JPH - Year 8, Volume 7, Number 1, 2010

ITALIAN JOURNAL OF PUBLIC HEALTH

Antimicrobial susceptibility and glycopeptide-resistance of enterococci in vegetables

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Abstract

Background: Vancomycin-resistant enterococci (VRE), often responsible for nosocomial infections, have frequently been isolated from animal and vegetable foods. In our study we evaluated the antibiotic susceptibility of enterococci isolated from eight types of vegetables randomly selected from grocery stores in Naples.

Methods: From July to November 2008, we analyzed 150 samples: the bacteria were isolated with standardized methods and antibiotic susceptibility was determined using the disc diffusion method. The resistance to vancomycin versus other antibiotics was assessed by the Kappa test.

Results: 70% of the samples, mainly parsley (96.2%), showed enterococci. Of these, 59.1% belonged to the species *Enterococcus faecium*. Strains resistant to vancomycin and teicoplanin were isolated respectively in 47.6% and 49.5% of the samples: the first one mainly in curly endive (72.7%) and the second one in parsley (76.9%). Almost all the isolated strains showed resistance to methicillin (89%), kanamycin (82%) and cephalothin (68%). The Kappa test showed statistically significant associations between resistance to vancomycin and resistance to teicoplanin, erythromycin, methicillin, tetracycline and chloramphenicol.

Conclusions: Because of the possible involvement of food in the transmission of resistant micro-organisms to human intestinal microbiota, our data may provide the basis for future studies.

Key words: enterococci, antimicrobial-resistance, GRE, vegetable

Introduction

Bacteria of the genus *Enterococcus*, or enterococci (formerly the "faecal" or Lancefield group D Streptococci) are Gram-positive, catalase-negative cocci. They are ubiquitous, commensal inhabitants of the gastrointestinal tract of humans and animals.

Because of their high tolerance to heat and strong survival capacity under adverse environmental conditions, enterococci can also colonize soil, surface water, plants and large numbers of animal and vegetable food products [1- 3].

A range of intrinsic and acquired antibiotic resistances is characteristic of enterococci. Natural resistances include cephalosporines, polymyxins, beta-lactams and low-levels of clindamycin and aminoglycosides [4].

Enterococci have always been considered low pathogenic bacteria, but in the last two decades *E. faecium* and *E. faecalis* have emerged as important pathogens responsible for hospital acquired infections [5, 6].

The attention towards this has increased with the incidence of high-levels of resistance to multiple antimicrobial drugs related to the use of chloramphenicol, erythromycin, tetracycline and high levels of clindamycin, aminoglycosides, beta-lactams, quinolones and glycopeptides, such as vancomycin [7].

Since 1986, when strains of vancomycin-resistant enterococci (VRE) were isolated from clinical samples for the first time in England and France [8], the incidence of nosocomial infections caused by these pathogens has gradually increased.

From 1989 to 1999, the National Nosocomial Infections Surveillance (NNIS) System of the Centres for Disease Control and Prevention reported an increase in the percentage of VRE associated with nosocomial infections in intensive care units from 0.4% to 25% [9].

In Italy VRE isolation in hospitalised patients has been reported since 1993 [10]. A large multicentre study carried out from 1993 to 1995 in 20 Italian hospitals reported that the average isolation frequency of vancomycin-resistant *Enterococcus faecalis* was 1.1%, and that vancomycin-resistant *Enterococcus faecium* was 8.5% [11]. According to the European Antibiotic Resistance Surveillance System (EARSS), in Italy the percentage of VRE among bloodstream-infecting isolates has risen by 10% from 2001 to 2002 [12].

VRE has also been frequently isolated from farm animals and foods of animal origin [13, 14]. Their presence in meat products could be associated with the therapeutic use of antimicrobials in veterinary medicine or the use of antibiotics in animal feed as growth promoters (antibiotic growth promoters, AGPs) [7, 12, 15, 16].

Some authors also believe that the use of untreated water or manure slurry on croplands could result in the spread of resistance to indigenous soil bacteria through horizontal transfer, which could in turn transfer resistance back to animals or humans via crops [15, 17].

