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International Journal of Food Microbiology 54 (2000) 197–204

INTERNATIONAL JOURNAL OF
Food Microbiology

www.elsevier.nl/locate/ijfoodmicro

Vancomycin-resistant enterococci isolated from animals and food

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Received 21 March 1999; received in revised form 7 July 1999; accepted 20 November 1999

Abstract

One hundred and one chicken products, boiled ham and turkey cold meat were acquired from 18 different supermarkets in Spain during October 1997 to June 1998 and were analyzed for vancomycin-resistant enterococci (VRE). In the same way, 50 intestinal chicken samples from a slaughterhouse were also studied. VRE were detected in 25 of 92 samples of food of chicken origin (27.2%), but no VRE were found in cooked pork or turkey products. VRE were also detected in 8 of 50 intestinal chicken samples from the slaughterhouse (16%). VRE were identified as *Enterococcus durans* ($n = 11$), *Enterococcus faecalis* ($n = 10$), *Enterococcus faecium* ($n = 10$) and *Enterococcus hirae* ($n = 2$). All these strains were characterized as belonging to the *vanA* genotype by polymerase chain reaction. Ampicillin, quinupristin/dalfopristin and high level aminoglycoside resistance were frequently found among these strains. Heterogeneity was observed in susceptibility patterns among VRE strains, even in those of the same species. The high rate of colonization of chicken products by *vanA* containing enterococci detected 6 months to 1 year after the banning of avoparcin as a growth promoter, supports other studies suggesting that the food chain could be a source of VRE colonization in humans and thus a source of VRE infections. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Enterococcus; Vancomycin-resistance; Chicken; Identification

1. Introduction

Enterococci are considered as human pathogens. Treatment of enterococcal infections, particularly endocarditis, requires the use of aminoglycosides in addition to a bacterial cell wall synthesis inhibitors, such as penicillin or vancomycin. The acquisition by *Enterococcus* strains of specific mechanisms of resistance precludes the synergistic effect of the combined antibiotics. In Europe, recent studies have consistently found vancomycin resistant enterococci (VRE) in healthy volunteers (Jordens et al., 1994; Klare et al., 1995b; Devriese et al., 1996; van der

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0168-1605/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0168-1605(99)00195-6

Auwera et al., 1996; Endtz et al., 1997) and in the environment, including sewage (Klare et al., 1993; Torres et al., 1994), animal feces (Bates et al., 1994; Aarestrup, 1995; Klare et al., 1995a; Devriese et al., 1996; Quednau et al., 1998; Butaye et al., 1999) and animal products (Klare et al., 1995b; Kirk et al., 1997; Wegener et al., 1997), and the risk of transferring VRE from animals to humans has caused great concern.

Avoparcin, a glycopeptide similar to medically important glycopeptides, has been used as a growth promoter in animal feeds in the European Community since the middle 1970s. Studies have indicated that the glycopeptide avoparcin selects for VRE (Aarestrup et al., 1996; Bager et al., 1997), and an association between the rates of vancomycin resistance in humans and avoparcin usage in animals has been suggested (Murray, 1995; Klare et al., 1995b; Aarestrup et al., 1996; van den Bogaard and Stobbergingh, 1996). For this reason, the European Community banned the use of avoparcin in animals as of April 1997.

The present study was designed to determine the level of VRE isolation in animal products, mainly chicken products, obtained from different supermarkets and from a slaughterhouse in Spain, after the banning of avoparcin.

2. Materials and methods

2.1. Samples and sample processing

One hundred and one randomly selected chicken products (thighs, breasts, necks, carcasses, skin, minced chickens, ground chickens, chicken sausages and chicken croquettes), boiled ham and turkey cold meat were studied for detection of VRE. They were acquired from 18 different supermarkets in Logroño, Spain, in the period October 1997 to June 1998. Fifty intestinal chicken samples from a slaughterhouse, located 30 miles from Logroño city, were also studied. Sample processing was as follows: approximately 1.5 g of sample was suspended in 3 ml of sterile saline and was heavily vortexed; a 100-μl aliquot of this suspension was added to 5 ml of M-*Enterococcus* broth (BioMérieux, Marcy-l'Etoile, France) supplemented with vancomycin (4 μg/ml) and was incubated at 37°C during 48 h. A 100-μl aliquot of this culture was used to inoculate a M-*Enterococcus* agar medium plate (BioMérieux) supplemented with vancomycin (4 μg/ml). Plates were incubated at 37°C and examined at 24 and 48 h. Colonies with the appearance of enterococci were studied.

