ORIGINAL STUDIES

Microbiological assessment of house and imported bottled water by comparison of bacterial endotoxin concentration, heterotrophic plate count and fecal coliform count

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Consumers increasingly use bottled water and home water treatment systems to avoid direct tap water. According to the International Bottled Water Association (IBWA), an industry trade group, 5 billion gallons of bottled water were consumed by North Americans in 2001. The principal aim of this study was to assess the microbial quality of in-house and imported bottled water for human consumption, by measurement and comparison of the concentration of bacterial endotoxin and standard cultivable methods of indicator microorganisms, specifically, heterotrophic and fecal coliform plate counts. A total of 21 brands of commercial bottled water, consisting of 10 imported and 11 in-house brands, selected at random from 96 brands that are consumed in Puerto Rico, were tested at three different time intervals. The Standard *Limulus* Amebocyte Lysate test, gel clot method, was used to measure the endotoxin concentrations. The minimum endotoxin concentration in 63 water samples was less than 0.0625 EU/mL, while the maximum was 32 EU/mL. The minimum bacterial count showed no growth, while the maximum was 7,500 CFU/mL. Bacterial isolates like *P. fluorescens, Corynebacterium sp. J-K, S. paucimobilis, P. versicularis, A. baumannii, P. chlororaphis, F. indologenes, A. faecalis and P. cepacia* were identified. Repeated measures analysis of variance demonstrated that endotoxin concentration did not change over time, while there was a statistically significant ($p< 0.05$) decrease in bacterial count over time. In addition, multiple linear regression analysis demonstrated that a unit change in the concentration of endotoxin across time was associated with a significant ($p<0.05$) reduction in the bacteriological cell count. This analysis evidenced a significant time effect in the average log bacteriological cell count. Although bacterial growth was not detected in some water samples, endotoxin was present. Measurement of Gram-negative bacterial endotoxin is one of the methods that have been suggested as a rapid way of determining bacteriological water quality.

Key words: Bottled water, Endotoxin, Limulus amebocyte lysate, Microbiological water indicators, Fecal coliform count, Bacterial heterotrophic plate count.

Endotoxins are of immediate concern in many pharmaceutical industry water systems producing parenteral products because of their pyrogenic effects (1). Exposure to endotoxins in treated drinking water can occur through ingestion, dermal abrasions, inhalation of water vapor, intravenous injection or during dialysis (2). Outbreaks of endotoxin-related illness associated with drinking water have been documented infrequently, probably because many outbreaks of fever-related illness from water are not commonly identified by routine medical and bacteriological analyses (2).

Endotoxins are a component of the lipopolysaccharide (LPS) complexes which make up a part of the outer layer of the cell walls of most Gram-negative bacteria (3), and some cyanobacteria (4). The presence and concentration of endotoxin can be determined using aqueous extracts from blood cells (amoebocytes) of the horseshoe crab (*Limulus polyphemus*). This test is known as the *Limulus* amoebocyte lysate (LAL) assay and was first described by Levin and Bang in 1960's.

In the 1970's, the feasibility of LAL assay was investigated as a potential assay that can be easily and relatively inexpensively applied to the examination of drinking water, surface water or waste waters (5-7). Analysis of a variety of potable water samples and waste

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waters using LAL assay by Jorgensen et al. (1976) reported endotoxin concentrations of 0-1,250 µg/l, as was formerly reported (7). Evans et al. (1978) found that total endotoxins could be correlated to bacteriological plate counts methods with coefficients of determination (r^2) in excess of 0.68 (6).

Since 1975, the Federal Food, Drug, and Cosmetic Act (FDA) ensures that the quality standards for bottled water are compatible with U.S. Environmental Protection Agency (EPA) standards for quality and safety of tap water (8). Bottled water production plants conduct monitoring and testing for detection of standard water microbial indicators. Heterotrophic plate counts and total and fecal coliforms bacterial counts are routinely assayed and recorded to fulfill quality control requirements. The greatest disadvantage of these methods is the time needed to obtain results related to the water quality (9). The LAL test is easy to perform and relatively inexpensive. Particularly important has been the ability to conduct in-process testing of raw materials and of water in the pharmaceutical industry (1).

