ACTA VET. BRNO 2003, 72: 123–129

Agricultural and Municipal Waste Water as a Source of Antibiotic-Resistant Enterococci

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Received September 6, 2002
Accepted March 25, 2003

Abstract

Cupáková, Š., J. Lukášová: *Agricultural and Municipal Waste Water as a Source of Antibiotic-Resistant Enterococci*. Acta Vet. Brno 2003, 72: 123-129.

The occurrence of enterococci in different waste waters from two cattle farms in south and north Moravia and sewage treatment plant of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic was investigated. Resistance and/or sensitivity of selected isolates was tested to various antibiotics. The samples were collected in the course of two years (1999-2000). The total counts of enterococci varied from 10^3 to 10^5 colony-forming units (CFU) per ml. Among 100 isolated strains 60 strains were identified as *Enterococcus faecalis*, 10 as *Ent. durans* as well as *Ent. hirae*, 8 strains were allotted to *Ent. faecium*, 5 to *Ent. mundtii*, 3 to *Ent. gallinarum*, 2 to *Ent. casseliflavus*, one strain was identified as *Enterococcus* spp. and one strain was not specified as enterococcus. The susceptibility to antibiotics was tested by both, the agar disk diffusion method and the microdilution method. The majority of enterococci (95%) was resistant to more than one antibiotic tested, especially to clindamycin, penicillin, cephalotin, ofloxacin and tetracycline. No vancomycin-resistant strain was found. Our results confirm that agricultural and municipal waste waters might be an important source of antibiotic-resistant enterococci.

Enterococci, waste water, silage, liquid manure, cattle, antibiotic-resistance

Enterococci are a part of the common microflora in the gastrointestinal tract of warm-blooded animals and birds. In addition they can occur in soil, surface or waste waters, on plants and vegetables (Franz et al. 1999).

Enterococci are now considered as the third most common pathogens causing nosocomial infections and "superinfections" in hospitalized patients, accounting for nearly 12% of these infections (Linden et al. 1999). The most common enterococci-associated infections are infections of the urinary tract, bacteremia, endocarditis, intra-abdominal and pelvic infections. The very consequential ones are neonate meningitis and bacteremia and central nervous system infections in adults (Murray 1990; Franz et al. 1999).

Vancomycin-resistant enterococci (VRE) represent a serious problem at present. They are usually isolated from environment, waste waters, animals, foods of animal origin. They still represent the worldwide problem for hospitalized patients, where the glycopeptide antibiotics vancomycin and teicoplanin are used in the therapy of several infections caused by Gram-positive bacteria (Klare et al. 1993; Borgen et al. 2001). The emergence of VRE in Europe is attached to the long-time use of the glycopeptide antibiotic avoparcin as a growth promoter for farm animals with the possibility of inducing cross-resistance to vancomycin. In 1997 it was banned in EU (Grosso et al. 2000). Many studies indicate food animals as reservoirs of resistant enterococci which might be transmitted to humans through the food chain and represent a potential risk for the consumers (Bates et al. 1994; Bager et al. 1997; Klein et al. 1998; Grosso et al. 2000).

The aim of this study was to investigate the occurrence of enterococci in different kinds of agricultural and municipal waste waters and to test their sensitivity to selected antimicrobial agents.

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Materials and Methods

Samples

The occurrence of enterococci was studied in different waste waters during the years 1999–2000. The following samples were collected:

- 12 samples of mixed waste waters from litter bed and milking parlor and 5 samples of silage waste waters from a cattle farm in the district of Blansko, south Moravia;
- 6 samples of cattle liquid manure from a cattle farm in the district of Nový Jičín, north Moravia;
- 10 samples of waste waters affluent to the sewage treatment plant of the Veterinary university Brno, Czech Republic.

Bacterial strains

A total of 100 bacterial strains were isolated during the microbiological analysis of agricultural and municipal waste water. The samples were diluted according to ČSN ISO 7218.The total numbers of enterococci were determined by inoculation of 0.1 ml of the respectived dilution of the sample onto Slanetz-Bartley agar (HiMedia, Mumbai, India), which contains NaN_3 as a selective agent for enterococci. The plates were incubated at 37 C for 24 h. Three colonies with different but "enterococcal" colony morphology from all tested samples were picked up. The purity of the isolated strains was checked by the streak plate technique on blood agar (Blood agar base No. 2, HiMedia, and sheep blood, Bioveta, Ivanovice na Hané, CZ) and Slanetz-Bartley agar (HiMedia).