2.2. Strain identification by phenotypical and genotypical methods

Identification was performed by the API 20 Strep system (BioMèrieux), supplemented by biochemical tests as previously recommended (Facklam and Collins, 1989). To corroborate the identification to the species level, isolates were tested for the presence of genes coding for *E. faecalis* antigen A (EfaA) (Singh et al., 1998), chromosomal *E. faecium* aminoglycoside acetyltransferase-6′ [AAC(6′)-Ii] (Costa et al., 1993), and muramidase-2 which was derived from *E. hirae* (Chu et al., 1992), by colony lysis hybridization. Intragenic probes for *efaA* (730 bp) and *aac(6′)-li* (323 bp) were generated by polymerase chain reaction (PCR) from *E. faecalis* TX4002 and from *E. faecium* TX0016. The muramidase gene of *E. hirae* cloned into *Escherichia coli* pUC19 (Chu et al., 1992) (kindly provided by Lolita Daneo-Moore) was used as a gene probe for *E. hirae*. Plasmid DNA was prepared using the Wizard Plus Minipreps kit (Promega, Madison, WI, USA) and was digested with *Eco*RI and *Eco*RV. These probes were cleaned and labeled with ^{32}P for hybridization purposes (Singh et al., 1998). Preparation of colony lysates containing denatured enterococcal genomic DNA and hybridizations under high stringency conditions were carried out using modified standard protocols (Singh et al., 1998). *E. gallinarum* and *E. casseliflavus* species were identified by PCR amplifications of *vanC1* and *vanC2* genes, respectively (Dutka-Malen et al., 1995; Miele et al., 1995). In those cases in which discrepancies between phenotypic and genotypic methods were observed, genotypic results were considered more likely to be correct.

2.3. Susceptibility testing

Susceptibility testing was performed with colonies obtained from each vancomycin containing plate. Only one isolate of a given species per specimen was

further studied after susceptibility testing, unless a different antibiotic resistance phenotype was observed. Minimal inhibitory concentrations (MICs) of vancomycin (Eli Lilly, Indianapolis, IN, USA), avoparcin (Roche), teicoplanin and erythromycin (Hoechst Marion Roussel, Paris, France), tylosin, streptomycin, kanamycin, gentamicin, ampicillin (Sigma, St. Louis, MO, USA), quinupristin/dalfopristin (Rhône-Poulenc Rorer, Vitry sur Seine, France) and ciprofloxacin (Bayer, Leverkusen, Germany) were determined by the agar dilution method according to the National Committee for Clinical Laboratory Standards (NCCLS) (1997). An *Enterococcus* isolate with an MIC ≥ 2000 μg/ml for streptomycin or kanamycin and >500 μg/ml for gentamicin was considered as high-level resistant to these aminoglycosides. β-Lactamase was determined by using nitrocefin disks (Beckton Dickinson Microbiology Systems, Cockeysville, MD, USA).

2.4. Characterization resistance genes

PCRs were performed with VRE strains to amplify *vanA* (Woodford et al., 1993), *vanB* (Clark et al., 1993), *vanC1* (Miele et al., 1995) and *vanC2* (Dutka-Malen et al., 1995) genes. PCRs were also performed with high-level kanamycin or gentamicin resistant *Enterococcus* strains to amplify genes coding for the APH(3′) and AAC(6′)/APH(2″) enzymes (van de Klundert and Vliegenthart, 1993) and quinupristin/dalfopristin resistant *E. faecium* strains to amplify the *satA* gene (Rende-Fournier et al. 1993).