This study investigated the application of endotoxin monitoring to bottled water for human consumption by comparing endotoxin concentration and bacteriological cell counts in 21 brands of commercially available bottled water in Puerto Rico. Comparisons were made between determinations of endotoxin concentration and bacterial counts, and changes over time of these parameters were also described.

Methods

The study was conducted in two phases. The initial phase consisted of determining the weekly concentration of endotoxin and bacterial count in serial dilutions of gram-negative bacteria over a four-week period. The aim of the second phase of this study was to determine the concentration of endotoxin and bacterial count in samples of commercially available bottled water. The presence of fecal coliform bacteria in bottled water was also tested.

Phase 1

Endotoxin concentrations associated with serial dilutions of stock concentrations of *Escherichia coli and Pseudomonas aeruginosa* (Gram-negative bacteria that served as positive controls) and *Staphylococcus aureus* (Gram-positive bacteria that served as the negative control) were measured. In general, endotoxins are not characteristic components of Gram-positive bacteria cell wall.

Over a four-week period, serial dilutions of stock concentrations of *E. coli and P. aeruginosa* were tested once a week for endotoxin concentration, using LAL gel clot, and standard growth media for bacterial colony counts. On the other hand, *S. aureus* was tested over a two-week period following the same procedure, because concentration of endotoxin was below LAL gel clot method detection limit.

Summary measures were computed to describe the logarithm (log) concentration of endotoxin and bacteriological cell count. Repeated measures analysis of variance was used to evaluate variation across time in the average log of the bacteriological cell count and the average log concentration of endotoxin.

Phase 2

To select a random sample of bottled water brands, the sampling frame was constructed to include 96 brands of commercially available bottled water that are consumed in the island and monitored by the Puerto Rico Department of Health (10). A simple random sample of 21 brands - 10 imported and 11 in-house - of bottled water was selected for analysis. These bottled water brands were bought in different local food markets.

Drinking water samples were tested at three different time intervals to measure endotoxin concentration, fecal coliforms and heterotrophic plate counts. The initial sampling and testing (T_0) was followed by the storage of water samples for one week at 4ºC when second testing (T_1) was performed. Third testing (T_2) was performed after one year of storage. Three water samples of 1 mL each per brand were defined as the minimum number of replicates needed to explore the within-variation of endotoxin concentration and bacteriological cell count among bottled water brands.

The Standard LAL test, gel clot method, was used to measure the endotoxin concentration. Endotoxin-free water and glassware were used to perform the assay. The test relies on a biochemical reaction between lysed blood cells of the horseshoe crab *Limulus polyphemus* and cell envelop (LPS) of Gram-negative bacteria. LAL clots in a test tube in the presence of a sufficient amount of endotoxin, and this reaction is the basis for the endotoxin test. In this study, sensitivity of the gel clot method was 0.0625 EU/mL.

Monitoring and detection of indicator and disease-causing microorganisms are a major part of sanitary and public health microbiology. Cultivable methods of indicator microorganisms, standard heterotrophic and fecal coliform plate counts were performed.

Coliforms, including *Escherichia coli,* are members of the family *Enterobacteriaceae*. These bacteria make up approximately 10% of the intestinal microorganisms of humans and other animals and have found widespread use as indicator organisms (3). Coliforms are defined as

facultatively anaerobic, gram-negative, nonsporing, rod-shaped bacteria that ferment lactose with gas formation within 48 hours at 35 ºC. Fecal coliforms most commonly derived from the intestines of warm-blooded animals can grow at the more restrictive temperature of 44.5 ºC (3).

