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Heterotrophic plate counts and the isolation of bacteria from mineral waters on selective and enrichment media

CÉLIA M. MANAIA, OLGA C. NUNES, PAULA V. MORAIS & M.S. DA COSTA* *Centro de Biologia Celular, Departamento de Zoologia and Instituto de Investigação da Água, Universidade de Coimbra, 3049 Coimbra Codex, Portugal*

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The heterotrophic plate counts of 15 brands of bottled non-carbonated mineral waters were examined and found to be generally high and variable. Four selective or enrichment media for the enumeration of coliforms (m-Endo LES and m-lauryl sulphate agar) and *Pseudomonas aeruginosa* (cetrimide-nalidixic acid agar and malachite green broth) were used to isolate several species of Gram-negative bacteria. Strains identified as CDC gr IVc-2 and *Comamonas* (*Ps.*) *acidovorans* were the two most commonly isolated. Considerable variation in populations was seen between the brands, as well as between two batches of the same mineral water.

Bottled non-carbonated mineral waters generally have high heterotrophic plate counts (HPC), since the elimination of micro-organisms by disinfection or sterilization is not permitted (Anon. 1980). Limits for the HPC at 22°C and 37°C apply only to bottles stored at 4°C for 12 hours after bottling. There are strict standards, however, that prohibit the sale of mineral waters containing coliforms, faecal coliforms, faecal streptococci, sulphide-reducing clostridial sporeformers, *Pseudomonas aeruginosa*, and pathogens (Anon. 1980).

The bacterial populations of mineral waters are diverse, and brand-dependent. These populations often include species of *Pseudomonas*, *Achromobacter*, *Alcaligenes*, *Acinetobacter*, *Flavobacterium*, *Aeromonas*, *Caulobacter*, *Arthrobacter*, *Corynebacterium* and sometimes iron bacteria (Gonzalez *et al.* 1987). Most studies indicate, but do not conclusively show that the bacteria in bottled mineral waters are derived from strains present in the source water in low numbers, and which grow in the bottles (Schmidt-Lorenz 1976).

* Corresponding author.

There is concern that the growth of HPC bacteria could interfere with the detection of small numbers of faecal indicators (Geldreich *et al.* 1975), which may also occasionally produce atypical colonies on isolation media. Non-pigmented *Ps. aeruginosa* strains have also been found by special investigations (Havelaar *et al.* 1985). These would have been missed by routine examination techniques. It has also been noted that some quality control laboratories incubate cultures for the enumeration of coliforms or faecal coliforms for 48 h, instead of the 'standard' 24 h, in an attempt to recover stressed organisms. This has resulted in false-positive 'presumptive coliforms' and temporary refusal of permission for sale of batches.

In this study the heterotrophic plate counts of 15 brands of bottled mineral water were compared. Random colonies from m-Endo LES and m-Lauryl sulphate agar (m-LSA) used for enumeration of coliforms in drinking water (Anon. 1982, 1985) and centrimide-nalidixic acid agar (CNA) and malachite green enrichment broth (MGB) used for the enumeration of *Ps. aeruginosa* (Shubert & Blum 1974; Cian & Devoucoux 1981) were isolated to assess the occurrence of

atypical colonies, as well as to identify other mineral water bacteria capable of growth on these media.

Methods and Materials

SAMPLES

Fifteen brands of non-carbonated mineral waters were examined (Table 1). Thirteen were purchased at retail outlets in Portugal, France (F, O, P) and Belgium (L). Mineral water D was packaged in 0·8 l returnable glass bottles, water N was packaged in 1 l Tetrabrik containers, and the remainder in polyvinylchloride (PVC) 1·5 l bottles. Two brands, B and J, had been removed from sale because they contained *Ps. aeruginosa*. Where possible, two batches were examined at intervals of 2–4 months. Each sample consisted of three bottles with the same expiry date or bottling code on the label.

