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Hospital-associated *Enterococcus faecium* in the water chain

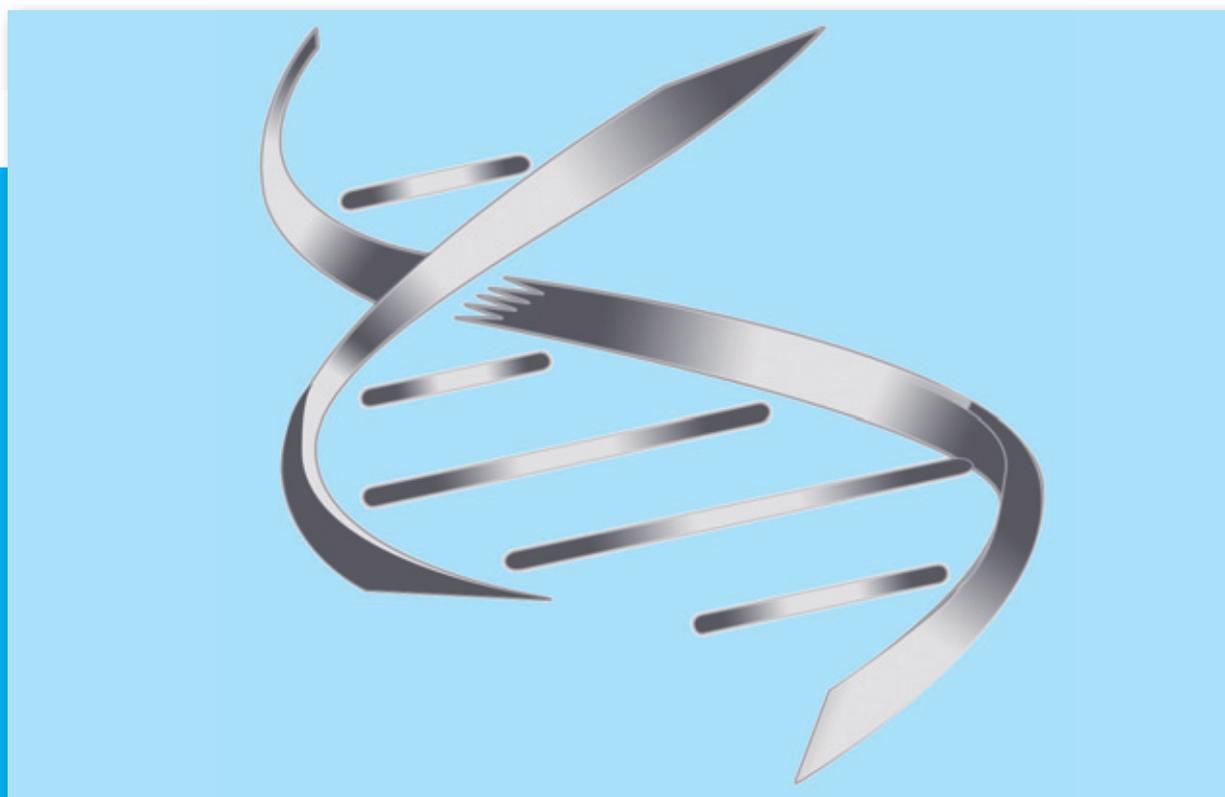**Iacovos Anastasiou, Heike Schmitt**
Utrecht University/IRAS, The Netherlands