The membrane filtration technique was used to test for fecal coliforms. A volume of 100 mL bottled water was passed through a membrane filter in accordance with test methods based on American Public Health Association (APHA) standard methods for the examination of water and wastewater (11). Filter was transferred to M-FC growth medium. After 24 hours of incubation, plates were observed for colony forming units.

Heterotrophic bacteria are those that require organic carbon rather than carbon dioxide as a carbon source (3). The EPA has suggested that the heterotrophic bacterial counts in drinking water should not exceed 500 CFU/mL.

Hetrotrophic bacteria were enumerated by the pour-plate technique employing Trypticase Soy Agar (TSA) medium. Dilutions were made in 9-mL blanks of phosphate buffer, and inoculation of 1 mL of each dilution was done by the pour-plate method in the TSA medium. The plates were inverted, and incubated for 48 hours at 35 ºC and counted with the aid of a colony counter. Gram stains were performed to isolated colonies. Bacterial isolates were then identified by genera and species using the Manual Conventional Method and API 20NE.

Summary measures were computed for the log concentration of endotoxin and bacteriological cell count. To assess differences in bottled water samples obtained from imported and in-house brands, the log concentration of endotoxin and bacteriological cell count at baseline was compared using the Wilcoxon two-sample test. Simple linear regression analysis was performed to test the effect of the average log concentration of endotoxin on the average log bacteriological cell count between bottled water samples (between effect model) :

$$\overline{Y}_j = \beta_0 + \beta_1 \overline{X}_j + \varepsilon_j$$

The model was also used to test the time effect of the log concentration of endotoxin on the average log bacteriological cell count - this was performed by regressing deviations of individual values from the bottled water mean (within effect model) :

$$(Y_{ij} - \overline{Y}_j) = \beta'_0 + \beta'_1 (X_{ij} - \overline{X}_j) + \varepsilon'_{ij}$$

A two way group effect regression model was then employed to test the effect of the average log concentration of endotoxin between bottled waters ( ) adjusting for the time effect ( ). We used the Stata statistical package (version 8) to perform these analysis (14).

Results

Phase 1 of the Study

The endotoxin concentration and bacterial cell count for *E. coli* and *P. aeruginosa* (positive controls) were measured according to the serial dilutions from stock (Tables 1 and 2). The average log concentration of endotoxin of *E. coli* ranged from 2.66±1.61 EU/mL in the first week to 2.89±1.61 EU/mL during the third and fourth weeks; however, the average log bacteriological cell count of *E. coli* varied from 7.10±1.91 CFU/mL in the first week to 2.93±2.14 CFU/mL in the third week (data not shown). The average log concentration of endotoxin of *P. aeruginosa* ranged from 4.04±1.61 EU/mL in the first week to 3.81±1.99 EU/mL during the fourth week; however, the average log bacteriological cell count of *P. aeruginosa* varied from 8.19±1.69 CFU/mL in the first week to 4.15±3.81

Table 1. Endotoxin concentration associated with Standard Plate Count of E.coli CFU/mL over a four-week period.

First Week (01/24/00)		Second Week (01/31/00)		Third Week (02/07/00)		Fourth Week (02/14/00)	
CFU/mL	**EU/mL**	**CFU/mL**	**EU/mL**	**CFU/mL**	**EU/mL**	**CFU/mL**	**EU/mL**
16,950	128	5,300	128	106	128	390	128
4,050	64	350	64	73	64	81	64
3,100	16	815	32	156	31	71	32
562	8	116	8	18	16	17	16
242	4	19	4	2	4	6	4
109.5	2	24	2	1	2	0	2

CFU= Colony Forming Units per mililiter EU/mL= Endotoxin Units per mililiter

Table 2. Endotoxin concentration associated with Standard Plate Count of *P. aeruginosa* CFU/mL over a four-week period.