ENUMERATION, ISOLATION AND IDENTIFICATION OF STRAINS

Heterotrophic plate counts (HPC) of water samples and decimal dilutions thereof were made in duplicate by the pour plate method on plate count agar (PCA) at 37°C for 48 h, and at 22°C for 72 h (Anon. 1982; Anon. 1985). Samples, 25 ml and 250 ml, were also filtered in duplicate through Gelman type GN-6 (0.45 μm pore size, 47 mm diameter) membrane filters, washed with phosphate dilution water (Anon. 1985) and placed on m-Endo LES (Difco), m-LSA and CNA (Institute Pasteur) media, and into 50 ml of MGB in 100 ml wide-mouth screw-capped bottles. The plates and bottles were incubated for up to 48 h at 37°C. Several colonies with different morphologies were taken from each filter, or in the case of MGB, after transferring a loopful of turbid cultures to PCA and incubating at 37°C for 24 h. After purification, the bacteria were stored at −80°C in Nutrient Broth (Difco) containing 15% glycerol.

All strains were initially screened by Gram stain, cytochrome oxidase test (Gerhardt *et al.* 1981), and identified with the API 20NE Test System for non-fermenters or the API 20E for fermentative bacteria (API System, S.A., La Balme les Grottes, France) according to manufacturers' instructions. Additional tests included flagella staining (Kodaka *et al.* 1982), pigment

Table 1. Heterotrophic plate counts (HPC) at 22°C and 37°C of bottled non-carbonated mineral water.

		Heterotrophic plate count (cfu/ml)*	
Brand	Lot	22°C	37°C
A	1	$1{\cdot}5 \times 10^4 \pm 3{\cdot}4 \times 10^3$	$4{\cdot}9 \times 10^3 \pm 4{\cdot}9 \times 10^3$
	2	$1{\cdot}9 \times 10^3 \pm 8{\cdot}5 \times 10^2$	$1{\cdot}6 \times 10^2 \pm 8{\cdot}4 \times 10^1$
B	1	$3{\cdot}4 \times 10^3 \pm 1{\cdot}2 \times 10^3$	$8{\cdot}3 \times 10^2 \pm 8{\cdot}9 \times 10^2$
C	1	$7{\cdot}8 \times 10^3 \pm 2{\cdot}7 \times 10^3$	$5{\cdot}5 \times 10^1 \pm 5{\cdot}0 \times 10^1$
	2	$3{\cdot}9 \times 10^2 \pm 4{\cdot}8 \times 10^2$	$7{\cdot}4 \times 10^1 \pm 4{\cdot}0 \times 10^1$
D	1	0	0
	2	0	0
F	1	$6{\cdot}7 \times 10^1 \pm 3{\cdot}0 \times 10^1$	N.D.
F	1	$5{\cdot}3 \times 10^3 \pm 4{\cdot}7 \times 10^3$	$5{\cdot}2 \times 10^2 \pm 1{\cdot}7 \times 10^2$
G	1	$2{\cdot}0 \times 10^4 \pm 3{\cdot}1 \times 10^3$	$8{\cdot}8 \times 10^3 \pm 1{\cdot}5 \times 10^3$
	2	$8{\cdot}3 \times 10^3 \pm 1{\cdot}9 \times 10^3$	$1{\cdot}2 \times 10^3 \pm 6{\cdot}2 \times 10^2$
H	1	$1{\cdot}4 \times 10^4 \pm 6{\cdot}1 \times 10^3$	$5{\cdot}7 \times 10^3 \pm 4{\cdot}0 \times 10^3$
	2	$2{\cdot}5 \times 10^4 \pm 9{\cdot}0 \times 10^3$	$1{\cdot}1 \times 10^4 \pm 3{\cdot}1 \times 10^3$
I	1	$9{\cdot}1 \times 10^3 \pm 5{\cdot}9 \times 10^3$	$1{\cdot}4 \times 10^3 \pm 8{\cdot}0 \times 10^2$
	2	$1{\cdot}4 \times 10^3 \pm 1{\cdot}0 \times 10^2$	$6{\cdot}9 \times 10^3 \pm 1{\cdot}1 \times 10^3$
J	1	$1{\cdot}9 \times 10^4 \pm 3{\cdot}4 \times 10^3$	$6{\cdot}6 \times 10^3 \pm 7{\cdot}4 \times 10^3$
L	1	$8{\cdot}0 \pm 0{\cdot}0$	$1{\cdot}2 \times 10^1 \pm 4{\cdot}9$
M	1	$1{\cdot}1 \times 10^4 \pm 7{\cdot}4 \times 10^3$	$1{\cdot}0 \times 10^4 \pm 9{\cdot}2 \times 10^3$
	2	$2{\cdot}6 \times 10^4 \pm 1{\cdot}9 \times 10^4$	$5{\cdot}4 \times 10^4 \pm 5{\cdot}3 \times 10^3$
N	1	0	0
	2	0	0
O	1	$5{\cdot}1 \times 10^3 \pm 7{\cdot}4 \times 10^2$	$1{\cdot}1 \times 10^3 \pm 1{\cdot}2 \times 10^2$
P	1	$3{\cdot}6 \times 10^4 \pm 9{\cdot}9 \times 10^3$	$2{\cdot}3 \times 10^4 \pm 1{\cdot}6 \times 10^4$