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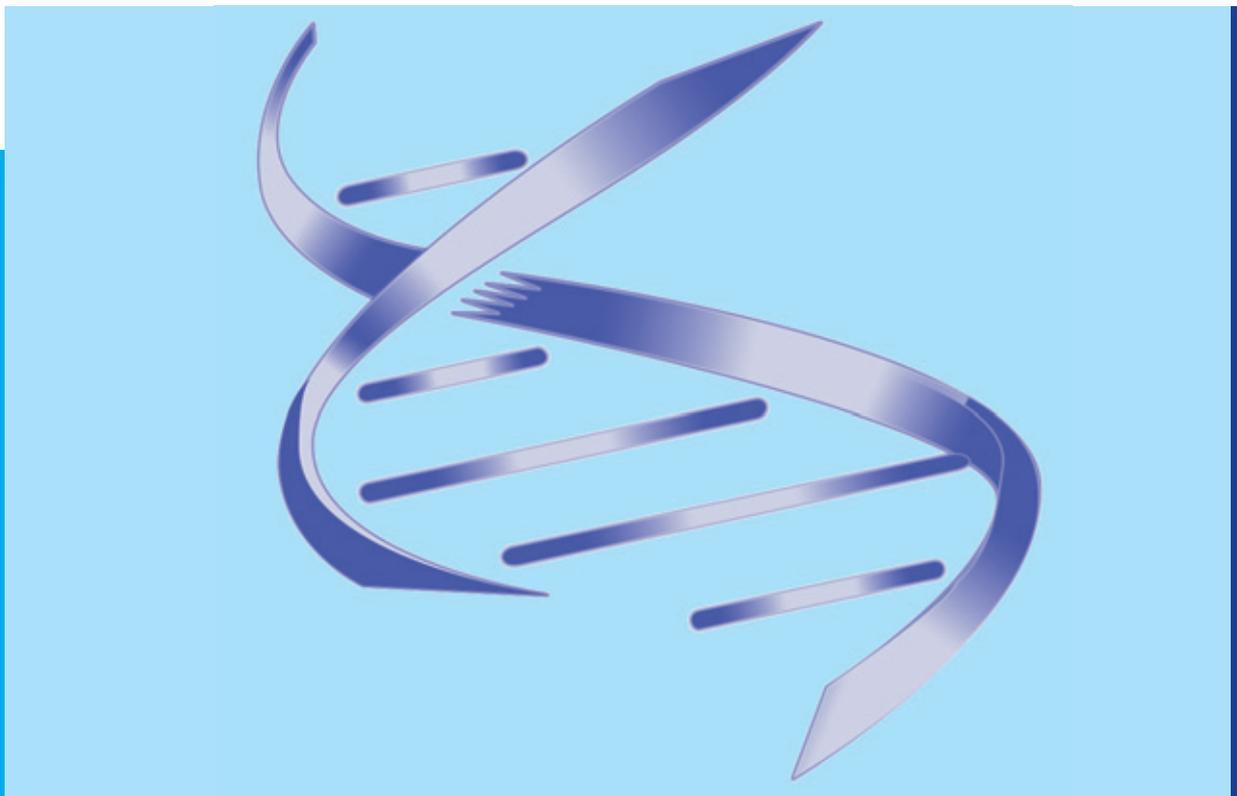**Utrecht University/IRAS, The Netherlands**

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Abstract

In this study, we investigated hospital effluent as possible source of antibiotic resistant bacteria in the environment. Molecular epidemiological studies of human and animal derived *Enterococcus faecium* strains had previously shown that *E. faecium* from outbreaks and infections worldwide are characterized by ampicillin resistance and a high prevalence of the *esp* (enterococcal surface protein) gene. Using ampicillin resistance and the *esp* gene as specific markers, we detected hospital-associated *E. faecium* (HA-Efm) in effluent from the University Medical Center in Utrecht (UMCU), influent and effluent from the sewage treatment plant in Utrecht (STPU) and in surface water from Nieuwegein and Lobith (Dutch Rhine). As expected for a nosocomial-derived organism, we found high levels in hospital sewage (~10^4-10^6 CFU/100ml). Dilution with municipal sewage led to lower counts (~10^4-10^5 CFU/100ml). Still, counts in STP influent suggested that the majority of hospital-associated *E. faecium* originated from the community. We found a slightly lower elimination of hospital-associated enterococci versus susceptible enterococci during sewage treatment. Absolute concentrations of hospital-associated *E. faecium* were much lower in surface water (<1 - 200 CFU/100ml) than in the STPU effluent (~10^2-10^3 CFU/100ml). Further characterization of the obtained isolates with multiple locus variant analysis (MLVA) showed a high frequency of MLVA type 159 and 12, again suggesting that antibiotic resistant *E. faecium* typical for hospital outbreaks might spread from the hospital into the environment. Hospital association was also suggested by multi locus sequence typing (MLST) of selected isolates. The detection of hospital-associated *E. faecium* in surface water shows that exposure to water contaminated by sewage effluent might represent a transmission route for community acquisition of this bacterium.