First Week (01/26/00)		Second Week 02/02/00)		Third Week 02/09/00)		Fourth (02/16/00)	
CFU/mL	**EU/mL**	**CFU/mL**	**EU/mL**	**CFU/mL**	**EU/mL**	**CFU/mL**	**EU/mL**
39,500	512	775	512	2,080	512	2,150	512
10,500	128	200	256	1,070	256	400	256
5,750	64	970	64	405	64	115	64
2,700	32	1,010	32	284	32	85	32
920	16	315	16	0	16	20	16
370	8	104	8	0	4	14	2

CFU= Colony Forming Units per mililiter EU/mL= Endotoxin Units per mililiter

CFU/mL in the third week. As expected, endotoxin concentration for *S. aureus* (negative control) was less than the detection limit, <0.0625 EU/mL (Table 3). The

Table 3. Endotoxin concentration associated with Standard Plate Count of *S. aureus* over a two-week period.

First Week (01/28/00)		Second Week (02/07/00)	
CFU/mL	**EU/mL**	**CFU/mL**	**EU/mL**
13,600	0.1	8,950	0.1
6,650	0.1	4,000	0.1
3,300	0	1,955	0
830	0	585	0
750	0	440	0
335	0	261	0

CFU= Colony Forming Units per mililiter EU/mL= Endotoxin Units per mililiter

average log bacteriological cell count of *S. aureus* varied from 7.60±1.44 CFU/mL in the first week to 7.17±1.38 CFU/mL in the second week. Analysis of variance with a repeated measures design revealed that the average log concentration of endotoxin of *E. coli* and *P. aeruginosa* did not change (p>0.05) over the four–week period; however, the average log bacteriological cell count significantly (p<0.05) decreased over time (Table 4).

Table 4. Repeated measures analysis: average log concentration of endotoxin of *E. coli* and *P. aeruginosa*

Outcome	Degrees of freedom	Mean squares	F	P
Average log concentration of endotoxin of *E. coli*	3	0.07	1.77	0.20
Average log concentration of endotoxin of *P. aeruginosa*	3	0.05	0.53	0.67
Average log bacterial count of *E. coli*	3	23.69	69.10	<0.0001
Average log bacterial count of *P. aeruginosa*	3	19.13	8.54	0.0015

Phase 2 of the Study

No significant (p>0.05) differences were observed in the median log concentration of endotoxin and the median log bacteriological cell count by bottled water brand at baseline (data not shown). The minimum endotoxin concentration in 63 water samples was less than 0.0625 while the maximum was 32 EU/mL. Fecal coliforms were not recovered from any of bottled water samples.

The minimum bacterial heterotrophic count showed no growth, while the maximum was 7,500 CFU/mL. Bacterial isolates including *P. fluorescens, Corynebacterium sp. J-K, S. paucimobilis, P. versicularis, A. baumannii, P. chlororaphis, F. indologenes, A. faecalis and P. cepacia* were identified from the positive bottled water brands by the Manual Conventional Method and API 20NE (Table 5).

The between effect regression model revealed that the average log bacteriological cell count significantly (p<0.05) increased 0.59 units per unit increase in the average log concentration of endotoxin (Figure 1). On the

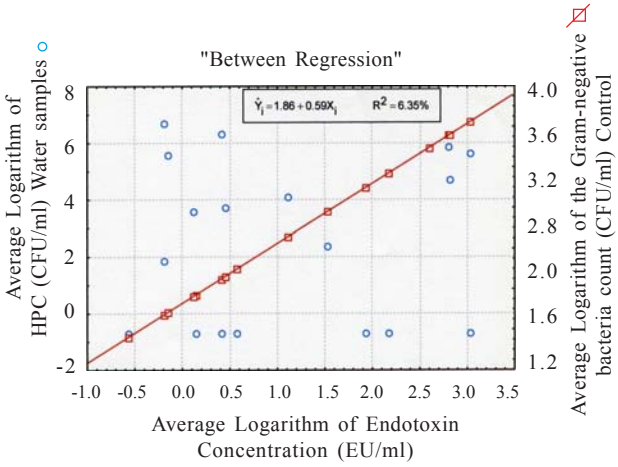

Figure 1. Average logarithm of the heterotrophic count as a function of the average log concentration of endotoxin (between regression).