* Values are the mean of 3 bottles ± standard deviation, N.D. not determined.

production on Pseudo P and Pseudo F media (Difco) after incubation at 22°C for 10 d, growth in nutrient broth at 41·5°C for 24 h, lecithinase production, and growth in 6·5% NaCl (Gilardi 1971), hydrolysis of Tween 80, acetamide hydrolysis, and the presence of poly-β-hydroxybutyrate inclusions (Gerhardt *et al.* 1981) after growth in Palleroni & Doudoroff (1972) medium. Reference strains for these tests, obtained from the American Type Culture Collection (Rockville, Md. USA), were *Pseudomonas aeruginosa* (ATCC 10145[t]), *Ps. cepacia* (ATCC 25416[t]), *Ps. fluorescens* (ATCC 13525[t]), *Ps. maltophilia* (ATCC 13637[t]), *Acinetobacter calcoaceticus* (ATCC 23055[t], ATCC 9957) and *Aeromonas hydrophila* (ATCC 7965).

Results

HETEROTROPHIC PLATE COUNTS OF BOTTLED NON-CARBONATED MINERAL WATERS

The non-carbonated mineral waters showed a wide variation in heterotrophic plate counts (Table 1). Two waters, one bottled in glass (D) and the other in Tetabrik containers (N) exhibited no recoverable heterotrophic bacteria in both batches tested; all other brands gave HPC ranging from $0{\cdot}8 \times 10^1$ to $3{\cdot}6 \times 10^4$ colony forming units (cfu)/ml at 22°C and $1{\cdot}1 \times 10^1$ to $5{\cdot}4 \times 10^4$ cfu/ml at 37°C. In most cases there were large differences in the heterotrophic plate counts between replicates of each batch, as well as between batches of the same brand (Table 1).

IDENTIFICATION OF STRAINS FROM SELECTIVE AND ENRICHMENT MEDIA

The 317 strains isolated from the selective or enrichment media were all Gram-negative and with the exception of two strains, all were oxidase positive (Table 2). No bacteria were isolated from brand F irrespective of the selective or enrichment medium used.

Most strains were isolated on several media, although some organisms were consistently isolated from one medium alone: *Comamonas* (*Ps.*) *testosteroni* was isolated on m-Endo LES, *F. meningosepticum* and *Ps. paucimobilis* on CNA, and *Ps. alcaligenes* and *Acinetobacter* spp. on m-LSA. Typical coliform colonies were not detected on m-Endo LES or m-LSA. One atypical strain was isolated from m-LSA and identified as *Enterobacter agglomerans*.