Todate, it has been demonstrated that only glycopeptide-resistant enterococci (GRE) isolated from meat products (chicken and pork meat) are able to overcome the gastric barrier and actively multiply in the human intestine [14, 18, 19].

Considering this, in addition to the limited attention given to enterococci isolated from vegetables as well as the limited information available about the different resistance phenotypes of enterococci isolated from foods, in this study we report the isolation, identification and antibiotic susceptibility profiles for members of the *Enterococcus* genus isolated from fresh produce samples. The antimicrobial agents tested represented the major groups of antibiotics used in health care centres such as β-lactams, cephalosporins, glycopeptides and quinolones.

Methods

From July to November 2008, 150 samples from eight different types of vegetables were analyzed. Fresh raw vegetables, non pre-packaged, were collected from randomly selected grocery stores in Naples.

In particular, we analyzed 39 units of lettuce, 27 of celery, 27 of parsley, 18 of curly endive, 9 of large-leaved endive, 9 of spinach, 12 of iceberg lettuce and 9 of chicory. After washing the samples with tap water (as vegetables are normally treated before eating them), 10 g of the sample was added to 90 ml buffered Peptone Water (Oxoid SpA) and homogenized in a stomacher for one minute. Then 0.1 ml of the homogenate was plated on Enterococcosel Agar (Oxoid SpA) and incubated aerobically at 37°C for 24 hours. Presumptive colonies were identified on the basis of esculin hydrolysis, Gram stain and catalase reaction. Enterococci were identified to the species level by the Api 20 Strep test (Biomérieux SpA) [16].

Antimicrobial susceptibility tests were performed using the disk diffusion method (Kirby-Bauer method). The MICs and the level of resistance were determined according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS, 2002) [20].

The following antimicrobials were tested: β-lactams (amoxycillin / clavulanic acid [30 µg], ampicillin [10 µg], penicillin [10 U.I.] and methicillin [5 µg]), cephalosporins (ceftriaxone [30 µg] and cephalothin [30 µg]), glycopeptides (vancomycin [30 µg] and teicoplanin [30 µg]), quinolones (ciprofloxacin [5 µg], norfloxacin [10 µg] and levofloxacin [5 µg]), chloramphenicol (30 µg), aminoglycosides (gentamicin [10 µg], kanamycin [30 µg] and streptomycin [10 µg]), macrolides (erythromycin [15 µg]); carbapenems (imipenem [10 µg]), tetracyclines (tetracycline [30 µg]) and others (linezolid [10 µg], nitrofurantoin [300 µg] and trimethoprim / sulfamethoxazole [25 µg]).

All statistical analyses were performed with SPSS version 10.0. Kappa statistic was used to evaluate associations of resistance to vancomycin with other antimicrobials.

This test allowed us to calculate the correlation between ordinal values excluding the effect of blind chance. The Kappa index take values between 1 (maximum agreement) and -1 (maximum disagreement). Generally, it is possible to say that there is a good level of agreement if the Kappa value is higher than 0.75 [21].

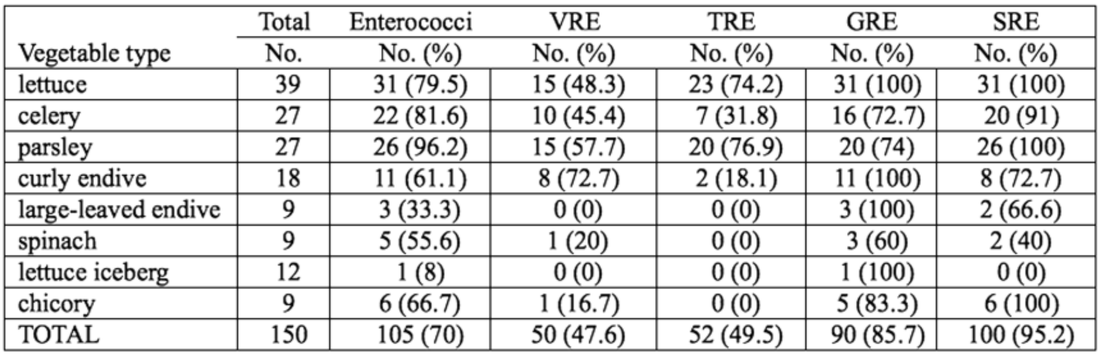
Results

Enterococci were isolated from 105 samples out of the 150 samples collected (70%). Table 1 shows the frequency of enterococci that were isolated for each type of vegetable and its resistance to aminoglycosides and glycopeptides.

