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Isolation, Identification and Antibiotic Resistance Patterns of Enterococci from Drinking Water in Canton Ticino

Key words: Enterococci, Isolation, Antibiotic resistance, Vancomycin, Drinking water

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Introduction

The genera *Streptococcus* and *Enterococcus* both consist of Gram-positive, spherical or ovoid cells, which are typically arranged in pairs or chains. They are non-sporing, facultatively anaerobic, catalase negative, homofermentative and have complex nutritional requirements. Since the transfer of *S.faecalis* and *S.faecium* to the revised genus *Enterococcus* (1), the total number of species included here is seventeen. The species identification using standardised commercial biochemical test kits that include a combination of rapid preformed enzyme detection tests with more traditional biochemical tests has been proven to be reliable (2).

Enterococci are widely distributed, mainly on mucosal surfaces of man and animals, including the gastrointestinal tract, but some are also found in water, soil, dairy products (3–6) and other foods (7, 8), and on plants.

The involvement of enterococci in human clinical infections has been thoroughly reviewed recently by *Murray* (9). The most common infection are those involving the urinary tract, in which enterococci are implicated in about 10% of all cases (10). Enterococci, especially *E.faecalis*, are the major cause for a significant proportion (5–20%) of cases of bacterial endocarditis (11).

One of the main problems of medicine in the nineties is the emergence of bacterial strains showing antibiotic resistance. This is due to the presence of an enormous number and variety of genes encoding resistance which are present in the bacterial genomes and which can be horizontally transferred or clonally disseminated even to taxonomically distant species (4). Because of the acquisition of resistance to the glycopeptide vancomycin by strains already multi-drug resistant, enterococci belong nowadays to the most alert pathogens responsible for nosocomial infections. This renders the infection caused by such strains untreatable. All

enterococci exhibit relative resistance to ß-lactam antibiotics due to decreased affinity of penicillin-binding proteins (PBPs) and to the presence of β-lactamase. Most *E.faecium* strains have an increased intrinsic resistance to penicillin and ampicillin, which renders them clinically resistant. Enterococci also show low levels of intrinsic activity to aminoglycosides, which are therefore unsuitable as monotherapy-drug to cure enterococcal infections. Combined with a cell-wall active drug (e.g. penicillin, ampicillin, piperacillin, vancomycin or teicoplanin) a synergistic, bactericidal effect is however observed. Therefore the standard therapy for severe, life-threatening enterococcal infection consists of a combination of ampicillin and gentamycin. However, *E.faecalis* and *E.faecium* expressing high-level resistance to the aminoglycosides gentamycin and streptomycin (HLAR) which eliminates the synergy seen with cell-wall acting antibiotics, are being isolated with increased frequency world-wide. The emergence of resistance to β-lactams and high-level of aminoglycosides has limited the choice of treatment to the glycopeptides vancomycin or teicoplanin. Vancomycin-Resistant Enterococci (VRE), first reported in Europe in 1988, are however emerging as a global threat to public health (12). VRE infections have increased very rapidly among hospitalised patient (13), but colonisation of patients outside health-care setting also appears to occur frequently (14). An important factor associated with VRE in Europe has been avoparcin, a glycopeptide antibiotic used to fatten farm animals such as poultry, pigs, cattle and calves. The German Government passed a national regulation in 1996 with the ban of avoparcin, while in 1997 the European Community, prohibiting the use of this feeding antibiotic, adopted a similar regulation. The use of other drugs like apramycin, other aminoglycosides, tetracyclines, β-lactams and quinolones in animal farming and veterinary medicine in sub-therapeutic concentrations have however been shown to create additional selection of resistant mutants (15).

Although there is a great deal of information available on the antibiotic susceptibility of enterococci isolated from clinical sources (16), such characteristics have not yet been thoroughly investigated – with some exception (3, 4) – in enterococci isolated from foods.

The present study was therefore undertaken to investigate the resistance to vancomycin and other antibiotics important in human therapy as well as in veterinary medicine of enterococci isolated from water, in a Swiss region where water is in 90% of the cases not treated at all.