The most abundant bacteria were found in both batches of each brand (Table 3). The most frequently isolated organisms were Centers for Disease Control (CDC) group IVc-2 strains and *Comamonas* (*Ps.*) *acidovorans*. Some strains were isolated only once; *Aeromonas hydrophila*, *E.*

Table 2. Isolation of strains from bottled mineral waters on the four selective or enrichment media

Strains	No. of strains				
	CNA*	MGB	m-Endo LES	m-LSA	Total
Acinetobacter sp.	0	0	0	3	3
Aeromonas hydrophila	0	0	1	0	1
Alcaligenes denitrificans subsp. *dentrificans*	1	2	9	15	27
dentrificans subsp. *xylosoxidans*	0	1	3	2	6
CDC gr IV c-2	0	7	9	42	58
Comamonas (*Ps.*) *acidovorans*	0	3	23	9	35
testosteroni	0	0	8	0	8
Enterobacter agglomerans	0	0	0	1	1
Flavobacterium meningosepticum	2	0	0	0	2
Hafnia alvei	0	1	0	0	1
Pseudomonas aeruginosa	10	6	15	14	45
alcaligenes	0	0	0	11	11
cepacia	1	1	1	1	4
fluorescens	15	2	1	7	25
maltophilia	0	0	5	2	7
paucimobilis	3	0	0	0	3
pickettii	3	0	9	13	25
putida	2	0	4	5	11
stutzeri	2	5	9	8	24
Not identified	0	0	0	4	4

* CNA, cetrimide-nalidixic acid agar; MGB, malachite green broth; m-LSA, membrane-lauryl sulphate agar.

Table 3. Distribution of strains (% per batch) isolated on selective and enrichment media from 12 bottled mineral waters

	Bottled mineral water brands																	
	A		B	C		E	G		H		I		J	L	M		O	P
	Batch																	
	1	2	1	1	2	1	1	2	1	2	1	2	1	1	1	2	1	1
Acinetobacter sp.	—	—	—	—	—	—	—	—	—	—	—	—	6·7	—	—	—	—	—
Aeromonas hydrophila	—	—	—	—	—	—	—	—	—	—	3·1	—	—	—	—	—	—	—
Alcaligenes denitrificans																		
subsp. *dentrificans*	15·8	—	—	—	9·1	—	28·6	84·6	8·7	—	—	4·0	—	—	—	6·7	—	—
supsp. *xylosoxidans*	—	—	—	—	—	—	—	—	—	—	—	24·0	—	—	—	—	—	—
CDC gr. IV c-2	10·5	68·8	—	30.8	90.9	—	25·0	—	60·9	40·0	—	—	—	—	—	—	—	—
Comamonas (Ps.) acidovorans	31·6	25·0	—	—	—	—	—	15·4	8·7	—	—	12·0	—	—	100·0	60·0	—	—
testosteroni	5·3	—	—	—	—	—	—	—	—	8·0	—	—	11·1	—	—	—	—	—
Enterobacter agglomerans	5·3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Flavobacterium meningosepticum	—	—	—	15·4	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Hafnia alvei	—	—	—	—	—	—	3·6	—	—	—	—	—	—	—	—	—	—	—
Pseudomonas aeruginosa	—	—	59.3	—	—	—	—	—	—	—	—	—	64·4	—	—	—	—	—
alcaligenes	—	—	—	—	—	—	—	—	—	—	—	12·0	6·7	—	—	33·3	75·0	—
cepacia	15·8	—	—	—	—	—	—	—	—	—	—	4·0	—	—	—	—	—	—
fluorescens	—	6·3	—	—	—	—	—	—	—	—	34·4	20·0	4·4	—	—	—	—	100·0
maltophilia	—	—	—	—	—	—	3·6	—	—	4·0	—	8·0	6·7	—	—	—	—	—
paucimobilis	—	—	—	—	—	100·0	—	—	—	—	—	—	—	—	—	—	—	—
picketti	—	—	—	53·8	—	—	39·3	—	21·7	36·0	—	—	—	100·0	—	—	—	—
putida	—	—	29·6	—	—	—	—	—	—	12·0	—	—	—	—	—	—	—	—
stutzeri	—	—	11·1	—	—	—	—	—	—	—	62·5	16·0	—	—	—	—	—	—
Not identified	15·8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	25·0	—

agglomerans, *Hafnia alvei* and *F. meningosepticum*.

