Survival of allochthonous bacteria in still mineral water bottled in polyvinyl chloride (PVC) and glass

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L. MOREIRA, P. AGOSTINHO, P. V. MORAIS AND M. S. DA COSTA. 1994. The mortality of *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, based on the culturability of these bacteria, was assessed in non-carbonated mineral water, bottled in polyvinyl chloride (PVC) containing the indigenous flora, sterile mineral water bottled in PVC, sterile mineral water in glass containers, and sterile tap water in glass containers. There was a general decrease in the culturability of these organisms in the four test waters, except that *Ps. aeruginosa* grew in sterile tap water. *Escherichia coli* and *Kl. pneumoniae* had the highest mortality rates under the conditions tested, while *Ent. cloacae* had a very low and constant mortality rate that would have resulted in the persistence of this organism in mineral water for a long period of time. After a sharp initial decrease in culturability, *Ps. aeruginosa* also had a very low mortality rate in mineral water bottled in PVC.

INTRODUCTION

Information about the survival capacity of bacterial indicators of faecal pollution and pathogenic bacteria in different water habitats is crucial to public health for the evaluation of possible risks. Coliforms and faecal coliforms are used as indicators of water quality and the absence of these bacteria as culturable cells is an indication of good quality water (Anon. 1981).

The bacteriological quality of bottled mineral water is based on the presence/absence of indicator bacteria for faecal pollution (*Escherichia coli*, faecal streptococci and sporulating sulphite-reducing anaerobes), surface water contaminants (total coliforms, sporulating sulphite-reducing anaerobes) and pathogenic bacteria such as *Pseudomonas aeruginosa* (Anon. 1980). The data available on the survival of bacteria in superficial waters cannot be extrapolated to bottled mineral waters and several workers who studied the survival of bacteria recognized the impact of experimental bottling stress on the population dynamics of these organisms (Flint 1987; Overbeek *et al.* 1990; Barcina *et al.* 1990; Korhonen and Martikainen 1991). The chemical and microbiological characteristics of mineral waters can also affect allochthonous bacteria in different ways (Schmidt-Lorenz 1976). In bottled mineral water allochthonous bacteria must cope with an enclosed environment of very low

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nutrient content and interact with the autochthonous population present in the bottle, which can reach considerable numbers a few days after storage (Morais and da Costa 1990; Hunter 1993). To understand the significance of the presence of coliforms or *Ps. aeruginosa* in bottled mineral water it is necessary to determine the survival capacity of these bacteria in water bottled in different types of containers during normal storage conditions.

Bottled water can occasionally become contaminated with bacteria, some of which could be pathogens, indicators of surface water contamination or of faecal contamination. Although the aquifer of a bottled mineral water can become contaminated, the bottling system is the most likely source of contamination. EC directives (Anon. 1980) recommend the microbiological quality control of bottled mineral water only immediately after bottling. Among other considerations, this would be the most likely time to detect indicators of faecal pollution or pathogenic bacteria, because of the presumed mortality of some of these organisms in bottled water. Furthermore, high heterotrophic plate counts of autochthonous bacteria after storage of bottled water can interfere with the detection indicators of faecal pollution (Geldreich *et al.* 1975; Lamka *et al.* 1980). In this study the survival of *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were determined in a bottled still mineral water in the presence and absence of the autochthonous bacterial flora. The influence of the bottling material (polyvinyl chloride or glass) on

the survivorship of the allochthonous bacteria was also investigated.

MATERIALS AND METHODS

Test strains

The test strains used were *E. coli* (ATCC 8677), *Ent. cloacae* (ATCC 13047), *Kl. pneumoniae* (ATCC 13833) and *Ps. aeruginosa* (ATCC 27853), obtained from American Type Culture Collection (Rockville, MD, USA) and maintained at $-80°C$ in Tryptic Soy Broth (TSB, Difco) plus 15% (v/v) glycerol.