3. Results

3.1. VRE detection and identification

VRE strains were detected in 25 of 92 samples of chicken origin (27.2%) obtained in supermarkets (Table 1). A higher level of detection (38.5–40%) was observed in samples as ground chickens (5 of 13 samples), thighs and breasts (4 of 10 samples) and carcasses (10 of 26 samples). No VRE strains were isolated from croquette or minced chicken (0 of 9 samples). The number of colonies on vancomycin supplemented agar plates, after enrichment in M-

Table 1
Animal products studied and vancomycin resistant *Enterococcus* strains isolated

Samples		No. of samples containing containing VRE	VRE isolated	
Sources	No.		Species	No.
Ground chickens	13	5	*E. faecalis*	2
			E. faecium	2
			E. hirae	1
Chicken sausages	13	1	*E. faecalis*	1
Chicken thighs	5	2	*E. faecium*	1
			E. faecalis	1
Chicken breasts	5	2	*E. faecalis*	1
			E. faecium	1
Chicken necks	6	1	*E. faecium*	1
Chicken carcasses	26	10	*E. durans*	3
			E. faecalis	3
			E. faecium	3
			E. hirae	1
Chicken skin	15	4	*E. faecium*	2
			E. faecalis	2
Chicken intestines	50	8	*E. durans*	8
Minced chicken	7	0		
Chicken croquette	2	0		

Enterococcus broth with vancomycin 4 μg/ml, was very variable (1– > 100 colonies per plate) among the different samples, with 33.3% showing low colony counts (≤ 10 colonies per plate) and 66.7% showing high colony counts (> 100 colonies per plate).

VRE were detected in 8 out of 50 chicken intestinal samples from a slaughterhouse (16%) (Table 1).

Other food tested such as boiled ham and turkey cold meat (nine samples) were negative for VRE detection.

VRE isolated from chicken were identified as *E. faecalis* ($n = 10$), *E. faecium* ($n = 10$), *E. durans* ($n = 11$) and *E. hirae* ($n = 2$) (Table 2). Some differences were found using phenotypic or genotypic identification. All 10 isolates identified as *E. faecalis* by API 20 STREP were confirmed by colony lysis hybridization using the *efaA* probe. Discrepancies were observed in eight *E. faecium* [which gave a positive result with the *aac(6′)* probe] that were identified by API 20 STREP as *E. durans* (one strain, biotype 1) and as *E. casseliflavus* (seven strains, biotype 2); neither of these last seven strains gave a positive PCR amplification product using specific primers for *vanC2* gene, specific for *E.casseliflavus* identification. Discrepancies were also observed between two strains that were identified as *E. durans* by phenotypic methods but as *E. hirae* by the genotypic one (Table 2).

3.2. PCR results and antibiotic susceptibility

Positive PCR amplification of *vanA* and negative results for *vanB*, *vanC1* and *vanC2* genes were shown in all vancomycin-resistant *Enterococcus* isolates obtained from this study.

All *vanA* containing *E. hirae*, *E. durans*, and *E. faecium* isolates were resistant to vancomycin, avoparcin, teicoplanin and erythromycin (Table 3). Two *E. hirae* and four *E. faecium* isolates showed high-level streptomycin and kanamycin resistance (MIC ≥ 2000 μg/ml), and the gene coding for APH(3′) enzyme was detected by PCR in all of these strains (Table 3). Three *vanA* containing *E. faecium* strains were highly resistant to kanamycin and gentamicin and the gene coding for the bifunctional AAC(6′)-APH(2″) enzyme was detected by PCR (Table 3). Four and five of 10 *vanA* containing *E. faecium* strains were resistant to ampicillin and quinupristin/dalfopristin, respectively. In all quinupristin/dalfopristin resistant strains, a negative result was obtained when amplification of *satA* gene was performed by PCR, using *E. faecium* strain B-303 as

Table 2
Phenotypes (biotypes) of *vanA* containing enterococci from chicken

Biotype	Test result												Identification	Number of isolates included in each biotype
	Sugar fermentation[a]								API 20 STREP	Hybridization				
	Ara	Sac	Raf	Sbs	Sbt	Mnt	Mns	Lac		*aac(6′)*	efa_{fs}	*mur-2*		
1	+	+	−	−	−	−	+	+	*E. durans* (low discrimination)	+	−	−	*E. faecium*	1
2	+	+	+	−	−	+	+	+	*E. casseliflavus* (very good to the genus)	+	−	−	*E. faecium*[b]	7
3	+	+	−	−	−	+	+	+	*E. faecium* (very good identification)	+	−	−	*E. faecium*	2
4	−	+	−	−	+	+	+	+	*E. faecalis* (good identification)	−	+	−	*E. faecalis*	10
5	−	+	+	−	−	−	+	+	*E. durans* (very good identification)	−	−	+	*E. hirae*	2
6	−	+	−	−	−	−	+	+	*E. durans* (low discrimination)	−	−	−	*E. durans*[c]	11

[a] Ara, arabinose; Sac, sacarose; Raf, raffinose; Sbs, sorbose; Sbt, sorbitol; Mnt, mannitol; Mns, mannose; Lac, lactose.
[b] *vanC2* negative.
[c] These strains were identified as *E. durans* by specific molecular methods.