Usually, several species were found in each brand of water, but *Ps. paucimobilis*, *Ps. pickettii* and *Ps. fluorescens* were the only bacteria isolated from brands E, L and P.

The two brands removed from sale because of the presence of *Ps. aeruginosa* also contained other bacteria. All *Ps. aeruginosa* strains produced pyocyanin and pyoverdin, easily recognized on CNA because of the bluish-green pigment; MGB, on the other hand, gave high proportions of false positive presumptive results (73% of the samples) based on turbidity of cultures rather than on pigmentation.

Discussion

The bottled non-carbonated mineral waters examined in this study gave HPCs comparable to those reported in other studies (Warburton *et al.* 1986; Gonzalez *et al.* 1987; Oger *et al.* 1987). High numbers of heterotrophic bacteria in non-carbonated mineral waters are considered to be the result of a natural biological process resulting primarily from the multiplication of those bacteria that were present in low numbers in the source water, although sometimes contamination within the bottling plant is considered to be responsible. The absence of recoverable heterotrophic bacteria may suggest the addition of bactericidal agents, such as silver ions. This may explain our failure to recover bacteria from brands D and N.

Previous studies have shown that media for the enumeration of coliforms also recover minor components of the heterotrophic flora which are not recovered in the HPC tests because of the large numbers of other common bacteria (Duquino & Rosenberg 1987). In the present study, four media for the enumeration of coliform bacteria and *Ps. aeruginosa* facilitated the isolation of several bacterial species, primarily pseudomonads. Most of the organisms we isolated have been found previously in other studies on bottled mineral waters, or at their sources. In some cases, species such as *Ps. fluorescens*, *Ps. stutzeri*, *Ps. alcaligenes* and *Ps. maltophilia* are the major components (Quevedo-Sarmiento *et al.* 1986). Others, such as CDC group IVc-2, have not so far been reported in bottled mineral waters. These strains are similar to strains of *Bordetella bronchiseptica* and share several diagnostic biochemical characteristics, but appear to be unrelated (Oberhofer 1985; Pickett & Greenwood 1986; Weaver *et al.* 1983).

The isolation of the same bacterium from two batches of a brand suggests that some species are consistently present for several months in the source or in the bottling plant. Other species were found only in one batch, suggestive of contamination during bottling, but some of these were found in low numbers and may have been missed in the other batch tested. *Enterobacter agglomerans* was isolated from a random selection of colonies on m-LSA. This would make the water unsuitable for consumption according to the EC directive (Anon. 1980) but failure to recover it in the routine tests for coliforms would not alert suppliers to possible contamination. Previous studies have occasionally reported coliforms (Warburton *et al.* 1986) and *Ps. aeruginosa* (Rivilla & Gonzalez 1988) in small numbers in bottled waters.

The isolation of *Ps. aeruginosa* in two brands was expected as it was known that these brands had been removed from sale for that reason. Nonetheless their presence, as well as the presence of coliforms in low numbers in some bottled mineral waters, reinforces the value for continued monitoring of mineral waters for evidence of contamination at the source or during the bottling process.

Current legislation is adequate for the examination of indicator bacteria and *Ps. aeruginosa* in bottled mineral water (Anon. 1980). The media utilized for the examination of the microbiological quality varies, and some may not be adequate, especially if the heterotrophic bacteria are numerous. In this study malachite green broth gave a high percentage of false-positive presumptive tests. These would have to be confirmed during routine work, leading to added costs.

The *Ps. aeruginosa* strains isolated in this study were all pigmented and easily recognized on CNA. Non-pigmented strains have been isolated (Havelaar *et al.* 1985) and can be missed. Care must be taken to confirm the strains which grow on selective media but lack pigmentation.

Selective or enrichment media may aid the isolation of minor population components which cannot be detected because of large numbers of other heterotrophic bacteria in non-carbonated mineral waters.

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