Preparation of microcosms

Tap water was obtained from the public water distribution system of Coimbra. The mineral water used was collected at a still mineral water bottling plant in Central Portugal and had the following physical and chemical characteristics: temperature at the source, 27°C; pH, 5·64; conductivity, $5·44 \times 10^{-5}$ Ohm^{-1} cm^{-1}; dry residue at 180°C, 42·2 mg l^{-1}; dissolved oxygen, 0·4 mg l^{-1}; and free CO_2, 50 mg l^{-1}.

The four test waters were prepared as follows:

(1) *Mineral water in PVC bottles with autochthonous flora.* Mineral water in 330 ml polyvinyl chloride (PVC) bottles was obtained immediately after bottling in factory and maintained at room temperature ($\pm 22°C$) in the dark for 3 months to allow the autochthonous flora to reach high numbers before inoculating the test strains. Mineral water was examined for the presence of coliforms by membrane filtration on m-Endo LES (Difco) and *Ps. aeruginosa* on Cetrimide Agar (Difco) of 250 ml of water (Anon. 1981).

(2) *Sterile mineral water in PVC bottles.* Empty PVC bottles (330 ml) were capped with ethylene-oxide sterilized polyethylene caps immediately after production. Sterility of PVC bottles was assessed by shaking the bottles with 5·0 ml of sterile phosphate dilution buffer (Anon. 1985). The number of colony-forming units (cfu) was determined in 1·0 ml by the pour plate method on Plate Count Agar (Anon. 1985) after incubation of the plates at 22°C for 72 h and at 37°C for 24 h. Mineral water was sterilized through 0·2 μm pore size nylon filters (Pall Ultipore, Portsmouth, UK) and then placed in a boiling water bath for 20 min (Colbourne *et al.* 1988). Sterility of the mineral water was assessed as described above.

(3) *Sterile mineral water in glass containers.* Glass-stoppered Pyrex Erlenmeyer flasks (300 ml) were washed overnight with concentrated HCl (37%), followed by a wash in perchloric acid (70%) for 24 h. The flasks were then rinsed several times with hot tap water, deionized water and three times in ultra pure water (Millipore). The flasks and the glass stoppers were heated overnight at 250°C, cooled and covered with sterile aluminium foil. The flasks were filled with sterile mineral water, prepared as described above, immediately before inoculation with the test strains.

(4) *Sterile tap water in glass containers.* Sterile glass stoppered Pyrex flasks, prepared as above, were filled with filtered and heated tap water as described above for mineral water.

Survival experiments

The test strains were grown with shaking in a basal medium containing (g l^{-1}): yeast extract, 0·76; K_2HPO_4, 15·1; $(NH_4)_2SO_4$, 2·2; $MgSO_4 . 7H_2O$, 0·2; $FeSO_4 . 7H_2O$, 6·0 mg l^{-1}; pH 6·8 (Pastan and Perlman 1968) supplemented with 2·0% glucose at 37°C until the cultures reached 0·5 O.D. units at 610 nm (Milton Roy Spectrophotometer 601, Milton Roy, Stafford, UK), harvested by centrifugation (2000 *g*) for 10 min at 4°C, and washed twice with sterile mineral water.

The washed suspensions were inoculated into flasks containing sterile mineral water and maintained for 24 h at 25°C in the dark. The starved bacterial cells were inoculated in duplicate in the four test waters to give the final suspension of about 10^5 colony-forming units (cfu) ml^{-1} determined by plate count (see below). The inoculated test waters were maintained at 25°C in the dark, without shaking, for the experimental period. Two independent experiments were performed in duplicate for each microcosm.