Experimental

Isolation of enterococci

Of the 3110 samples of drinking water brought in by inspectors or sent by local authorities during 1997 to the Cantonal Laboratory, 2121 have been investigated from the microbiological point of view. In accordance with the Swiss legislation

(17) they have been tested, among other parameters, for the presence of enterococci and *E.coli.*

For counting and isolating enterococci, one hundred millilitres of water were filtered through a 0.45 µg sterile 47 mm cellulose acetate membrane (Millipore EZ-Pack MSP000813). The membrane was then placed directly on the surface of m-Enterococcus Agar (m-Ent, Difco 0746-17-0), inoculum side up. After incubation at 37 °C±1 °C for 48 h, the membrane was then transferred onto Bile Esculine Agar (BEA, Biolife 401014) for confirmation according to the method proposed by *Figueras* et al. (18). The following modifications were adopted: incubation for esculine hydrolysis was carried out at 42 °C±1 °C during 4 hours. A representative number of colonies, appearing as pink to dark maroon, from 0,5–3 mm in diameter after incubation onto m-Enterococcus and which turned brown with brown halo onto Bile Esculine Agar, were subcultured on Tryptic Soy Agar (TSA, Biolife 4021502) for purification. After incubation at 37 °C±1 °C for 24 h, they were tested for absence of catalase. Confirmed isolates were stored at –20 °C until species identification tests were performed. In general, 10% but maximum 5 typical enterococci colonies were subcultured for further testing.

E.coli counts were determined according to the Swiss Food Manual (19).

Identification

Species identification was carried out with API 20 Strep (Biomérieux 20600), according to the manufacturer's instructions.

Antibiotic resistance patterns

Antibiotic resistance was performed with the Kirby-Bauer disk diffusion test. Inoculum preparation (Tryptose Broth, Difco 0062-01-4), inoculation of test plates (Müller-Hinton Agar, Biomérieux 51075), application of disks to inoculated agar plates, incubation of plates, reading of the results were carried out according to international standards (20). The following drugs were tested: augmentin (AMC bio-disk Biomérieux 54632, consisting of 20 µg amoxicillin and 10 µg clavulanic acid), ampicillin (AM bio-disk Biomérieux 54002, consisting of 10 µg ampicillin), ciprofloxacin (CIP bio-disk Biomérieux 54932, consisting of 5 µg ciprofloxacin), erythromycin (E bio-disk Biomérieux 54192, consisting of 15 µg erythromycin), teicoplanin (TEC bio-disk Biomérieux 54302, consisting of 30 µg teicoplanin), vancomycin (VA bio-disk Biomérieux 54912, consisting of 30 µg vancomycin), imipenem (IPM bio-disk Biomérieux 54832, consisting of 10 µg imipenem), gentamycin (GM bio-disk Biomérieux 54262, consisting of 10 µg gentamycin), nitrofurantoin (FM bio disk Biomérieux 54222, consisting of 300 µg nitrofurantoin), streptomycin (S bio disk Biomérieux 54642, consisting of 10 µg streptomycin), rifampicin (RA bio-disk Biomérieux 54572, consisting of 30 µg rifampicin) and tetracyclin (TE bio disk Biomérieux 54882, consisting of 30 µg tetracyclin). Interpretation of the results was performed according to the scheme provided by

disk-manufacturer. Quality control of antimicrobial disks was performed batchwise with reference strain *E.coli* ATCC 25922, while each lot of test plates was checked with reference strain *P.aeruginosa* ATCC 27853 according to the international standard (20). In addition, minimum inhibitory concentrations (MIC) for the aminoglycosides gentamycin and streptomycin were tested with E-test strips (GM E-test Merck 5100-1258 and SM E-test Merck 5100-2688) allowing the detection of high level resistance between 0.064–1024 µg/ml.

Results

Isolation and identification of enterococci

Seventy-three of 2121 drinking water samples (3.4%) were contaminated with enterococci. One hundred and six colonies have been isolated, purified and taxonomically identified.

