beverage of choice (Bullers, 2002; FDA, 2002). Such explosive growth in the bottled water industry is presumably a result of people’s perception of purity, safety, better taste, convenience, and increasing public awareness of fitness and beneficial effects of drinking water on health. Furthermore, because bottled water contains no caffeine, calories, or sugar, it is used as a diet drink and is often viewed as a statement of lifestyle.

Americans are drinking bottled water in record numbers—approximately 18 bil. L (5 bil. gal) in the year 2001 (Bullers, 2002). More than one-half of all Americans drink bottled water, and approximately one-third of the public consumes it regularly (NRDC, 1999). Once the bottle is opened, it is stored at varied temperatures, ranging from room temperature (in indoor places, such as offices, homes, and schools) to extreme hot or cold temperatures (in outdoor places, such as cars, garages, and sports fields). Furthermore, bottled water, once opened, is often consumed over several hours or days at room or outdoor temperatures.

The bottled water industry is regulated on three levels: federal, state, and trade association (FDA, 1997; IBWA, 2004; U.S. EPA, 1996). Bottled water is self-regulated by the International Bottled Water Association (Alexandria, Virginia). However, regulations are less stringent compared with U.S. Environmental Protection Agency (U.S. EPA) regulations for tap water (NRDC, 1999; U.S. EPA, 1996). Bottled water is considered a food product and is also regulated by the Food and Drug Administration (FDA) (Rockville, Maryland). All bottled water products must comply with FDA’s quality standards, labeling regulations, and Good Manufacturing Practices (FDA, 1997; IBWA, 2004). In addition, the bottled water industry is subject to state regulatory requirements. All of these regulations are directed to the quality of water in a sealed bottle. However, currently, there are no recommendations or guidelines regarding the temperature of storage and the duration for storage once the bottle water is opened and used or regarding the use of preservatives to retard microbial growth.

Because many people use water bottles over extended periods of time and store them at room temperature or in extreme heat outdoors, one potential concern is that there may be bacterial growth in bottled water that may have potential health implications. Therefore, it is important to investigate whether there is bacterial growth over time in bottled water, once opened and used, and study the effects of temperature on the quality of bottled water with regard to microbial contamination. Based on these observations and rationale, a hypothesis was generated and experiments were designed to address the following objectives.

Hypothesis

Once opened and used, bottled water may undergo contamination with oral microflora, which could grow and limit its usage life, potentially requiring guidelines for its mode of use and storage.

Objectives

The objectives of the study were to (1) determine if there is a time-dependent bacterial growth in bottled water once opened and used, (2) study the effect of different ways of storage (room temperature versus cold temperature) on bacterial growth in bottled and tap water, and (3) identify the types of microorganisms in the bacterial growth.

Materials and Methods

Materials. Sixty-eight drinking water bottles (250 mL each) from the same manufacturer and lot were purchased from a supermarket. Samples of tap water were collected in sterile bottles (250 mL) from the laboratory where the experiments were conducted (University of Texas M. D. Anderson Cancer Center, Houston, Texas). Blood agar plates (n = 30), MacConkey culture plates (n = 36), Columbia CNA culture plates (n = 36), and 0.22-μm Nalgene filters (n = 90) were purchased from Fisher Scientific (Hampton, New Hampshire). The microbiology laboratory was fully equipped with all of the instruments and equipment required for experiments carried out in this study, including sterile hoods, an incubator, cold room, autoclave room, sonicator, microscope for colony counts, colony counter, and laboratory supplies, including large flasks, sterile gloves, pipettes, Pipetman (Gilson, Inc.,
Middleton, Wisconsin), and bacteria incinerator. Statistical significance was determined by a paired t test.

Procedure. A simplistic schematic of the study is shown in Figures 1 to 3.

Determining the Bacterial Colony Count in the Unused (Uncontaminated) Bottled Water. After checking the volume of water, the contents of 12 unopened bottles were filtered in a sterile hood through Nalgene filters. Using a sterile technique, the filters were carefully cut with a scalpel blade and placed on the blood agar plates (three regular and one large), covered with lids, and labeled with the appropriate time point (baseline). The agar plates were then incubated with the “agar up” position at 37°C in the incubator. After 48 hours, the agar plates were removed from the incubator, and a colony count was performed using a microscope and colony counter. The numbers of colonies were recorded as colonies per milliliter by dividing the total colonies by the volume of water filtered.