Viability determinations

Samples or dilutions of the experimental waters containing the test bacteria were spread in triplicate on Tryptic Soy Agar (TSA, Difco), and incubated at 37°C for 24 h. Phosphate buffer dilution water, pH 7·2, was used for dilution of the test and endogenous bacteria for viable counts (Anon. 1985). Presumptive coliform colonies were confirmed by production of gas in brilliant green bile broth (Anon. 1985). *Pseudomonas aeruginosa* colonies were confirmed by hydrolysis of casein and the production of pyoverdin and pyocyanin on Skim Milk Agar (Difco) after incubation at 30°C for 48 h (Havelaar *et al.* 1985).

The endogenous bacterial flora of the mineral water was enumerated preparing all samples and decimal dilutions in phosphate buffer dilution water. Samples or dilutions (0·1 ml) were spread plated in triplicate on R_2A Agar (Reasoner and Geldreich 1985). The plates were incubated in plastic bags in humidified incubators at 22°C for 15 d.

Data analysis

The experimental results were fitted with a theoretical model of the kinetics of cell death under starvation conditions described by:

$$dN/dt = kN^{\alpha+1} \qquad (1)$$

where N is the number of viable cells at time t, and k and α are constants (Kumada *et al.* 1985). This equation was obtained by making the growth constant dependent on N where $K(N) = kN^{\alpha}$. To adjust the mortality curves, we used the equation for the initial conditions where $N = N_0$ and $t = t_0$ because it minimizes errors during the calculations of the mortality rates. With the same equation, k and α were calculated by non-linear adjustment by the method of least-squares. The mortality rate was calculated as $d = kN^{\alpha}$.

RESULTS

The mineral water samples used in this study contained no coliforms or *Ps. aeruginosa* in 250 ml. The coliform bacteria and *Ps. aeruginosa* added to the test waters were easily detected in the presence of the indigenous bacteria because, within the first 24 h at 37°C, only the test bacteria formed visible colonies on TSA. During the experimental period

the heterotrophic plate counts (HPC) of the indigenous bacteria of the bottled mineral water (test water 1) varied between 1.09×10^5 cfu ml^{-1} and 1.03×10^6 cfu ml^{-1} at 22°C and there was no indication that the allochthonous bacteria had any effect on the indigenous population.

The viable counts of the three test enterobacteria decreased under all experimental conditions, but the decrease depended on the organism and the conditions in which they were examined. The population of *E. coli* decreased rapidly in mineral water, especially in mineral water bottled in PVC irrespective of the presence or absence of autochthonous bacterial flora. In sterile tap water, after an initial decrease, the viable counts remained almost constant during the experimental period (Fig. 1a).

Of the three enterobacteria tested, *Ent. cloacae* had the slowest decrease in viable counts in any of the conditions, although the decrease in viable counts was slightly more pronounced in sterile mineral water bottled in PVC than in the other test conditions (Fig. 1b).