Table 3
MICs of different antibiotics for *vanA* containing enterococci isolated from chicken products

Enterococcus strains	No.	Antibiotic MIC (mg/ml)[a]										
		Van	Avo	Tei	Cip	Amp	Ery	Tyl	Q/D	Str	Kan	Gen
E. hirae[b]	2	64	64	8–16	0.5	8	> 128	> 128	1	2000	> 2000	≤ 32
E. durans	11	256	256	8–32	0.5–1	≤ 0.5–8	> 128	> 128	ND[d]	≤ 64	≤ 64	≤ 32
E. faecium[b]	3	256	256	32	0.5–1	8	> 128	> 128	1–2	> 2000	> 2000	≤ 32
E. faecium[b]	1	> 256	> 256	128	1	256	> 128	> 128	0.5	> 2000	> 2000	≤ 32
E. faecium[c]	3	> 256	> 256	64–128	2	8	> 128	> 128	16	128	> 2000	> 2000
E. faecium	3	128– > 256	128– > 256	32–256	1–2	16–32	> 128	> 128	2–16	≥ 2000	≤ 64	≤ 32
E. faecalis	6	> 256	> 256	256	≤ 0.5–1	≤ 0.5	4	≤ 0.5	ND	≤ 64–256	≤ 64	≤ 32
E. faecalis	4	> 256	> 256	256	≤ 0.5–1	≤ 0.5–2	> 128	> 128	ND	256	≤ 64	≤ 32

[a] Abbreviations: Van, vancomycin; Avo, avoparcin; Tei, teicoplanin; Cip, ciprofloxacin; Amp, ampicillin; Ery, erythromycin; Tyl, tylosin; Q/D, quinupristin/dalfopristin; Str, streptomycin; Kan, kanamycin; Gen, gentamicin.
[b] A positive PCR reaction for *aph(3′)* gene was detected.
[c] A positive PCR reaction for *aac(6′)-aph(2″)* gene was detected.
[d] ND, not determined.

a positive control (characterized as containing the *satA* gene by PCR followed by sequencing).

All *vanA* containing *E. faecalis* isolates were resistant to vancomycin, avoparcin and teicoplanin, and four were resistant to erythromycin. None of these 10 isolates showed high-level aminoglycoside resistance (Table 3).

4. Discussion

A high rate of *vanA* containing *Enterococcus* isolates was detected in food samples of chicken origin in this study (27.2%), 6 months to 1 year after banning avoparcin as a growth promoter in animals, and could be an important source of VRE for humans. Isolation of *vanA* containing *E. faecium* strains in thawing liquids from frozen poultry, turkey meat and fresh slaughtered chicken samples has been previously reported in Germany (Klare et al., 1995b) and high-level glycopeptide resistant VanA-type strains of *E. faecium* and *E. faecalis* from supermarket-purchased chicken carcasses were detected in England during the period when avoparcin was used as growth promoter (Kirk et al., 1997).

The lack of detection of VRE in boiled pork ham and turkey cold meat is not surprising because these kind of products have been previously boiled. A low incidence of VRE (0.5%) has been detected in raw minced beef and pork in Germany (Klein et al., 1998). Although enterococci are used in the dairy industry, particularly as a starter for cheese, there has been no evidence of VRE in dairy products to date (Giraffa and Sisto, 1997).