Identification of isolated strains

The following tests were used for the identification of isolated strains: Gram staining, appearance of colonies on blood agar, catalase production, growth at 45 °C, in broth (tryptone glucose yeast extract broth, HiMedia) with 6.5% NaCl and at pH 9.6 (Havlová et al. 1993), growth on bile-esculin agar (Oxoid, Basingstoke, Hampshire, England). Phenotyping of selected isolates was done using the Api Rapid ID 32 Strep system (BioMerieux, L'Etoile, France) and completed by production of pyrrolidonyl arylamidase (PYRAtest, Pliva-Lachema, a.s., Brno, Czech Republic), motility and production of yellow pigment.

Tests of sensitivity to antimicrobial agents

The susceptibility to antibiotics was tested by the agar disc diffusion method (DD) and the microdilution method (MD) to show the minimal inhibition concentration of antibiotic used (MIC) as well.

Agar disc diffusion method

These antimicrobial discs were used: ampicillin (AMP), cephalotin (CLT), clindamycin (CLI), erythromycin (ERY), gentamycin (GEN), chloramphenicol (CMP), ofloxacin (OFL), penicillin (PEN), teicoplanin (TEI), tetracycline (TTC) and vancomycin (VAN, Oxoid). DD method was performed according to Urbášková (1999), the Mueller-Hinton agar (Oxoid) was used. Based on the measurement of inhibition zone diameters the strains were classified as "susceptible", "intermediate resistant" or "resistant" using the interpretation criteria given in NCCLS (1999) and for TEI according to Urbášková (1999).

Microdilution method

The microtitre plates Gram-plus Strepto for MIC testing (obtained from Trios, spol. s r.o., Prague, Czech Republic) were used. The following antimicrobial drugs and their breakpoints were used: 0.125 $mg \cdot l^{-1}$ for benzylpenicillin (PEN), 0.25 mg l^{-1} for clindamycin (CLI), 0.5 $mg \cdot l^{-1}$ for erythromycin (ERY), 2 $mg \cdot l^{-1}$ for cephalotin (CLT), tetracycline (TTC) and ofloxacin (OFL), 4 $mg \cdot l^{-1}$ for chloramphenicol (CMP) and vancomycin (VAN), 8 $mg \cdot l^{-1}$ for ampicillin (AMP), ampicillin+sulbactam (AMS) and teicoplanin (TEI), 32 $mg \cdot l^{-1}$ for nitrofurantoin (FUR). After inoculation of 10 µ of standardized bacterial suspension into each well, the microtitre plates were incubated for 24 h at 37 °C and the growth of bacteria was measured at 630 nm using the Plate Reader (Morwell Diagnostics GmBH, Zurich, Switzerland). MIC value, MIC_{50}, MIC_{90} and MIC range were defined for each tested strain and antimicrobial drug. The isolates were classified as "susceptible" or "resistant" using the interpretation criteria for MIC given in NCCLS (1995).

Results

Total counts of enterococci in samples varied from 10^3-10^4 (waste waters from the farms) to 10^5 CFU per ml (area of the university). Among 100 isolated strains, the following species were identified; 60 strains of *Enterococcus faecalis* (60%), 10 strains of *Ent. durans* (10%) as well as of *Ent. hirae* (10%), 8 strains of *Ent. faecium* (8%), 5 strains of *Ent. mundtii* (5%), 3 strains of *Ent. gallinarum* (3%), two of *Ent. casseliflavus* (2%) and one strain (1%) of *Enterococcus* spp. One isolated strain was not specified as enterococcus. All isolated strains

were Gram-positive and catalase negative cocci. They grew at 45 °C, in 6.5% NaCl broth, at pH 9.6 and on bile-esculin agar. All strains produced pyrrolidonyl arylamidase. Enterococcal species isolated from various kinds of agricultural waste waters are summarized in Table 1.