Determining the Bacterial Colony Count in Water After Drinking Once from the Bottle (Contaminated). Ten bottles for time point 0 hours and five bottles for each of the other time points (2, 8, 24, and 48 hours) from the stock of unopened bottles were analyzed after normally drinking one time by one of the three healthy volunteers. After one sip, the bottles were vortexed and sonicated for 1 minute for uniform mixing of the inoculate. Water from these bottles was then pooled into a large (4-L) autoclaved flask for thorough mixing. The pooled water was poured into sterile bottles, the volume was recorded, and the bottles were labeled and incubated at 37°C for different time points (0, 2, 8, 24, and 48 hours). The bottles, after the appropriate duration of incubation (“time point 0” samples were analyzed immediately), were pooled in an autoclaved flask after vortexing and sonicating and filtered through Nalgene filters, as described above. The filters were cut out, placed on the blood agar plates, labeled appropriately, and incubated at 37°C. After 48 hours of incubation, colonies were counted and the number of colonies per milliliter was calculated, as before.

Determining the Effects of Temperature of Storage on Bacterial Colony Growth of Bottled Water versus Tap Water. The above experiments were repeated with two sets of bottles containing bottled water and two sets of bottles containing tap water that was uncontaminated (unused) and contaminated (after one time use). The bottles containing bottled water and tap water were incubated for different time points (baseline, 0, 24, and 48 hours) at two different temperatures (room temperature [23°C] and cold temperature [4°C]). The appropriate dilution volume was filtered, and the agar plates were incubated at 37°C. All of the experiments were conducted in triplicate. The colony count was conducted after 48 hours, and the number of colonies per milliliter was calculated by multiplying by the appropriate dilution factor.

Characterizing the Types of Organisms and Identification of Bacteria. The above experiments were conducted using special media (MacConkey agar plates for Gram-negative organisms and Columbia CNA agar plates for Gram-positive organisms) instead of the usual blood agar plates. For further identification of species, the colonies from the blood agar were streaked on blood and nutrient agar for Gram staining and biochemical testing.
chocolate agar, and species were identified by morphological and biochemical tests, routinely performed in a clinical microbiology laboratory (Murray et al. [Eds.], 2003) or by 16S rDNA sequencing analysis of the clones, as described by Han et al. (2002). Briefly, genomic DNA from pure culture colonies was extracted and subjected to amplification by a polymerase chain reaction for a 593-base pair fragment of the 16S ribosomal RNA gene (16S rDNA). A set of universal bacterial primers—5' TGCCAGCAGCGCGGTATAC 3' and 5' CGCTGTTGCGGACTTACC 3' (515-1107 of Escherichia coli J01859)—was used for the amplification. The amplification was sequenced by the dye-terminator method in an ABI 377 sequencer (Applied Biosystems, Foster City, California), and sequence analysis was performed through a GenBank database (National Center for Biotechnology Information) BLAST query.

Results

Effect of Time on Bacterial Growth in Bottled Water After Drinking One Time. To determine the effect of storage time on bacterial growth after one sip, the bottles were incubated over different time points at 37°C to simulate body temperature and outdoor temperatures. As shown in Figure 4, at baseline, unused bottled water was free of bacterial colonies. After one time use, the bacterial colony count remained low for 2 hours. However, after 8 hours, there was a significant increase in the number of bacterial colonies, from <1 col/mL at baseline and 0 time point to 3, 28, 3014, and 37,938 col/mL at 2, 8, 24, and 48 hours, respectively ($p < 0.0001$) (Figure 5).

Effects of Temperature on Bacterial Growth Over Time in Bottled versus Tap Water. Because people store bottled water at different temperatures, to determine the effects of storage temperature on bacterial growth, the bottles were incubated at room temperature (23°C) and cold temperature (4°C) over various time points. As shown in Figure 6, there was a progressive increase in the number of bacterial colonies over 48 hours, especially after 8 hours. In contrast, the bacterial growth was significantly reduced at the cold temperature. At the cold temperature (4°C), the number of bacterial colonies was approximately 50% lower at 24 hours (mean ± standard error of the mean, 4486 ± 256 versus 8027 ± 308 col/mL; $p = 0.003$) and more than six times lower at 48 hours (3800 ± 808 versus 25,667 ± 3950 col/mL; $p = 0.03$) compared to room temperature (23°C). As shown in Figure 7, the bacterial growth was very minimal in tap water, even at room temperature. The number of bacterial colonies in the tap water 48 hours after one sip were 40 times lower than in the bottled water at room temperature (683 ± 96 versus 25,667 ± 3950 col/mL; $p = 0.012$) and 80-fold lower at the cold temperature (53 ± 12 versus 3800 ± 808 col/mL; $p = 0.02$).