A small constant decrease in the viable counts of *Kl. pneumoniae* was observed in mineral water bottled in PVC with indigenous flora and in sterile tap water. On the other hand, this strain was rapidly inactivated in sterile mineral water bottled in PVC and glass resulting in very low viable counts after the 20 d experimental period (Fig. 1c).
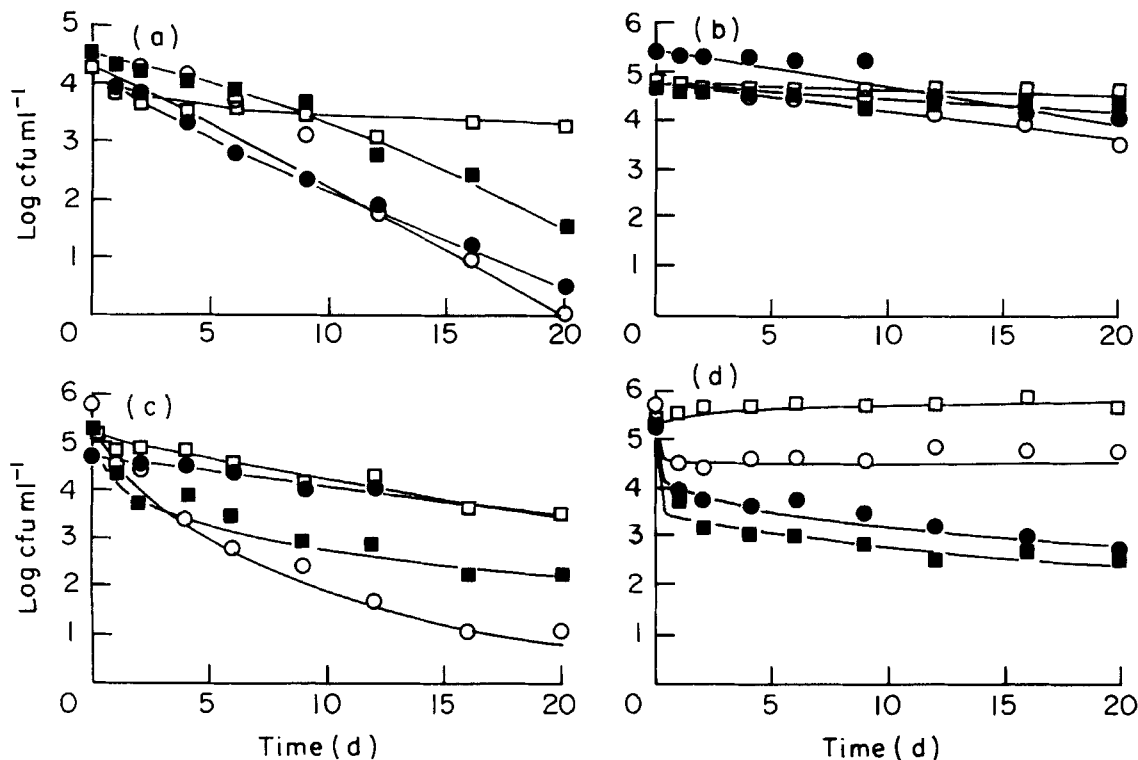

Fig. 1 Survival determined by viable counts (mean of two independent experiments) on tryptic soy agar of (a) *Escherichia coli*, (b) *Enterobacter cloacae*, (c) *Klebsiella pneumoniae* and (d) *Pseudomonas aeruginosa* inoculated in mineral water in PVC bottles with autochthonous flora (●), in sterile mineral water in PVC bottles (○), in sterile mineral water in glass containers (■) and in sterile tap water in glass containers (□)

In contrast to the other test bacteria, *Ps. aeruginosa* grew in tap water. Immediately after inoculation of this organism in mineral water there was a sharp decrease in the viable counts. Afterwards, there was a very slow decrease in viable counts in PVC bottled mineral water with autochthonous flora and sterile mineral water in glass containers, but the viable counts in sterile mineral water bottled in PVC remained constant for the duration of the experiment (Fig. 1d).

The theoretical curves calculated from eqn 1 fit the experimental results, and described the bacterial survival during starvation. For each bacterium, in each microcosm, the mortality rate varied, either decreasing or increasing during the experimental period.

The mortality rates of *E. coli* in mineral water microcosms were very similar and they were almost constant during the experimental period (Fig. 2a). On average it had the highest mortality rate of all allochthonous bacteria, although in tap water *E. coli* showed a high initial mortality rate followed by a low mortality rate which was similar to other enterobacteria in tap water.

Enterobacter cloacae had a low and almost constant mortality rate during the experimental period in all microcosms (Fig. 2b). The behaviour of *Kl. pneumoniae* was different from that of the other allochthonous bacteria examined, showing a high mortality rate immediately after being inoculated in sterile mineral water followed by a sharp decrease

in the mortality rate after this initial shock. On the other hand, the mortality rate of *Kl. pneumoniae* was very low and constant in the presence of the autochthonous bacterial flora of the mineral water (Fig. 2c).