Table 1
Numbers of strains of *Enterococcus* spp. isolated from different agricultural waste waters

Species	Mixed waste water farm I.	Silage waste water farm I.	Cattle liquid manure farm II.	Waste water from area of the university
Ent. faecalis	30	6	19	5
Ent. durans	8	2	0	0
Ent. hirae	5	1	3	1
Ent. faecium	2	4	2	0
Ent. mundtii	1	1	2	1
Ent. gallinarum	1	0	2	0
Ent. casseliflavus	0	2	0	0
Enterococcus spp.	1	0	0	0

Resistance to antibiotics of isolated enterococci performed by agar disc diffusion method is shown in Table 2. The majority of tested isolates were resistant to CLI (98.99%) and OFL (90.91%). More than a half of the isolates was resistant to CLT and PEN (66.67%). No strain was resistant to vancomycin and teicoplanin; only 32 strains shown VAN-intermediate resistance. The strains were resistant at least to one and not more than to seven antibiotics tested. The majority of strains was simultaneously resistant to four (37.37%) or five (26.26%) drugs. The most frequently occurring resistant phenotypes were CLI-CLT-OFL-PEN (27 strains) and CLI-CLT-OFL-PEN-TTC (21 strains).

In terms of the MIC for the individual anti-microbial drugs, resistance to CLT, PEN and CLI was demonstrated in more than 95% of enterococcal strains and more than a half of them

Table 2
Survey of antibiotics resistance of isolated enterococci (agar disc diffusion method)

Antimicrobial agent	Disc content [μg]	Zone diameter for susceptible strain [mm]	Susceptible strains		Intermediate resistant strains		Resistant strains	
			No.	%	No.	%	No.	%
Ampicillin	10	≥ 17	95	95.96	-	-	4	4.04
Cephalotin	30	≥ 18	13	13.13	20	20.20	66	66.67
Clindamycin	2	≥ 21	1	1.01	0	0	98	98.99
Erythromycin	15	≥ 23	72	72.73	19	19.19	8	8.08
Gentamycin	10	≥ 15	76	76.77	12	12.12	11	11.11
Chloramphenicol	30	≥ 18	88	88.89	3	3.03	8	8.08
Ofloxacin	5	≥ 21	9	9.09	-	-	90	90.91
Penicillin	10 units	≥ 15	33	33.33	-	-	66	66.67
Teicoplanin	30	≥ 14	99	100.00	-	-	0	0
Tetracycline	30	≥ 19	33	33.33	23	23.23	43	43.44
Vancomycin	30	≥ 17	67	67.68	32	32.32	0	0

interpretation criteria NCCLS (1999) and for TEI Urbášková (1999)

Table 4.
MIC_{50}, MIC_{90} and MIC range of antimicrobial agents for enterococcal species

		MIC characteristics for individual antibiotics [mg l^{-1}]											
		PEN	AMP	AMS	CLT	CMP	TTC	ERY	CLI	VAN	TEI	FUR	OFL
Enterococcus faecalis	MIC_{50}	2	0.5	2	16	4	8	1	>4	2	<0.5	16	4
(n = 60)	MIC_{90}	>2	4	2	16	32	16	2	>8	2	<0.5	16	4
	range	1->2	0.25-4	1-4	8->16	2-64	0.5-16	0.063-8	4->8	0.25-2	<0.5-2	<1-64	0.5-4
Enterococcus durans	MIC_{50}	2	0.5	2	16	4	0.5	0.063	>4	1	<0.5	32	2
(n = 10)	MIC_{90}	>2	1	2	>16	4	16	1	>8	2	1	64	4
	range	1->2	0.5-4	2-4	16->16	2-8	0.5-16	0.063-1	4->8	0.5-2	<0.5-1	8-64	1-4
Enterococcus hirae	MIC_{50}	2	0.5	2	16	4	0.5	0.063	>4	1	<0.5	32	2
(n = 10)	MIC_{90}	>2	4	2	16	4	1	0.063	>4	1	<0.5	64	2
	range	0.25->2	0.125-4	0.5-2	4->16	4	0.25-16	0.063	>4->8	0.5-1	<0.5	32-64	1-4
Enterococcus faecium	MIC_{50}	>2	0.5	2	>16	4	0.5	0.125	>4	1	<0.5	64	4
(n = 8)	MIC_{90}	>2	0.5	2	>16	4	0.5	>4	>4	1	<0.5	64	8
	range	2->2	0.5-4	2-4	16->16	1-4	0.5	0.063->4	>4->8	1	<0.5-0.5	64	4-8
Enterococcus mundtii	MIC_{50}	>2	0.5	2	>16	4	0.5	2	>4	1	<0.5	64	8
(n = 5)	MIC_{90}	>2	1	2	>16	4	4	4	>4	1	<0.5	64	8
	range	2->2	0.25-1	1-2	16->16	4	0.5-4	0.063-4	<0.03->4	1	<0.5	64	4-8
Enterococcus gallinarum	MIC_{50}	>2	0.5	4	>16	4	0.5	0.125	>4	1	1	64	4
(n = 3)	MIC_{90}	>2	4	4	>16	4	0.5	0.25	>4	1	2	64	8
	range	>2	0.5-4	2-4	>16	4	0.5	0.06-0.25	>4	0.5-1	<0.5-2	32-64	4-8
Enterococcus casseliflavus	MIC_{50}	1	0.25	1	4	4	0.5	4	0.063	1	<0.5	8	1
(n = 2)	MIC_{90}	>2	0.5	4	>16	4	1	>4	>4	4	2	32	4
Enterococcus spp.	MIC_{50}	2	4	2	>16	4	0.5	0.063	>4	1	<0.5	32	2
(n = 1)	MIC_{90}	2	4	2	>16	4	0.5	0.063	>4	1	<0.5	32	2