These microorganisms were most frequently isolated from celery and parsley (respectively 81.6% and 96.2%), whereas the broad leaf endive was the least contaminated vegetable (8%).

The majority of the samples that were positive for enterococci were resistant to aminoglycosides (95.2% to streptomycin and 85.7% to gentamicin). Furthermore, 47.6% and 49.5% were contaminated by VRE and teicoplanin resistant strains (TRE) respectively and in particular a high

Table 1. Distribution of VRE, TRE, SRE and GRE according to vegetable type.



Vegetable type	Total No.	Enterococci No. (%)	VRE No. (%)	TRE No. (%)	GRE No. (%)	SRE No. (%)
lettuce	39	31 (79.5)	15 (48.3)	23 (74.2)	31 (100)	31 (100)
celery	27	22 (81.6)	10 (45.4)	7 (31.8)	16 (72.7)	20 (91)
parsley	27	26 (96.2)	15 (57.7)	20 (76.9)	20 (74)	26 (100)
curly endive	18	11 (61.1)	8 (72.7)	2 (18.1)	11 (100)	8 (72.7)
large-leaved endive	9	3 (33.3)	0 (0)	0 (0)	3 (100)	2 (66.6)
spinach	9	5 (55.6)	1 (20)	0 (0)	3 (60)	2 (40)
lettuce iceberg	12	1 (8)	0 (0)	0 (0)	1 (100)	0 (0)
chicory	9	6 (66.7)	1 (16.7)	0 (0)	5 (83.3)	6 (100)
TOTAL	150	105 (70)	50 (47.6)	52 (49.5)	90 (85.7)	100 (95.2)

VRE= vancomycin-resistant enterococci; TRE= teicoplanin-resistant enterococci;
GRE= gentamycin-resistant enterococci; SRE= streptomycin-resistant enterococci.

prevalence of VRE was isolated from parsley (57.7%) and curly endive (72.7%), while TRE strains were found mainly in lettuce (74.2%) and parsley (76.9%).

Table 2 shows micro-organisms isolated, to the species level, in terms of resistance to glycopeptides and aminoglycosides. The most frequent isolates were *Enterococcus faecium* (59.1%), whereas *Enterococcus avium* represented 3.8%, *Enterococcus durans* 19% and *Enterococcus faecalis* 18.1% of the isolates.

A high prevalence of resistance to glycopeptides was found in the strains of *Enterococcus faecium* (respectively 53.2% to vancomycin and 93.5% to teicoplanin) and *Enterococcus faecalis* (47.4% both to vancomycin and teicoplanin). Enterococcus avium was the only isolated species not resistant to vancomycin.

Table 3 shows the antibiotic susceptibility profiles of the Enterococcus strains isolated. Many strains were sensitive to ampicillin (90.2%), amoxycillin / clavulanic acid (97%), imipenem (92%), penicillin (88%), trimethoprim / sulfamethoxazole (87%) and chloramphenicol (82%). Almost all of the isolated strains demonstrated resistance to methicillin (89%), kanamycin (82%) and cephalothin (68%). The Kappa test showed statistically significant associations between resistance to vancomycin and teicoplanin ($k = 0.479$, $p < 0.0001$), erythromycin ($K = 0.442$, $P < 0.0001$), methicillin ($K = 0.121$, $P = 0.031$), tetracycline ($K = 0.320$, $P < 0.0001$) and chloramphenicol ($K = 0.187$, $P = 0.027$) (Table 4).

Discussion

Glycopeptide-resistance of enterococci has been increasingly associated with nosocomial infections. The European Antibiotic Resistance Surveillance System (EARSS) reported the percentage of VRE among enterococcal clinical isolates in Italy was higher than 10% in 2001 and 2002. In particular, the proportion of VRE among bloodstream-infecting *E.faecium* isolates in 2002 (19%) was one of the highest in Europe, while the percentage of VRE among bloodstream-infecting *E.faecalis* isolates (4%) was similar to that found in other European countries [8, 11, 12].