Of the 17 species currently recognised as *Enterococci*, only 4 were identified, namely *Enterococcus faecalis* (38 isolates), *E.faecium* (31 isolates), *E.durans* (25 isolates) and *E.casseliflavus* (12 isolates).

Antibiotic resistance patterns

The overall results of the susceptibility test of 106 isolates and 12 antibiotics are reported in Table 1a [augmentin (AMC), ampicillin (AM), ciprofloxacin (CIP), erythromycin (E), teicoplanin (TEC) and vancomycin (VA)] and Table 1b [imipenem (IPM), gentamycin (GM), nitrofurantoin (FM), streptomycin (S), rifampicin (RA) and tetracyclin (TE)].

None of the 106 isolated strains was found to be resistant against augmentin, ampicillin, teicoplanin, vancomycin and imipenem. Two strains of *Enterococcus casseliflavus* and one of *E.durans* were found to react «intermediately» against vancomycin and imipenem, respectively.

Table 2 shows the *E.coli* counts, the enterococci counts and their ratio in contaminated drinking waters as well as the antibiotic resistance patterns of those isolates which exhibited resistance to one of the tested drugs. Seven (6.7%; 6 *E.faecium* and 1 *E.faecalis*), 12 (11.3%; 9 *E.faecium*, 2 *E.faecalis* and 1 *E.casseliflavus*), 2 (1.9%; both *E.durans*), 4 (3.8%; all *E.faecium*) and 5 strains (4.7%; 2 *E.faecalis* and one strain each of *E.faecium*, *E.durans* and *E.casseliflavus*) were found to be resistant against ciprofloxacin, erythromycin, nitrofurantoin, rifampicin and tetracyclin, respectively.

One strain of *E.faecalis* showed simultaneous resistance against three antibiotics: erythromycin, tetracyclin and streptomycin. Four strains of *E. faecium* were simultaneously resistant to two antimicrobials: against ciprofloxacin and rifampicin

Table 1a. Average, maximum and minimum diameter of inhibition zones measured by resistance testing of 106 enterococci strains isolated from drinking water with the agar diffusion method and 6 different antibiotics (R = resistant; I = intermediate; S = sensitive)

	Tested antibiotics											
Diameter of inhibition zones in mm	Augmentin 20/10 µg ø (mm) R = ≤ 13 I = 14–17 S = ≥ 18		Ampicillin 10 µg ø (mm) R = ≤ 16 S = ≥ 17		Ciprofloxacin 5 µg ø (mm) R = ≤ 15 I = 16–20 S = ≥ 21		Erythromycin 15 µg ø (mm) R = ≤ 13 I = 14–22 S = ≥ 23		Teicoplanin 30 µg ø (mm) R = ≤ 10 I = 11–13 S = ≥ 14		Vancomycin 30 µg ø (mm) R = ≤ 14 I = 15–16 S = ≥ 17	
Average	28.1		27		20.3		21		20		19.9	
Maximum	*35*	*S*	*35*	*S*	*28*	*S*	*29*	*S*	*27*	*S*	*30*	*S*
Minimum	*22*	*S*	*19*	*S*	*13*	*R*	*10*	*R*	*16*	*S*	*16*	*I*
Standard deviation	3		3.11		3.07		5.3		2.1		2.26	
Variance	9		9.7		9.45		28		4.6		5.11	

Table 1b. Average, maximum and minimum diameter of inhibition zones measured by resistance testing of 106 enterococci strains isolated from drinking water with the agar diffusion method and 6 different antibiotics (R = resistant; I = intermediate; S = sensitive)