Pseudomonas aeruginosa had a high mortality rate immediately after inoculation in mineral water with flora and in mineral water bottled in washed glass but, afterwards, the mortality rates decreased almost to zero in all the mineral water microcosms (Fig. 2d). When inoculated in tap water *Ps. aeruginosa* had a negative mortality rate, which showed that this organism grew under the experimental conditions.

DISCUSSION

This study demonstrates that, in general, the test bacteria examined show a progressive decrease in culturability in mineral water bottled in glass or PVC. The results do not rule out the existence of viable cells in a non-culturable state. However, microbiological quality control relies on the culturability of bacteria on selective media, and not on the determination of viability by staining methods. The utilization of selective media would, no doubt, have a more pronounced effect on the culturability than the non-selective medium used here.

The capacity of survival of organisms under stress conditions depends, among other factors, on the age of the cultures. In this study all organisms were grown until the early
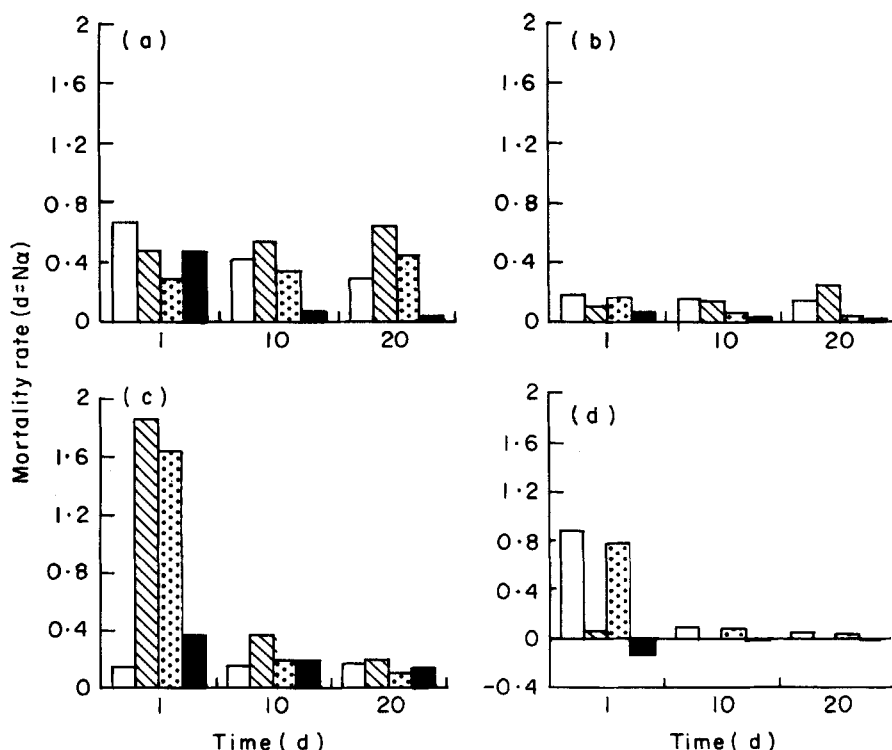

Fig. 2 Mortality rate (d = N^x) at 1, 10 and 20 d during the experimental period of (a) *Escherichia coli*, (b) *Enterobacter cloacae*, (c) *Klebsiella pneumoniae* and (d) *Pseudomonas aeruginosa* inoculated in mineral water in PVC bottles with autochthonous flora (☐), in sterile mineral water in PVC bottles (▨), in sterile mineral water in glass containers (▦), and in sterile tap water in glass containers (■)

stationary phase of growth, which previous studies show to be the most resistant phase of growth of *E. coli* and *Kl. pneumoniae* to survival in water (LeChevallier *et al.* 1988; Gauthier *et al.* 1992). The initial starvation of the test organisms was designed to deplete the bacteria of nutrient carryover and minimize the possibility of regrowth under the experimental conditions.