Table 5. Identification of Heterotrophic Bacteria Isolates from Bottled Water Brands

#Family	Identification Genus and species	Bottled Water Brand	Gram Stain
	Pseudomonas fluorescens	La Roca	Gram-negative rods
	Pseudomonas fluorescens	*Volvic Natural Spring	Gram-negative rods
Pseudomonadaceae	Pseudomonas versicularis	*Evian Natural Spring	Gram-negative rods
	Pseudomonas chlororaphis	Pureza Real	Gram-negative rods
	Pseudomonas cepacia	Paradise	Gram-negative rods
Corynebacteriaceae	Corynebacterium Group J-K	*Volvic Natural Spring	Gram-positive rods
Sphingomonadaceae	Sphingomonas paucimobilis	Mejesty Premium Baby	Gram-negative rods
Moraxellaceae	Acinetobacter baumannii	Pureza Real	Gram-negative rods
Alcaligenaceae	Alcaligenes faecalis	*Dannon Natural Spring	Gram-negative rods
Flavobacteriaceae	Flavobacterium indologenes	Pureza Real	Gram-negative rods

According to the Second Edition of Bergey's Manual of Systemic Bacteriology
* Imported bottled water brands

contrary, the within effect model showed a significant time effect of the log concentration of endotoxin on the average log bacteriological cell count ($\hat{\beta}$ = -1.46; p<0.001) (Figure 2). The two way group effect regression model

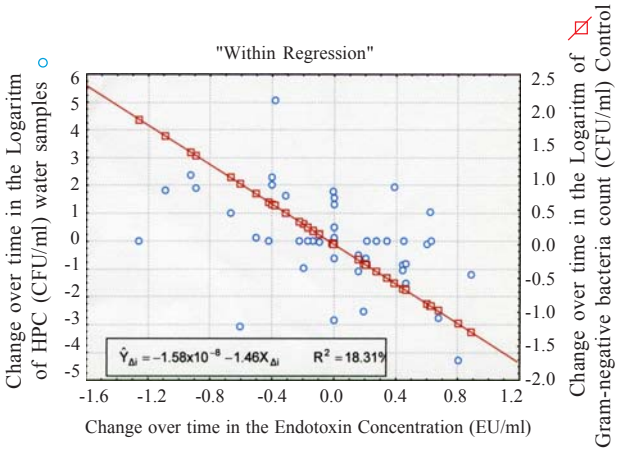

Figure 2. Change in the average logarithm of heterotrophic count as a function of the change in the average log concentration of endotoxin (within regression).

demonstrated that the average log bacteriological cell count did not change (p>0.05) with a unit increase in the average log concentration of endotoxin after adjusting for the time effect (Table 6). However, a significant (p<0.05) time effect in the log bacteriological cell count was observed. Although bacterial growth was not detected in some water samples, endotoxin was present.

Table 6. Average log bacteriological cell count as a function of the average log concentration of endotoxin adjusting for the change in the average log concentration of endotoxin across time: Multiple linear regression model.

Variable	$\hat{\beta}$	SE ($\hat{\beta}$)	p	R^2
Between regression	0.59	0.52	0.27	8.66%
Within regression	-1.46	0.66	0.038	
Constant	1.86	0.81	0.032	

Discussion

The standard methods for assessing microbiological bottled water quality are based on the growth of bacteria on laboratory media. Plate count methods have one major disadvantage in common, an incubation time of at least 24 hours. Also, viable counts select for the organisms that can grow in the chemical and physical conditions provided: e.g., type of medium, temperature of incubation and light intensity. Water bacteria have slow rates of growth (an adaptation to low nutrient concentrations) (1). Furthermore, cell division may even be inhibited by the relatively high nutrient concentrations in the growth medium (1).