Characterization and Identification of the Type of Microorganisms in Bacterial Growth. To determine the type of microbial organisms growing in the water after drinking one time, filters were incubated on special agar culture plates—Columbia CNA culture plates for Gram-positive and MacConkey culture plates for Gram-negative organisms. As shown in Figure 8, the predominant types of organisms were Gram-positive. After 48 hours of incubation at room temperature, the number of bacterial colonies on Columbia CNA was seven times higher than on the MacConkey culture plates (Figure 8). The growth of both Gram-positive ($p =$...
0.016) and Gram-negative organisms ($p = 0.02$) was significantly reduced at the cold temperature (Figure 8). The number of both Gram-positive (683 col/mL) and Gram-negative (287 col/mL) organisms was very low in tap water after 48 hours of incubation at room temperature compared with bottled water (Figure 9). At the cold temperature, the growth was further reduced for both Gram-positive (52 col/mL) and Gram-negative organisms (35 col/mL) at 48 hours (Figure 9). All these values were significantly lower compared with bottled water at similar temperature and time points.

To determine whether bacterial growth of the bottled drinking water could be reduced by pouring the bottled water into a cup rather than by touching the bottle to the mouth, bottles that were opened but uncontaminated were incubated at room and cold temperatures at different time points over 48 hours. There was approximately 50% reduced bacterial growth in the uncontaminated bottled water compared with bacterial growth observed after one-time drinking (11 133 ± 970 versus 25 667 ± 3950 col/mL; $p = 0.035$). Storage at the cold temperature greatly reduced this growth further, to 453 ± 58 col/mL compared with growth at room temperature ($p = 0.03$) at 48 hours (Table 1). Thus, bottled water that is opened and used without touching the bottle to the mouth and subsequently stored at the cold temperature showed significantly reduced bacterial amplification. Similarly, tap water that was not touched to the mouth (uncontaminated) had strikingly minimal growth (Table 1) at room temperature (up to 132 col/mL at 48 hours) or no growth at the cold temperature (2 col/mL at 48 hours). Thus, drinking bottled or tap water either by using a cup or without touching the bottle to the mouth can greatly reduce bacterial growth by avoiding contamination with oral microflora.

**Identification of Bacteria.** To further identify the organisms, pure colonies were streaked on blood and chocolate agar and species were further identified by morphology and biochemical tests. These tests (Murray et al. [Eds.], 2003) resulted in identification of Gram-positive organisms, consisting mainly of coagulase-negative bacteria, *Stomatococcus mucilaginosus*, Coagulase-negative *Staphylococcus*, and *Staphylococcus aureus*. For Gram-negative organisms that were difficult to identify by routine tests, bacterial clones from chocolate agar were subjected to 16S-ribosomal RNA gene-sequencing analysis (Han et al., 2002). Molecular gene sequencing led to identification of *Neisseria subflava* and *Neisseria pharyngis*.

**Discussion**

There has been a dramatic increase in bottled water use in the United States in recent years (Ferrier, 2002; NRDC, 1999). The bottled water industry is one of the fastest growing businesses in the United States, with annual consumption of more than 18 bil. L (5 bil.gal) of water. Total bottled water sales have increased from approximately 6% per year to more than 13% per year over the last five years. Accordingly, it has been predicted that bottled water may soon become the nation’s second most popular beverage, after soft drinks. One of the reasons people choose to drink bottled water instead of tap water is because of the perceived purity of bottled water. People are willing to spend a lot of money for what they consider a purer, safer, and tastier drink. Therefore, it is important to ask the following question: “Bottled Water: How Safe Is It?”

In the United States, bottled water and tap water are regulated by two different agencies; the FDA regulates bottled water and U.S. EPA regulates tap water. U.S. EPA has issued extensive regulations on the production, distribution, and quality of drinking water. The
FDA regulates bottled water as a food under the Federal Food, Drug, and Cosmetic Act. The FDA describes bottled water as water that is intended for human consumption and that is sealed in bottles or other containers with no added ingredients, except that it may contain optional fluoride or safe and suitable antimicrobial agents, such as ozone. However, the FDA does not specifically require that bottlers use antimicrobial agents in bottled water, as long as the water is safe for human consumption. The FDA does not have any specific regulations requiring chlorination of water. Furthermore, bottled water is considered to have an indefinite safe shelf life, if it is produced in accordance with current good manufacturing processes (CGMP) and quality standard regulations and is stored in an unopened, properly sealed container. Therefore, FDA does not require an expiration date for bottled water. Manufacturers are not required to include on their label the chloride levels or the expiration time or storage temperature once the bottle is opened and used.

The purpose of this study was to determine if there was time-dependent bacterial growth in bottled water and study the effects of storage temperature on bacterial growth, once the bottled water has been opened and used. The results of this study indicate that, after one sip only, bottled water is contaminated by oral microflora, which multiplies into a significant bacterial growth that far exceeds the 500-col/mL limit proposed by the Natural Resources Defense Council and 100 col/mL limit mandated by the European Council (European Council, 1998; NRDC, 1999). The number of bacterial colonies increased to more than 3000 col/mL at 24 hours and approximately 38 000 col/mL at 48 hours at 37°C. Storage at a cold temperature significantly retarded the rate of microbial growth compared with storage at room temperature. In sharp contrast, tap water had only minimal bacterial growth over the entire storage time and virtually no growth at a cold temperature, which was most likely a result of the chlorination process.