Antibiotic resistance leads to serious clinical problems related to, on one hand, the difficulty in finding effective drugs for treatment of infections caused by multi-drug-resistant micro-organisms and, on the other hand, the possibility that genetic coding for glycopeptide-resistance may be transferred by pheromone-mediated, conjugative (often multi-resistant) plasmids or transposons to both enterococci and more virulent pathogens, such as *Staphylococcus aureus* and *Streptococcus pneumoniae* [15, 22].

This issue is a real and growing threat to human health. Evidence from Europe shows there have been numerous reports of VRE isolated from animals used for food as well as food products [23, 24].

Some authors believe that water and food are also possible vectors of strain transmission to human intestinal flora [25]. To date, the transfer of resistance genes to intestinal bacteria has only been confirmed in rats, in which the passage of glycopeptides-resistance from *E. faecalis* to *E. coli* has been proven [26].

In our study 105 (70%) of the 150 samples collected were contaminated by *Enterococcus spp.* In particular, of the samples examined, parsley was the vegetable most frequently shown to be positive for contamination (96.2%).

The values of contamination from our study were similar to those found by Klein et al., who

Table 2. Distribution of VRE, TRE, SRE and GRE according to the microorganisms isolated.

Microorganism	Total No. (%)	VRE No. (%)	TRE No. (%)	GRE No. (%)	SRE No. (%)
Enterococcus faecium	62 (59.1)	33 (53.2)	58 (93.5)	59 (95.2)	58 (93.5)
Enterococcus faecalis	19 (18.1)	9 (47.4)	9 (47.4)	18 (94.7)	18 (94.7)
Enterococcus avium	4 (3.8)	0 (0)	2 (50)	4 (100)	4 (100)
Enterococcus durans	20 (19)	8 (40)	3 (15)	9 (45)	18 (90)
TOTAL	105 (100)	50 (47.6)	52 (49.5)	90 (85.7)	98 (93.3)

VRE= vancomycin-resistant enterococci; TRE= teicoplanin-resistant enterococci;
GRE= gentamycin-resistant enterococci; SRE= streptomycin-resistant enterococci.

Note: Micro-organisms isolated to the species level and resistance to glycopeptides and aminoglycosides among positive samples for each species.

Table 3. Antibiotic susceptibility of enterococci in vegetables.

Antibiotic	Resistant	Intermediate	Susceptible
	%	%	%
Amoxycillin/clavulanic acid	3	-	97
Ampicillin	4.5	5.3	90.2
Ceftriaxone	25	59	16
Ciprofloxacin	21	47	32
Chloramphenicol	8	10	82
Cephalothin	68	13	19
Erythromycin	5	68	27
Imipenem	5	3	92
Kanamycin	82	10	8
Levofloxacin	10	30	60
Linezolid	17	11	72
Methicillin	89	5	6
Nitrofurantoin	8	24	68
Norfloxacin	16	37	47
Penicillin	12	-	88
Tetracycline	15	13	72
Trimethoprim/sulfamethoxazole	13	0	87

Table 4. Statistically significant associations of resistance to vancomycin with other antibiomicrobials.

Antimicrobials tested	Vancomycin	
	k	p
Chloramphenicol	0.187	0.027
Erythromycin	0.442	<0.0001
Methicillin	0.121	0.031
Tetracycline	0.320	<0.0001
Trimethoprim/sulfamethoxazole	0.479	<0.0001

isolated enterococci in almost all of the samples of raw minced beef and pork meat in Germany [27], while considerably higher than those reported by Pavia et al., who studied enterococci contamination in raw meat and found that only 45% of the samples were positive [16].

The most frequently isolated species were *Enterococcus faecium* (59.4%) and *Enterococcus* *durans* (19%), while *Enterococcus avium* represented only 3.8% of the isolates identified.

Enterococcus faecium was also the most frequently isolated species in a study carried out by Johnston and Jaykus, who reported on the isolation, identification and antibiotic susceptibility profiles for enterococci isolated from fresh produce samples collected in 15 farms in the United States [28].

