

## Survival of human pathogenic bacteria in different types of natural mineral water

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### ABSTRACT

The aim of this study was to determine the survival of human pathogens (*Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*) in five natural mineral waters (NMWs) with different properties and mineralization levels. Five NMWs from four Spanish spas with different dry residue at 110 °C were used: A = 76,935 mg/L; B = 1,827 mg/L; C = 808.4 mg/L; D = 283.8 mg/L; and E = 170.4 mg/L. An initial inoculum of  $1 \times 10^6$  colony forming units (cfu)/mL was used for survival studies. Distilled water, chlorinated tap water and Mueller–Hinton broth were used as controls. Colony counts in all different waters were lower than those achieved with Mueller–Hinton broth over all incubation periods. A direct effect between the bacterial survival and the level of mineralization water was observed. The NMW E with low mineralization level along with the radioactive properties showed the highest antibacterial activity among all NMWs.

**Key words** | enzymatic activity, *Escherichia coli*, natural mineral water, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, survival

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### INTRODUCTION

Water has been used from time immemorial for remedial purposes. The contamination of water, and particularly drinking water, has been a medical concern since Hippocrates pointed out that water contributes significantly to the individual and collective health. The World Health Organization (WHO) has been concerned with health aspects of the management of water resources for many years and publishes various documents concerning the safety of the water environment and its importance for health. These include a number of normative 'guidelines' documents, such as the Guidelines for Drinking Water Quality and the Guidelines for Safe Recreational Water Environments (WHO 2006, 2008).

A variety of microorganisms can be found in swimming pools and similar recreational water environments, which may be introduced in a number of ways. Fecal and non-fecal human shedding (e.g. from vomit, mucus, saliva or skin) in the water is a potential source of pathogenic

organisms (Yoder *et al.* 2004). In some natural spas utilizing thermal and mineral water it may not be possible to treat the water in the usual way (i.e. by recycling or disinfection) because the agents believed to be of benefit, such as sulfides, carbonates or the microorganisms involved in geochemical processes, would be eliminated or impaired. However, few outbreaks linked to natural spring water have been described (Hubert *et al.* 1991; Willke *et al.* 2009). Although survival of pathogenic bacteria in natural mineral waters (NMWs) has been little studied, it has been described that *Legionella pneumophila* cell populations can potentially survive in hot spring water as free organisms for long periods by maintaining metabolic activity (Ohno *et al.* 2003).

The main concern of the public health authorities is to control the dissemination of pathogens into spas and their transmission to users. For this reason, the aim of the study was to determine the survival of typical human pathogenic

species (*S. aureus*, *E. coli*, and *P. aeruginosa*) in five NMWs with different mineral content and properties.

## METHODS

### Strains

Three American Type Culture Collection (ATCC) control strains (*S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853), and three clinical isolates (*S. aureus*, *E. coli*, and *P. aeruginosa*) from patients with skin and soft-tissue infections were used. The strains were stored at  $-70^{\circ}\text{C}$  in skimmed milk and were subcultured on blood agar plates 3 days before each experiment.

### Natural mineral waters

Characteristics of the five NMWs from four Spanish spas are shown in Table 1. A sterility test was performed in all waters to test heterotrophic plate count (HPC). Water samples were inoculated on Mueller–Hinton agar plates (Difco Laboratories, Detroit, MI, USA) and incubated for 24 h at  $37^{\circ}\text{C}$ .

### Survival curves

A bacterial suspension from overnight individual cultures of *S. aureus*, *P. aeruginosa* and *E. coli* strains were adjusted to achieve a density of  $10^8$  colony-forming units (cfu)/mL, as measured by a UV spectrophotometer (Hitachi U-1100). One milliliter of this suspension was introduced into flasks with 100 mL of the different tested waters. Distilled water, tap water (chlorine concentration of 0.2–0.4 ppm and dry

residue at  $110^{\circ}\text{C} = 90$  mg/L) and Mueller–Hinton broth were used as controls. All experiments showed an initial inoculum of approximately  $10^6$  cfu/mL. Flasks were incubated at  $28^{\circ}\text{C}$  (average temperature at which the NMW E from the Spa of Alange emerges). Samples were collected for colony counting at 0, 1, 2, 3, and 24 h. Samples were serially diluted in 0.9% sodium chloride and plated onto Mueller–Hinton agar, and further incubated at  $37^{\circ}\text{C}$  for 24 h prior to colony counting. The detection limit was 50 cfu/mL. All experiments were repeated five times, and the data were expressed as the mean for each sample.