	Tested antibiotics											
Diameter of inhibition zones in mm	Imipenem 10 µg ø (mm) R = ≤ 13 I = 14–15 S = ≥ 16		Gentamycin 10 µg ø (mm) R = ≤ 12 I = 13–14 S = ≥ 15		Nitrofurantoin 300 µg ø (mm) R = ≤ 14 I = 15–16 S = ≥ 17		Streptomycin 10 µg ø (mm) R = ≤ 6 I = 7–9 S = ≥ 10		Rifampicin 30 µg ø (mm) R = ≤ 16 I = 17–19 S = ≥ 20		Tetracyclin 30 µg ø (mm) R = ≤ 14 I = 15–18 S = ≥ 19	
Average	29.1		16.9		22.3		9.4		29.3		27.7	
Maximum	*40*	*S*	*30*	*S*	*30*	*S*	*20*	*S*	*48*	*S*	*39*	*S*
Minimum	*15*	*I*	*11*	*R*	*12*	*R*	*6*	*R*	*13*	*R*	*8*	*R*
Standard deviation	3.7		4.21		3.45		4.2		7.6		5.42	
Variance	14		17.7		11.9		18		58		29.4	

Table 2. Microbial counts in different types of water and antibiotic susceptibility patterns of resistant enterococci strains (R = resistant; I = intermediate; S = sensitive)

		Microbial counts CFU/ 100 ml		Ratio	Tested antibiotics												Taxonomy
N°	*Type of water*	Enterococci	*E. coli*	*E. coli*/Enterococci	Ciprofloxacin 5 µg, Ø (mm), R = ≤ 15, I = 16–20, S = ≥ 21		Erythromycin 15 µg, Ø (mm), R = ≤ 13, I = 14–22, S = ≥ 23		Nitrofurantoin 300 µg, Ø (mm), R = ≤ 14, I = 15–16, S = ≥ 17		Rifampicin 30 µg, Ø (mm), R = ≤ 16, I = 17–19, S = ≥ 20		Tetracyclin 30 µg, Ø (mm), R = ≤ 14, I = 15–18, S = ≥ 19		Streptomycin E-test MIC 0.064–1024 µg/ml MIC µg/ml		*Enterococcus species*
1	*spring water (not particularly deep), in rural area*	2	5	2.5	19	I	27	S	30	S	32	S	10	R	16	LR	*casseliflavus*
2	*spring water (not particularly deep), in rural area*	17	6	0.4	21	S	10	R	15	I	20	S	30	S	32	LR	*faecium*
3	*spring water (not particularly deep), in rural area*	17	6	0.4	21	S	10	R	15	I	20	S	30	S	32	LR	*faecium*
4	*spring water with percolation from surface, in rural area*	2	1	0.5	18	I	12	R	18	S	32	S	8	R	1024	HR	*faecalis*
5	*spring water in urban area, fed by nearby river*	19	38	2.0	20	I	11	R	30	S	38	S	36	S	16	LR	*casseliflavus*
6	*underground water, in rural area*	1	1	1.0	19	I	12	R	20	S	22	S	28	S	32	LR	*faecium*
7	*spring water, in rural area*	64	32	0.5	23	S	26	S	12	R	20	S	30	S	48	LR	*durans*
8	*spring water, in rural area*	64	32	0.5	28	S	24	S	12	R	20	S	30	S	48	LR	*durans*

9	*spring water with percolation from surface, in rural area*	1	2	2.0	14	R	14	I	18	S	13	R	32	S	16	LR	*faecium*
10	*spring water with percolation from karstic surface*	2	1	0.5	13	R	15	I	20	S	22	S	30	S	16	LR	*faecium*
11	*spring water with percolation from surface*	12	2	0.2	15	R	12	R	17	S	21	S	24	S	8	LR	*faecium*
12	*spring water with percolation from surface, in rural area*	8	3	0.4	15	R	18	I	23	S	22	S	29	S	64	LR	*faecalis*
13	*spring water (not particularly deep), in rural area*	10	14	1.4	19	I	21	I	25	S	37	S	10	R	32	LR	*durans*
14	*spring water in urban area, fed by nearby river*	28	56	2.0	15	R	13	R	16	I	26	S	30	S	16	LR	*faecium*
15	*spring water in urban area, fed by nearby river*	28	56	2.0	20	I	27	S	20	S	19	I	11	R	12	LR	*faecium*
16	*spring water in landslide zone, in rural area*	1	2	2.0	16	I	11	R	19	S	25	S	31	S	64	LR	*faecium*
17	*spring water, presence of pest*	4	1	0.3	20	I	10	R	20	S	29	S	30	S	32	LR	*faecium*
18	*spring water with percolation from surface, in rural area*	26	2	0.1	14	R	14	I	24	S	30	S	26	S	96	LR	*faecium*
19	*spring water, in rural area*	1	1	1.0	19	I	24	S	24	S	25	S	8	R	64	LR	*faecalis*
20	*spring water with percolation from surface*	117	14	0.1	18	I	14	I	21	S	15	R	30	S	12	LR	*faecium*
21	*spring water*	1	1	1.0	23	S	13	R	18	S	15	R	32	S	24	LR	*faecium*
22	*Surface water, in rural area*	100	90	0.9	14	R	15	I	17	S	20	S	30	S	16	LR	*faecium*
23	*Surface water, in rural area*	43	20	0.5	24	S	12	R	18	S	22	S	30	S	24	LR	*faecium*
24	*Surface water, in rural area*	43	20	0.5	20	I	14	I	20	S	14	R	28	S	32	LR	*faecium*