With regards to antibiotic susceptibility, in our study most strains were resistant to methicillin (89%), kanamycin (82%) and cephalothin (68%). These results match the intrinsic antibiotic-resistance profile of enterococci [15].

The percentage of samples contaminated by VRE and TRE strains were 47.6% and 49.5% respectively compared to 29% and 30% of positive samples found by Pavia et al [16].

The VRE values detected in our study were even higher than the ones detected in pork meat in Denmark (15%) [29], and higher than the findings of Klare et al. [30] in minced pork meat in Germany. Instead, similar results to ours were detected in the United Kingdom in fresh and frozen chicken, with 58% of the *E. faecium* and 40% of the *E. faecalis* isolates identified proving to be vancomycin-resistant [31].

Alternatively, in the study conducted by Johnston and Jaykus [28] strain of VRE were not identified, instead results showed higher rates of resistance to ciprofloxacin and tetracycline.

Applying the panel of antibiotics used in our study, Hayes et al [32] reported that resistant *Enterococcus spp.* commonly contaminate retail meat.

The comparison of results between the two studies showed a higher prevalence of antibiotic-resistance in the strains isolated from Hayes et al. compared to ours. This is confirmed by other studies that have found the prevalence of antibiotic-resistant enterococci in farm animals and their meat to be higher than 60% [15, 29].

In another study on fresh vegetables conducted by Prazak et al., resistance patterns among *Listeria monocytogenes* isolated from cabbage farms was studied. Their results showed that 98% of the isolates were resistant to at least two drugs and 85% were found to be resistant to penicillin [33]. Instead, Hamilton-Miller and Shah [34], studied the antibiotic susceptibility of enterobacterial flora isolated from salad vegetables and found a high level of resistance to ampicillin.

A reassuring result that emerged from our study is that only 4.5% of isolated strains showed resistance to ampicillin, the antibiotic of first choice in human infections caused by enterococci, while in the Pavia et al. study the percentage of ampicillin-resistant strains was 17.8% [16].

In addition, Knudtson and Hartman, who studied enterococci contamination of pork carcasses and products, water and clinical samples in the United States, did not identify any ampicillin-resistant strains [35].

It is important to note that all the above mentioned studies, except for Johnston and Jaykus, focus on products treated with heat before being consumed [28]. In our study we analyzed fresh vegetables since cooking of animal products can inactivate enterococci. Vegetable foods, on the other hand, are most often consumed raw and therefore there is a risk that they may act as possible vectors in the transmission of antibiotic resistance traits to human intestinal flora [25, 36].

There is also strong epidemiological evidence regarding the correlation between the use of antibiotics in animal feed and in human medicine and the development of resistance to these drugs. For example, several studies have found high rates of resistance both in food of animal origin and in men demonstrating a possible cross-resistance between avoparcin, an antibiotic belonging to the glycopeptides group, and vancomycin [25, 28]. In Italy, a high resistance to avoparcin is still found in animal products [7, 12, 15, 16] although this antibiotic has not been used as a food additive since 1997 because it was suspected of *"... inducing resistance to glycopeptides used in human medicine through foods given to animals...."* [37]. This is not surprising since, in 1999, Langlois et al. demonstrated that animals resistant strains can be transferred from one generation to another, even long after the suspension of the antibiotics [38, 39].

Instead, the finding of antibiotic-resistant microorganisms in vegetables could be related to the fact that when manure slurry from farms is spread onto fields, resistant bacteria and antibiotic resistance genes are also transferred to the area, thus creating an environment conducive to the horizontal transfer of resistance genes to the indigenous soil bacteria [17, 36]. This specific source of contamination could explain the differences existing between the results of this study and other previously mentioned studies.

Without doubt the data reported in our study shows the presence of strains of glycopeptides-resistant enterococci (GRE) in fresh vegetables. This evidence is of great interest because of the new importance of microbial antibiotic-resistance and the possible involvement of food in the transmission of resistant micro-organisms to human intestinal bacterial flora, with subsequent deterioration of its normal functions. In order to confirm this hypothesis, further studies based on the molecular typing of bacterial clones, plasmids, translocatable elements, and resistance genes are necessary.

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