In 2002, Rapala et al. measured endotoxin concentrations at nine different full-scale drinking water treatments plants in Finland. They found that endotoxin concentrations ranged from 18 to 356 EU/mL at the plant intakes, and 3 to 15 EU/ mL at the finished water in the distribution system (4).

The bacterial isolates obtained in this study were similar to those reported in other studies (15,16). The most common Gram-negative rod species isolated were species of *Pseudomonas*. The genus *Pseudomonas* is ubiquitous in nature and is commonly found in the autochthonous microflora of mineral waters. Furthermore, *Pseudomonas* is frequently associated with low levels of available nutrients in aquatic environments.

While the bacterial growth was not detected in some water samples, endotoxin was detected. Two explanations for this observation may be offered. First, endotoxins are released into the environment after lysis of the bacterial cells, leaving the endotoxin molecule in the water and no viable cells. Second, limitations of cultivable methods may underestimate bacterial growth.

The results obtained demonstrated that endotoxin concentration did not change over time, while there was a statistically significant decrease in bacterial count over time. Moreover, the average log bacteriological cell count did not change with a unit increase in the average log concentration of endotoxin after adjusting for the time effect. However, a significant time effect in the log bacteriological cell count was observed. This finding strengthens the applicability of the endotoxin testing as an indicator of microbial water quality.

We conclude that LAL assay has application in and beyond the pharmaceutical industry in areas where endotoxin concentration has previously been of no concern. The measurement of Gram-negative bacterial endotoxin is one of the methods that have been suggested as a rapid way of determining bacteriological water quality.

Resumen

Los consumidores utilizan cada vez más el agua embotellada y los sistemas de tratamiento de aguas caseros para evitar el consumo del agua potable servida directamente del grifo. Según la Asociación Internacional del Agua Embotellada (IBWA, por sus siglas en inglés), los norteamericanos consumieron 5 billones de galones de agua en el año 2001. El objetivo principal de este estudio fue determinar la calidad microbiológica de aguas

embotelladas importadas y locales de consumo humano por medio de la medición y la comparación de la concentración de endotoxina bacteriana y los métodos estándares de contajes de indicadores bacterianos, específicamente el contaje heterotrófico y el contaje de coliformes fecales. Un total de 21 marcas de aguas embotelladas distribuidas comercialmente, compuesta por 10 importadas y 11 locales, fueron seleccionadas aleatoriamente de 96 marcas de consumo en Puerto Rico y fueron analizadas en tres intervalos de tiempo diferentes. La prueba estándar de *Limulus* Amebocyte Lysate, método del coágulo gelatinoso, fue utilizada para medir las concentraciones de endotoxina. De un total de 63 muestras analizadas, la concentración mas baja obtenida fue menor a 0.0625 EU/mL y la máxima fue de 32 EU/mL. El contaje microbiológico mínimo no demostró ningún crecimiento, mientras que el máximo fue de 7,500 CFU/mL. Del crecimiento de colonias en los contajes heterotróficos se aislaron e identificaron bacterias tales como *P. fluorescens, Corynebacterium sp. J-K, S. paucimobilis, P. versicularis, A. baumannii, P. chlororaphis, F. indologenes, A faecalis y P. cepacia.* El análisis de varianza de medidas repetidas reveló que la concentración de endotoxina no varió a través del tiempo, pero el contaje bacteriológico disminuyó significativamente ($p<0.05$). El análisis de regresión lineal múltiple reveló que un cambio de una unidad en la concentración de endotoxina a través del tiempo se asoció con una reducción significativa ($p<0.05$) en el contaje bacteriológico. Aún en la ausencia de crecimiento bacteriano en algunas muestras de agua, se pudieron detectar concentraciones de endotoxina. Se ha propuesto la medición de la endotoxina bacteriana como un método más rápido para la determinación de la calidad bacteriológica del agua.

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