A limited number of studies on the survival of faecal indicator organisms and pathogens in mineral water show marked discrepancies that are probably due to different test conditions, and different chemical and microbiological parameters of the mineral waters. In one study, 10^7 cfu ml^{-1} of *E. coli* inoculated in a gas-free mineral water became undetectable within 4 d but the addition of a small amount of faeces to the bottled mineral water enhanced the survival of *E. coli* to several weeks (Ducluzeau *et al.* 1976). Another study reported that *E. coli* declined by 1 log every 2 weeks in sterile spring water stored at 4°C (Karapinar and Gönül 1991). Mineral waters, however, are not generally stored at this temperature during transport and at retail outlets. In this study the decrease in culturability of coliform bacteria depended on the type of water (mineral or tap), the presence or absence of the normal flora of the mineral water and the type of container used (PVC or glass). While *E. coli* is the canonical indicator of faecal pollution, coliforms such as *Kl. pneumoniae* and *Ent. cloacae* are commonly found in tap water (Gavini *et al.* 1985) and are capable of growth in drinking water systems (Camper *et al.* 1991; Stewart and Olson 1992). The presence of the latter organisms in mineral water may not signify faecal contamination but clearly indicates that the aquifer and/or the bottling system are contaminated with non-mineral water sources. In this study *Ent. cloacae* had very low mortality rate and would have persisted in the mineral water for a long period of time irrespective of the test conditions. *Pseudomonas aeruginosa* is frequently isolated from water and several studies show it to grow even under conditions of very limited nutrient content (van der Kooij *et al.* 1982). It is also a major concern in mineral water bottling plants, and has been isolated from bottled mineral water which had already been removed from market (Manaia *et al.* 1990).

The autochthonous bacteria of mineral water have been reported to have an inhibitory effect on the survival of *E. coli* (Lucas and Ducluzeau 1990). In the present study, however, the autochthonous flora of the mineral water, which reached $1·03 \times 10^6$ cfu ml^{-1} during the experimental period did not appear to have an effect on the survival of *E. coli* and *Ent. cloacae*, but it did appear to have a negative effect on the survival of *Ps. aeruginosa* and a positive effect on the survival of *Kl. pneumoniae* compared to the other conditions tested.

The initial sharp decrease in culturability of *Ps. aeruginosa* in mineral water with the bacterial flora in PVC, in sterile mineral water in glass, as well as the initial decrease of *Kl. pneumoniae* in sterile mineral water can perhaps be attributed to a requirement for a period of physiological adaptation to stress conditions (Morita 1982; Roszak and Colwell 1987; Matin *et al.* 1989) after which the mortality rate decreases.

The type of container did not influence the survival capacity of the enterobacteria tested, but *Ps. aeruginosa* had a lower mortality rate in water bottled in PVC that may reflect the capacity of this bacterium to colonize this type of material (Vess *et al.* 1993). It has also been shown that the colonization of surfaces enhances the capacity of bacteria to resist disinfection and starvation (Fletcher and Marshall 1982; LeChevallier *et al.* 1988; Geesey and White 1990). In this study *Ps. aeruginosa* also showed a high degree of survivorship in mineral water bottled in PVC.

For every organism examined the mortality rate was lower in tap than in mineral water and *Ps. aeruginosa* even grew in tap water. This difference can be due only to differences in the chemical composition or in the carbon content of the waters.

This study confirms EC regulations that mandate the microbiological quality control of mineral water immediately after bottling, since the absence of *E. coli* several days after bottling is not proof that faecal contamination has occurred during bottling. The need for the microbiological testing of coliforms in bottled mineral water is important because organisms such as *Ent. cloacae* and *Kl. pneumoniae* will survive for very long periods of time, and their presence indicates contamination with surface water, even in the absence of faecal pollution, and may therefore indicate the presence of pathogens in the mineral water.

In view of the multiplication and growth of some organisms, in particular *Ps. aeruginosa* in water, it is important to conduct more studies into the survival and growth of potential waterborne pathogens to ensure the high quality and safety of bottled still mineral waters.

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