In Europe, the food chain has been suspected to be a source of VRE acquired by humans. VRE in food may originate in the enriched populations selected by the use of avoparcin as a food additive for animals (Leclercq and Courvalin, 1997). Based on this, it may be hypothesized that the primary transmission is from animals to humans and not the other way around. Horizontal exchange of VRE isolates and Tn*1546*-like elements, which contain vancomycin-resistant genetic determinants, between poultry, pigs and humans occurs (Jensen, 1998). Therefore, transfer of VRE into the hospital setting from the community is a realistic possibility (Bates et al., 1993). Avoparcin was associated with the occurrence of vancomycin-resistant *E. faecium* on Danish poultry and pigs farms suggesting that food animals constitute a potential reservoir of infection for VRE in humans (Bager et al., 1997). A case of VRE infection has been reported in a truck driver working at a factory packaging chickens where the probable source of VRE infection were animals colonized with VRE (Das et al., 1997). In contrast, meat associated nosocomial infection could not be demonstrated by Klein et al. (1998).

Consumption of meat has been associated with colonization by VRE of gastrointestinal tract of

humans. In one study, 9.7% of meat eaters carried VRE in their feces (Schouten et al., 1997). In contrast, van den Braak et al. found that *vanA* containing *Enterococcus* strains did not differ significantly between vegetarian and non-vegetarian groups, but there was only one *Enterococcus* isolated which had acquired (VanA-type) vancomycin resistance (van den Braak et al. 1997). Contamination of vegetables by VRE contained in sewage (Klare et al., 1995b; Torres et al., 1994) cannot be excluded.

Different *vanA* enterococcal species were isolated from chickens samples as *E. faecalis*, *E. faecium*, *E. durans* and *E. hirae*. Curiously, *E. durans* was the only species isolated from intestinal samples. We have not found any *E. gallinarum* or *E. avium* strain, which were originally described from chickens (Nowlan and Deibel, 1967; Barnes et al., 1978). Devriese et al. (1991) indicates that the composition of the intestinal flora of chickens is more complex that it was once thought; these authors described that *E. faecalis*, *E. faecium*, *E. cecorum*, *E. durans* and *E. hirae* were regularly isolated in the intestinal flora of chickens of different age groups but *E. gallinarum* and *E. avium* were rarely isolated.

Discrepancies observed between phenotypic and genotypic identification in *vanA* containing *Enterococcus* strains could be related with the fact that most phenotypic identification schemes are designed for clinical human strains. Differences in sugar fermentation have been reported for *E. faecium* isolated from chicken in relation with those isolated from humans; for example, most *E. faecium* isolates from chicken are raffinose positive, but most *E. faecium* isolates from humans are raffinose negative (Devriese et al., 1996). In this sense, biotype 2 identified as *E. casseliflavus* by API 20 STREP would be identified as *E. faecium* if the raffinose test were negative. The positive reaction with these strains by PCR with the primers for *aac(6′)-Ii* (specific for *E. faecium*) (Costa et al., 1993) and the negative result with the primers for *vanC2* gene (specific for *E. casseliflavus*) (Dutka-Malen et al., 1995) corroborate the importance and the usefulness of genotypic methods as a tool for identification of these strains.

High-level aminoglycoside resistance was detected more frequently in *vanA* containing *E. faecium* or *E. hirae* isolates than in *vanA* containing *E. faecalis* (0/10) or *E. durans* (0/11) isolates. Among 10 *vanA* containing *E. faecium* strains, five strains showed quinupristin/dalfopristin resistance. In none of these strains was the *satA* gene demonstrated by PCR, therefore a different and unknown mechanism could be implicated.

In conclusion, a high rate of detection of *vanA* containing enterococci was found in chicken samples 6 months to 1 year after the banning of avoparcin as a growth promoter. In general, current models predict that the decay time of resistance rates after cessation or decline in the volume of selective drug use may require a prolonged period of time (Austin et al., 1999). The food chain remains a potential source of VRE colonization in humans as well as a possible source of VRE infections. Objective risk assessment criteria to demonstrate such a possibility have been suggested by the Scientific Committee for Animal Nutrition of the European Union, but long and extensive research will be needed to fulfil those requirements (Klare and Witte, 1998). In the meantime, it is prudent to consider that antibiotics with cross-resistance with others used in humans should not be used as feed additives in animal husbandry.

Acknowledgements

This work has been supported by a grant of Fondo de Investigaciones Sanitarias of Spain (FIS 98/0282). B.R. has a FPI fellowship from MEC of Spain. We thank Yolanda Sáenz for helping us in sample collection.

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