In healthy people, the accelerated microbial growth in bottled water may not be a great health concern. However, some people may be more vulnerable to drinking contaminated bottled water than the general population. People with compromised immune systems, such as cancer patients receiving chemotherapy, people who have undergone organ transplants, people with Human Immunodeficiency Virus (HIV) or other immune disorders, frail elderly patients, or infants can be particularly at risk for infections.

Although organisms identified in this study can all be regarded as part of the normal flora, their pathogenic potential should be recognized (Davis, 2000). Dental caries, periodontal disease, abscesses, and endocarditis are hallmarks of infections in humans. For example, Staphylococcus aureus is the most common cause of food-borne illness (Burton and Engelkirk [Eds.], 2000). Coagulase-negative staphylococci, such as Staphylococcus epidermidis, are recognized as a significant cause of infection in immunocompromised patients with indwelling catheters (Von Eiff, 2001). Stomatococcus mucilaginosus, also considered to be normal oral flora, has been reported to be an opportunistic pathogen, causing catheter-related sepsis and endocarditis (Lemozy et al., 1990; Poirier and Graudreau, 1989). It is also thought to contribute to the pathogenesis of dental caries. The flora of gingival areas also causes dental caries in approximately 80% of the population (Davis, 2000).

Table 1—Bacterial growth in the uncontaminated bottled water versus tap water over different periods of time.*

<table>
<thead>
<tr>
<th>Samples</th>
<th>0 h (col/mL)</th>
<th>24 h (col/mL)</th>
<th>48 h (col/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bottled water</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Room temperature (23°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MacConkey</td>
<td>0.8 (0.3)</td>
<td>57 (3.5)</td>
<td>6833 (549)</td>
</tr>
<tr>
<td>Columbia</td>
<td>69.9 (2.5)</td>
<td>584 (79)</td>
<td>11 133 (970)</td>
</tr>
<tr>
<td>Cold temperature (4°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MacConkey</td>
<td>0.8 (0.3)</td>
<td>88.1 (45)</td>
<td>523 (78)</td>
</tr>
<tr>
<td>Columbia</td>
<td>69.9 (2.5)</td>
<td>237 (5)</td>
<td>453 (58)</td>
</tr>
<tr>
<td>Tap water</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Room temperature (23°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MacConkey</td>
<td>0 (0)</td>
<td>2.8 (0.3)</td>
<td>41.3 (2.2)</td>
</tr>
<tr>
<td>Columbia</td>
<td>0.14 (0.05)</td>
<td>1.9 (0.3)</td>
<td>132 (18.3)</td>
</tr>
<tr>
<td>Cold temperature (4°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MacConkey</td>
<td>0 (0)</td>
<td>0.3 (0.02)</td>
<td>1.97 (0.46)</td>
</tr>
<tr>
<td>Columbia</td>
<td>0.14 (0.05)</td>
<td>0.36 (0.02)</td>
<td>2.1 (0.37)</td>
</tr>
</tbody>
</table>

* Data shown represent mean ± standard error of the mean values.
Previous studies have examined and reported on the microbiological water quality of unused bottled water (Hernandez-Duquino and Rosenberg, 1987; Lalumandier and Ayers, 2000; Rosenberg, 1990). Observations made in the present study suggest the need for future research to investigate the pathologic significance of bacteria contaminating bottled water of patients with active infections. This would be especially important in patients that are immunocompromised, such as cancer patients with neutropenia and fever, patients infected with HIV, elderly patients, or young infants with pneumonia or bacteremia. One potential concern in these patients may be propagation of the infection by self-inoculation of potentially harmful bacteria with repeated use. Another concern in this setting may be the potential for cross-contamination, if bottles are shared among family members or others. The findings of this study also provide a future direction for finding a potential solution to the problem, such as a preservative that can retard microbial growth without compromising the taste of water. These findings suggest that the development of FDA guidelines for the storage temperature and usage life for bottled water, once opened and used, should be considered. The label should also display the need for refrigeration and expiration time once the bottle is opened and used. The results of this study also would be useful to increase public awareness about the potential concerns of drinking contaminated water that is inappropriately stored. Based on these findings, it might be prudent to not store opened and used bottled water for an extended period of time, especially at room temperature or high outdoor temperatures.

Conclusions

The results of this project provide new information about the potential for progressive bacterial growth in bottled water once opened and used. This information will be useful for increasing public knowledge of appropriately using and storing bottled water. This finding also has potential global health implications because bottled water is used worldwide. This research suggests the importance of the development of guidelines and appropriate labeling of bottled water for refrigeration and expiration time once the bottle is opened. Future research in this area may lead to waters of improved quality that is not only pleasant-tasting, but also is protective against microbes.

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