### Statistical analysis

$\log_{10}$  reductions ( $\log_{10}$  colony counts at time 0 –  $\log_{10}$  colony counts at each sampling time) were calculated. Comparisons between  $\log_{10}$  reductions were performed by analysis of variance (ANOVA) with the Tukey's test for multiple comparisons.

## RESULTS

### Sterility test

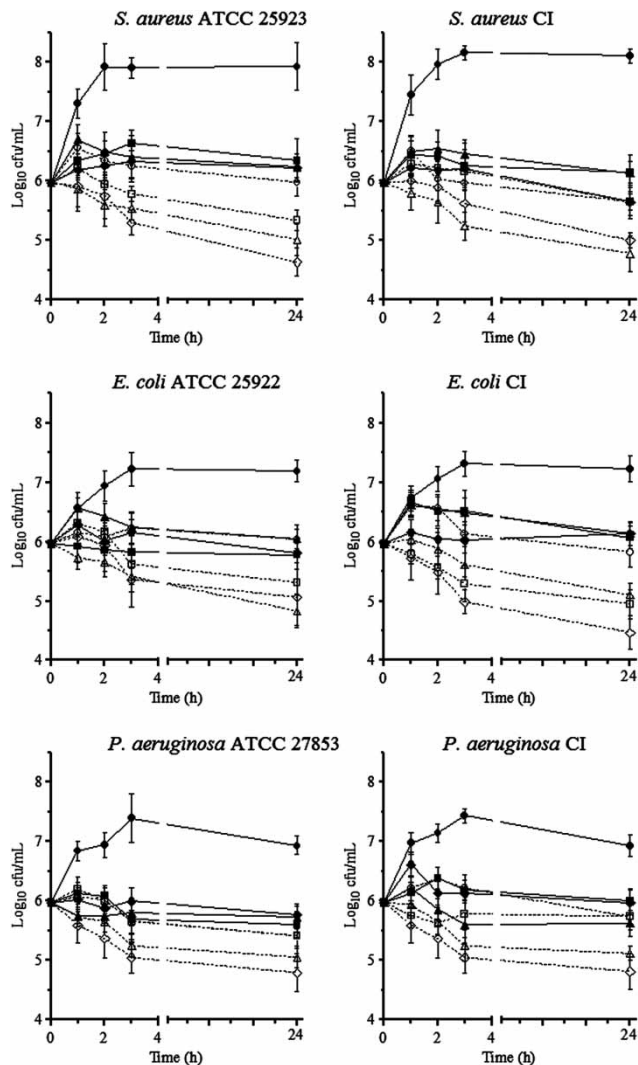
No viable counts were obtained in any NMW after 24 h incubation at  $37^{\circ}\text{C}$ .

### Survival curves

Figure 1 shows bacterial colony counts over 24 h obtained with the waters. Colony counts in all different waters were lower than those achieved with Mueller–Hinton broth over

**Table 1** | Characteristics of the natural mineral waters (NMWs) used in this study

NMW	Mineralization level	Predominant ions	Spa/city	Dry residue at $110^{\circ}\text{C}$ (mg/L)
A	High	Sulfate, sodium, and sulfurous	Carabaña/Madrid	76,935
B	High	Bicarbonate, sodium, and carbogaseous	Troncoso spring (Mondariz)/ Pontevedra	1,827
C	Medium	Bicarbonate, chloride, sodium, and carbogaseous	Gándara spring (Mondariz)/ Pontevedra	808.4
D	Low	Bicarbonate, calcium, and magnesium	Solán de Cabras/Cuenca	285.8
E	Low	Chloride, bicarbonate, sodium, and calcium	Alange/Badajoz	170.4