was resistant to OFL (56.56%). Resistance to ERY was found in 45.45% of isolates; TTC was resisted by 40.40% of bacteria and FUR by 21.21%. Only *Ent. faecalis* and *Ent. durans* were resistant to chloramphenicol. All strains were AMP, AMS, VAN and TEI sensitive. Out of the tested antibiotics the different resistance to TTC, ERY and OFL of enterococci isolated from the above mentioned sources was found (Table 3). The tested enterococci were simultaneously resistant at least to three and at the most to seven antibiotics examined; 41 strains were resistant to five antibiotics. The obtained values MIC_{50}, MIC_{90} and MIC range of various antibiotics is demonstrated in Table 4.

Discussion

Enterococcus faecalis and *Enterococcus faecium* are the predominant species in water environment. Švec et al. (1999) studied 630 bacterial strains isolated from surface waters and 135 strains (21%) were identified as *Ent. faecium*, 115 strains (18%) were identified as *Ent. faecalis*. They assumed that also other identified species – *Ent. mundtii*, *Ent. casseliflavus*, *Ent. gallinarum*, *Ent. hirae* and *Ent. durans* formed an important part of water environment. Similar re-

Table 3
Numbers of antibiotic-resistant strains of *Enterococcus* spp. isolated from different sources (microdilution method)

Antimicrobial agent	Mixed waste water farm I.		Silage waste water farm I.		Cattle liquid manure farm II.		Waste water from area of the university	
	n=48	%	n=16	%	n=28	%	n=7	%
Ampicillin	0	0	0	0	0	0	0	0
Ampicillin+sulbactam	0	0	0	0	0	0	0	0
Benzylpenicillin	48	100.0	16	100.0	28	100.0	6	85.7
Cephalotin	48	100.0	16	100.0	28	100.0	7	100.0
Chloramphenicol	6	12.5	1	6.3	6	21.4	1	14.3
Clindamycin	48	100.0	15	93.8	28	100.0	6	85.7
Erythromycin	25	52.1	10	62.5	8	28.6	2	28.6
Nitrofurantoin	6	12.5	8	50.0	6	21.4	1	14.3
Ofloxacin	18	37.5	13	27.1	21	75.0	4	57.1
Teicoplanin	0	0	0	0	0	0	0	0
Tetracycline	27	56.3	1	6.3	7	25.0	5	71.4
Vancomycin	0	0	0	0	0	0	0	0

sults were obtained in other studies, which showed distribution of enterococci in sewage or waste waters, e.g. Valdivia et al. (1996) isolated 45 strains of enterococci from municipal waste waters; 32 of them were *Enterococcus faecalis*, 10 isolates belonged to *Ent. faecium* and three to *Ent. hirae*. Lauková et al. (1997) studied the occurrence of enterococci in municipal sewage from different sewage treatment plants in the Eastern Slovakia. They analyzed 2 000 isolates and documented the predominant occurrence of *Ent. faecium* (50%), followed by *Ent. gallinarum* (25.5%) and *Ent. casseliflavus* (10.1%). These presented studies suggest a higher incidence of *Ent. faecium* in municipal sewage and waste waters. Our results show an expressive prevalence of *Enterococcus faecalis* strains in different types of agricultural waste waters, which could be explained by the fact that the gastrointestinal tract of farm animals is probably more colonized by this species.