(one strain), against ciprofloxacin and erythromycin (two strains) and against erythromycin and rifampicin (one strain).

As expected, most of the strains showed resistance against the aminoglycoside gentamycin (14.1%; 15 strains) and streptomycin (53%; 56 strains). As confirmed by determination of the minimum inhibitory concentration, all gentamycin-resistant isolates could however be classified as low resistant (MIC < 16 µg/ml). Higher resistance was observed with streptomycin, with one *E.faecalis* strain showing particularly high resistant behaviour (MIC<= 1024 µg/ml).

Discussion

Enterococci were once regarded as relatively harmless bacteria that are commonly encountered and rarely important. Colonisation with enterococci is limited to the gastrointestinal tract. Opportunistic infections exist when these Gram-positive cocci are found in sterile sites of the body. Some enterococci are pan-sensitive to antibiotics, although resistance can occur to ß-lactams, aminoglycoside and glycopeptide antimicrobial agents. In the USA, since 1993, enterococci have been the second most common cause of nosocomial infections and the third most common blood culture isolate (13). A growing number of reports from Europe suggest that colonisation with Vancomycin Resistant Enterococci (VRE) frequently occurs in the community as well (21–23). Reports also suggest that VRE exist elsewhere in the environment, including animal faeces and in foods of animal origin for human consumption (23–25). The link between VRE colonisation of animals slaughtered for food production and VRE in human was shown by *Bates* et al. (24). Genetically related VRE or tetracyclin-resistant enterococcal isolates have been found in livestock, animal carcasses, and foods such as cheese, outpatients, and hospitalised patients. These facts strongly suggest that interspecies transmission of genetic material carrying resistance genes can occur and may contribute to colonisation and infection in humans. *Perreten* reviewed and compared antibiotic resistance genes of bacteria isolated from food with those of known clinical origin (26).

Although distribution of VRE in the environment is quite well documented, no extensive and exhaustive studies have been carried out up to now on the role of food and water (especially drinking water) in the spreading of VRE.

In Southern Switzerland isolation of VRE from clinical specimen is uncommon: resistance patterns of human isolates are very similar to those observed in the present study on water isolates (27). In the present investigations, the contamination level ranged from 1 to 117 Colony Forming Units (CFU) of enterococci per 100 ml and from 1 to 90 CFU *E.coli* per 100 ml. Data reported by *Vismara* (28) suggests that ratios *E.coli*/enterococci could be used to assess the origin of contamination: the ratio coliforms/faecal streptococci is higher in humans (4.4) than in animals (in cattle 0.2; in sheep 0.4; in pig 0.02). In the present work, the ratios *E.coli*/enterococci were lower than 1 (arbitrarily taken as limit) in 17 of the 24 positive samples, thus leading to the interpretation that drinking water may have

been contaminated more likely with enterococci of animal rather than human origin. Contamination with resistant enterococci of human origin is however not excluded. This is supported also by the type of water that has been found contaminated. Most of the samples were indeed collected in rural areas where presence of cattle and other farming animals is abundant (with relatively low ratio *E.coli*/ enterococci). Some water samples from urban areas with potential contamination by sewage (with relatively higher ratio *E.coli*/enterococci) were also found to carry resistant strains. The necessary epidemiological methods to definitely prove this assumptions are however not available yet.