**Figure 1** | Mean  $\pm$  standard deviation bacterial counts ( $\log_{10}$  colony-forming units (cfu)/mL) over 24 h in curves with Mueller–Hinton broth (continuous lines with filled circles), NMW A (continuous lines with filled squares), NMW B (continuous lines with filled triangles), NMW C (continuous lines with filled rhombus), NMW D (dotted lines with open circles), NMW E (dotted lines with open squares), distilled water (dotted lines with open triangles), and tap water (dotted lines with open rhombus).

the whole incubation period and all microorganisms. In four NMWs (A, B, C, and D), colony counts similar to the initial inoculum were observed up to 3 h with a later growth inhibition. However, NMW E, distilled, and tap water showed a reduction in cell numbers at 3 h in five of six microorganisms (from 0.19 to 0.68  $\log_{10}$  cfu/mL), six of six microorganisms (from 0.36 to 0.68  $\log_{10}$  cfu/mL), and six of six microorganisms (from 0.60 to 0.98  $\log_{10}$  cfu/mL), respectively. Significant differences after 2 h were observed

between all NMWs, distilled and tap waters against Mueller–Hinton broth.

Table 2 shows  $\log_{10}$  cfu/mL reduction in cell numbers at 24 h for the different waters. Distilled and tap waters showed a greater reduction in cell numbers at 24 h in all microorganisms with a mean reduction of 0.99  $\log_{10}$  cfu/mL and 1.19  $\log_{10}$  cfu/mL, respectively. No statistically significant differences were observed between distilled and tap water. NMW E was the only spring water which showed a reduction in cell numbers at 24 h in all microorganisms with a mean reduction of 0.57  $\log_{10}$  cfu/mL. The other low mineralization water (NMW D) showed a reduction in cell numbers at 24 h in four of six microorganisms with a mean reduction of 0.20  $\log_{10}$  cfu/mL. The high mineralization waters (A and B) and the medium mineralization water C showed reductions at 24 h in two, three, and four of six microorganisms, respectively, with a mean reductions of  $-0.01$ ,  $-0.01$ , and 0.20  $\log_{10}$  cfu/mL, respectively. Reductions in cell numbers at 24 h in the low mineralization water E were significantly less ( $p < 0.001$ ) than those observed in tap and distilled waters for two of six microorganisms. However, in the rest of the NMWs, reductions at 24 h were significantly less than those observed in tap and distilled waters for all microorganisms except NMW D against *P. aeruginosa* ATCC 27853.

## DISCUSSION

Composition, properties, and indications of the NMWs are generally well known. However, the ability of bacteria to survive in NMWs, especially pathogenic bacteria, has been little studied. A great variety of human pathogens can contaminate thermal and mineral pools but few outbreaks linked to natural spring water have been described (Hubert et al. 1991; Willke et al. 2009). Limited transmission and spread of microbial infections in spring waters could be attributed to the inability of human pathogenic bacteria to survive in these environments. The present work is a study of the survival of clinical isolates in NMWs with different properties and mineralization levels. No colonies were recovered from any NMWs in the sterility tests (HPC) at 37 °C. The incubation time is the most important factor for isolating bacteria from mineral water (Leclerc & Moreau 2002).

**Table 2** | Reductions in cell numbers ( $\log_{10}$  cfu/mL  $\pm$  DS) at 24 h for the different waters and Mueller–Hinton broth