Most enterococci are naturally or inherently resistant to various drugs, including cephalosporins, oxacillin and to clinically achievable concentrations of clindamycin and aminoglycosides, a great part is relatively resistant to penicillin and ampicillin (Murray 1998; Urbášková 1999). Important is their resistance to tetracyclines, macrolides and chloramphenicol (Gray et al. 1991; Franz et al. 1999). Klare et al. (1993) showed that glycopeptide-resistant *E. faecium* can be found not only in clinical samples but also in environmental samples as well as in waste waters or sewage treatment plants. Our results are in agreement with these data. Schlegelová et al. (2002) described in their study resistance to TTC (MIC_{90} >32 mg l^{-1}) and to ERY (MIC_{90}>32 mg l^{-1}) among *Enterococcus faecalis* strains isolated from bulk milk samples. Compared to our results, MIC_{90} 16 mg l^{-1} for TTC and MIC_{90} 2 mg l^{-1} for ERY, a lower prevalence of resistant strains of *Ent. faecalis* was demonstrated.

Bates et al. (1994) isolated vancomycin-resistant *E. faecium* from a duck, a chick, a turkey, a dog, a pony and from pigs, but VRE were not isolated from cattle or sheep. Devriese et al. (1996) isolated *Van*A-resistant *Ent. faecium* strains from the intestines and faeces of horses, dogs, chickens and pigs, *Van*A-positive strains identified as *Ent. durans* from gallinaceous birds, *Ent. faecalis* in a horse and *Ent. gallinarum* in a pheasant. Furthermore, a prevalence of vancomycin-resistant *Ent. faecium* was studied in faecal samples from pigs in Spain. There, 43 from 240 pig farms represented in the sampling had

at least one VRE-positive faecal sample (Herrero et al. 2000). Kolář et al. (2000) described for the first time the occurrence of VRE in animals in the Czech Republic. The VRE were isolated from 2.5% of examined hens and 75% of them was diagnosed as *Ent. faecium* phenotype *Van*A. In our study noVRE strain from agricultural waste water samples was detected.

Multidrug-resistant enterococci are commonly isolated from humans, sewage, aquatic habitats, agriculture and animal sources. It indicates their ability to enter the human food chain. Lauková et al. (1997) found that enterococci isolated from waste waters were resistant at least to one (except vancomycin) and not more than to six of the examined antibiotics, most of them being biresistant. Similarly, Kolář et al. (2000) reported about multiresistant enterococci isolated from hens. These findings are in accordance with our results. In this study 95% multiresistant strains of *Enterococcus* spp. in waste water samples were determined.

Finally, a large spectrum of enterococci was isolated and identified from various agricultural waste waters. *Ent. faecalis* predominated among them. The majority of enterococci were resistant to more than one antibiotics tested, above all to CLI, PEN, CLT, OFL and TTC. But no VRE strain was demonstrated. Our results suggest that agricultural waste waters might be an important source of resistant enterococci, which could be taken into account in their further application in agriculture.

Odpadní vody jako zdroj antibotik-rezistentních enterokoků

Byl sledován výskyt enterokoků v různých typech odpadních vod a testována jejich citlivost k různým druhům antibiotik. Vzorky byly získávány ze dvou farem skotu, z jižní a severní Moravy, a z čistírny odpadních vod Veterinární a farmaceutické univerzity Brno, Česká republika, a to v průběhu let 1999-2000. Celkový počet enterokoků se pohyboval v rozmezí 10^3 až 10^5 CFU·ml^{-1}. Z celkového počtu 100 izolovaných kmenů jsme identifikovaly 60 kmenů jako *Enterococcus faecalis*, 10 jako *Ent. durans* a *Ent. hirae*, 8 kmenů jako *Ent. faecium*, 5 jako *Ent. mundtii*, 3 jako *Ent. gallinarum*, 2 jako *Ent. casseliflavus* a jeden kmen byl identifikován jako *Enterococcus* spp. Jeden izolovaný kmen nebyl enterokok. Citlivost k antibiotikům byla testována dvěma metodami, a to agarovou diskovou difúzní metodou a mikrodiluční metodou. Převážná část enterokoků (95 %) byla rezistentní k více než jednomu testovanému antibiotiku, především ke klindamycinu, penicilinu, cefalotinu, ofloxacinu a tetracyklinu. Nezachytily jsme žádný vankomycin-rezistentní kmen. Naše výsledky ukazují, že zemědělské a splaškové odpadní vody mohou být významným zdrojem enterokoků rezistentních k antibiotikům.

Acknowledgements

This study was supported by the grant of Ministry of Education, Youth and Sports of the Czech Republic No. 162700005.

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