Regarding vancomycin resistance, the results of the present investigation shouldn't be a matter of concern, while the molecular characterisation and the potential transfer mechanism of the detected resistance to some other antimicrobials (like tetracyclin and erythromycin) should be further investigated.

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Summary

Of 2121 samples of drinking water investigated from the microbiological point of view, 73 were found to be contaminated with enterococci. 106 enterococcal strains have been isolated and their antibiotic resistance investigated by disk susceptibility testing.

None of the strains was found to be resistant against augmentin, ampicillin, teicoplanin and vancomycin, while two strains of *Enterococcus casseliflavus* and one of *E.durans* were found to react «intermediately» against vancomycin and imipenem, respectively. Seven, twelve, two, four and five strains, respectively were found to be resistant against ciprofloxacin, erythromycin, nitrofurantoin, rifampicin and tetracyclin, respectively.

Most of the strains showed low resistance to the aminoglycosides gentamycin and streptomycin. Higher resistance to streptomycin was however observed in one *E.faecalis* strain.

Five strains showed simultaneous resistance against two antibiotics.

The results show that some acquired resistance to antibiotics may be present in enterococci isolated from drinking water. Vancomycin Resistant Enterococci (VRE) were however not isolated.

Zusammenfassung

Von 2121 mikrobiologisch untersuchten Trinkwasserproben waren 37 mit Enterokokken kontaminiert. Daraus wurden 106 Stämme isoliert und deren Resistenz gegenüber Antibiotika mittels Agardiffusionstest überprüft.

Es wurde keine Resistenz gegenüber Augmentin, Ampicillin, Teicoplanin und Vancomycin festgestellt; zwei Stämme *Enterococcus casseliflavus* und ein Stamm von *E.durans*

zeigten jedoch intermediäres Verhalten gegenüber Vancomycin bzw. Imipenem. Sieben, zwölf, zwei, vier und fünf Stämme erwiesen sich als resistent gegenüber Ciprofloxacin, Erythromycin, Nitrofurantoin, Rifampicin bzw. Tetracyclin. Die meisten Stämme waren gegenüber Gentamycin und Streptomycin schwach resistent: Ein einziger Stamm wies jedoch eine hohe Resistenz gegenüber Streptomycin auf.

Bei fünf Stämmen konnte eine Zweifachresistenz nachgewiesen werden.

Die Resultate zeigen, dass Resistenz gegenüber Antibiotika auch in aus Trinkwasser isolierten Enterokokken vorkommen kann. Vancomycin Resistente Enterokokken (VRE) konnten jedoch nicht isoliert werden.

Résumé

Des 2121 échantillons d'eau potable soumis à une analyse microbiologique, 73 étaient contaminés par des entérocoques. 106 souches ont été isolées et soumises au test de diffusion sur gélose afin de déceler des résistances antibiotiques.

Aucune résistance n'a pu être mise en évidence pour l'augmentine, l'ampicilline, la teicoplanine et la vancomycine; deux souches de *Enterococcus casseliflavus* et une de *E. durans* ont montré une réponse intermédiaire à la vancomycine et à l'imipenem. Sept différentes souches sont résultées résistantes à la ciprofloxacine, douze à l'erythromycine, deux à la nitrofurantoine, quatre à la rifampicine et cinq à la tetracycline. La pluspart des souches a montré une faible résistance à la gentamycine et à la streptomycine; pour une seule souche on a décelé une forte résistance envers le dernier de ces deux antibiotiques.

Cinq souches sont résultées résistantes à deux antibiotiques en même temps.

Les résultats montrent que même les entérocoques des eaux potables peuvent présenter des résistances vis-à-vis de certains antibiotiques. Il n'a par contre pas été possible d'isoler des entérocoques résistants à la vancomycine.

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