	<i>S. aureus</i> ATCC 25923	<i>S. aureus</i> clinical isolate	<i>E. coli</i> ATCC 25922	<i>E. coli</i> clinical isolate	<i>P. aeruginosa</i> ATCC 27853	<i>P. aeruginosa</i> clinical isolate
Mueller–Hinton broth	<b>-1.96 <math>\pm</math> 0.40<sup>d,e</sup></b>	<b>-2.14 <math>\pm</math> 0.11<sup>d,e</sup></b>	<b>-1.22 <math>\pm</math> 0.18<sup>d,e</sup></b>	<b>-1.26 <math>\pm</math> 0.22<sup>d,e</sup></b>	<b>-0.96 <math>\pm</math> 0.15<sup>d,e</sup></b>	<b>-0.96 <math>\pm</math> 0.17<sup>d,e</sup></b>
NMW A <sup>a</sup>	<b>-0.38 <math>\pm</math> 0.37<sup>d,e</sup></b>	<b>-0.18 <math>\pm</math> 0.17<sup>d,e</sup></b>	0.19 $\pm$ 0.24 <sup>d,e</sup>	<b>-0.10 <math>\pm</math> 0.19<sup>d,e</sup></b>	0.37 $\pm$ 0.22 <sup>d,e</sup>	0.03 $\pm$ 0.19 <sup>d,e</sup>
NMW B <sup>a</sup>	<b>-0.26 <math>\pm</math> 0.23<sup>d,e</sup></b>	<b>-0.16 <math>\pm</math> 0.30<sup>d,e</sup></b>	<b>-0.08 <math>\pm</math> 0.22<sup>d,e</sup></b>	<b>-0.17 <math>\pm</math> 0.20<sup>d,e</sup></b>	0.25 $\pm$ 0.19 <sup>d,e</sup>	0.35 $\pm$ 0.21 <sup>d,e</sup>
NMW C <sup>b</sup>	<b>-0.25 <math>\pm</math> 0.17<sup>d,e</sup></b>	0.30 $\pm$ 0.23 <sup>d,e</sup>	0.16 $\pm$ 0.16 <sup>d,e</sup>	<b>-0.16 <math>\pm</math> 0.18<sup>d,e</sup></b>	0.20 $\pm$ 0.18 <sup>d,e</sup>	0.01 $\pm$ 0.20 <sup>d,e</sup>
NMW D <sup>c</sup>	<b>-0.01 <math>\pm</math> 0.23<sup>d,e</sup></b>	0.31 $\pm$ 0.28 <sup>d,e</sup>	<b>-0.07 <math>\pm</math> 0.16<sup>d,e</sup></b>	0.14 $\pm$ 0.26 <sup>d,e</sup>	0.56 $\pm$ 0.21	0.24 $\pm$ 0.21 <sup>d,e</sup>
NMW E <sup>c</sup>	0.63 $\pm$ 0.16	0.32 $\pm$ 0.13 <sup>d,e</sup>	0.66 $\pm$ 0.21	1.02 $\pm$ 0.24	0.56 $\pm$ 0.20	0.23 $\pm$ 0.22 <sup>d,e</sup>
Distilled water	0.96 $\pm$ 0.26	1.19 $\pm$ 0.29	1.14 $\pm$ 0.28	0.87 $\pm$ 0.20	0.92 $\pm$ 0.17	0.86 $\pm$ 0.12
Tap water	1.33 $\pm$ 0.24	1.09 $\pm$ 0.39	0.90 $\pm$ 0.47	1.50 $\pm$ 0.27	1.18 $\pm$ 0.32	1.16 $\pm$ 0.29

Positive values of the log reduction represent killing, and negative values (given in bold) correspond to regrowth with respect to initial inoculum.

<sup>a</sup>High mineralization water.

<sup>b</sup>Medium mineralization water.

<sup>c</sup>Low mineralization water.

<sup>d</sup> $p < 0.001$  vs. distilled water.

<sup>e</sup> $p < 0.001$  vs. tap water.

An incubation of cultures for 14 days at 20 °C has frequently been used because many of these organisms are slow growing (Leclerc & Moreau 2002). In this study, we used an incubation of 24 h at 28 °C (average temperature at which the NMW from the Spa of Alange emerges) and an incubation of 24 h at 37 °C for colony counting. Thus, the effect of NMW on the clinical isolates can be observed avoiding a possible masking with slow growth bacteria from NMW water.

Fluctuation of external osmolarity is one of the most common types of environmental stress factors for all kind of cells, both of prokaryotic and of eukaryotic origin (Morbach & Krämer 2002). In our study, survival of pathogenic bacteria in all NMWs and distilled or treated water showed a similar behavior although significant differences among them were observed. Colony counts in all different waters were lower than those achieved in Mueller–Hinton broth for all microorganisms at all time points, showing statistically significant differences after 2 h. However, not all microorganisms were affected in the same way. In the case of *S. aureus*, a Gram-positive halophilic bacterium, it has been described that sodium chloride provides a favorable growth medium (Lo Nostro et al. 2005). In our study, a slight regrowth at 24 h in six of eight curves in NMWs with a greater mineralization level than 170.4 mg/L was observed. In the case

of *P. aeruginosa*, all waters (ions) adversely affected the bacterial survival.

In our study, a direct effect between the bacterial survival and the level of mineralization water seems to be observed. Thus, low levels of mineralization were associated with lower bacterial survival at 24 h (a greater reduction in cell numbers). Distilled and tap waters (dry residue at 110 °C = 0 and 90 mg/L, respectively) showed the highest reduction in cell numbers at 24 h in all microorganisms with values from 0.8 to 1.5  $\log_{10}$  cfu/mL. The low mineralization water E (dry residue at 110 °C = 170.4 mg/L) was the only NMW showing a reduction in cell numbers at 24 h in all microorganisms with colony counts similar to those showed by distilled and tap water. The other low mineralization water D (dry residue at 110 °C = 238.8 mg/L) showed a reduction in cell numbers for four of six strains. When the level of mineralization of NMW increased (A, B, and C water), lower reductions in cell numbers at 24 h were observed. It should be noted, however, that a longer incubation period of study would probably show greater reductions in cell numbers as has previously been observed in *Aeromonas hydrophila* (Pianetti et al. 2008).

In spite of the influence of mineralization level, other factors could affect the bacterial survival in water. The mineral content present in tap water is offset by specific

chemical compounds added to this water during the treatment process which act negatively towards bacterial survival. It is not surprising that the reduction in viable counts was greater in tap vs. distilled water, although no significant differences were observed. NMW A and B showed very similar reductions with a great difference in the dry residue at 110 °C (76,935 vs. 1,827 mg/L, respectively). However, NMW D and E with a similar dry residue at 110 °C (238.8 vs. 170.4 mg/L) showed significant differences in the bacterial survival at 24 h in three of six microorganisms.

The lack of clear correlation between bacterial survival and dry residue in the two previous cases could be due to the following: (i) a baseline amount of minerals (i.e. dry residue of NMW B = 1,827 mg/L) could be needed to maintain the bacterial metabolism; (ii) high proportions of some ions in NMW could be toxic for bacterial cells inhibiting the growth; and (iii) traces of Radon-222 present in water could contribute to bacterial killing. Among all NMWs tested, only NMW E with Radon-222 levels of 322 Bq/L can be considered radioactive according to Spanish regulations (Radon-222 levels higher to 67.3 Bq/L) (Maraver & Armijo 2010). Although the mutagenic effects of heavy ions in bacteria have been previously described (Horneck et al. 1994), the role of radon remains unclear. More studies are needed to evaluate the effect of radon on bacterial survival. Radon therapy enhances the antioxidant functions (superoxide dismutase and catalase) and immune suppression in humans (Yamaoka et al. 2004). Because of its good liposolubility, it has been hypothesized that radon could bind to lipids of cellular membrane and dampen the hyperexcitability with an antispasmodic, analgesic and normalizing effect in humans (Armijo 1968). In the same way, radon could bind to lipids of bacterial membrane affecting their growth. No evidence of pathogen contamination shown in the Spa of Alange, as we have confirmed over the years, could be explained by the inhibitory effect of this radioactive water on human pathogenic bacteria.

## CONCLUSIONS

A direct effect between the bacterial survival and the level of mineralization water was observed. The NMW

E with low mineralization level along with the radioactive properties showed the highest antibacterial activity.

## ACKNOWLEDGEMENTS

The authors are grateful to Professor Josefina San Martin Bacaicoa for her generous support and knowledge.

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First received 23 December 2011; accepted in revised form 17 July 2012. Available online 14 August 2012