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List of abbrevations

ampR	Ampicillin Resistant
ampS	Ampicillin Sensitive
ARE	Ampicillin-Resistant enterococci
AR-Efm	Ampicillin-Resistant *Enterococcus faecium*
ATCC	American Type Culture Collection
BD	Becton Dickinson
bp	Base Pair
CC-17	Clonal Complex-17
CFU	Colony Forming Unit
CI	Confidence Interval
CLSI	Clinical and Laboratory Standards
ddl	D-alanine-D-alanine ligase
DLV	Double Locus Variant
DNA	Deoxyribonucleic acid
E0980	Ampicillin-Resistant *Enterococcus faecium*
E1162	Ampicillin-Resistant *Enterococcus faecium* and *esp* positive
esp	Enterococcal surface protein
GW	Geriatric Ward
HA-Efm	Hospital-Associated *Enterococcus faecium*
ICU	Intensive Care Unit
IRAS	Institute of Risk Assesment Sciences
ISO	International Organization for Standardization
MLST	Multilocus Sequence Typing
MLVA	Multiple Locus Variable-Number Tandem Repeat Analysis
MT	MLVA type
NaOH	Sodium Hydroxide
PAI	Pathogenicity island
PBP	Penicillin-Binding Protein
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
RIVM	National Institute for Public Health and the Environment
S	Svedberg unit
SDS	Sodium Dodecyl Sulfate
ST	MLST type
STP	Sewage Treatment Plant
STPS	Sewage Treatment Plant Someren
STPU	Sewage Treatment Plant Utrecht
SLV	Single Locus Variant
TLV	Triple Locus Variant
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
UMCU	University Medical Center Utrecht
vanA	D-Ala-D-Lac ligase
vanB	D-Ala-D-Lac ligase
VNTR	Variable-Number Tandem-Repeat
VPH	Veterinary Public Health
VRE	Vancomycin Resistant enterococci
VREF	Vancomycin-Resistant *Enterococcus faecium*

Introduction

Antimicrobial resistance in the environment

Antibiotics are extensively used to prevent or to treat microbial infections in human and veterinary medicine. Most of these compounds are partially metabolized by patients and are then discharged to hospital sewage or to municipal waste water. In addition to antimicrobials and disinfectants, resistant bacteria themselves are excreted by humans and animals and are emitted into sewage or manure and other environmental compartments. Many organisms have always been resistant to a particular antibiotic agent by nature of their physiology or biochemistry. Susceptible organisms can also become insensitive by mutation or by incorporation of genetic information which encodes resistance (Kümmerer 2004). These resistance genes are located on the bacterial chromosome or on extra-chromosomal plasmids, and are transmitted to the next generation (vertical gene transfer). Genetic elements, such as plasmids, can also be exchanged among bacteria of different taxonomic affiliation (horizontal gene transfer). Horizontal gene transfer may be achieved by a) conjugation through cell to cell contact, b) transduction using bacterial viruses as a gene vector, or c) transformation when resistant plasmid DNA is transferred into bacteria (Madigan et al. 1997; figure 1). Horizontal gene transfer is common in systems where the density of bacteria is high and so, accordingly, is the chance of two suitable bacterial coming close to each other (Bates 1997; Harwood et al. 2001), i.e. in aerobic and anaerobic septic tanks of sewage treatment plants biofilms. While resistance in farm animals will be spread mostly to manure and soil, resistance in humans will mostly impact the water chain. Transfer of resistant bacteria from environmental compartments to humans may occur through surface water (if surface water is used for irrigation, or as recreational water), through manure (if manure is used as a fertilizer) or through food (if resistant bacteria are present in meat) (Perreten and Schwarz 1997; Schwartz et al. 2003). One additional concern is the possible presence of resistant pathogens or resistant bacteria in drinking water. This might occur if surface water is used for drinking water production and treatment is not sufficiently effective.
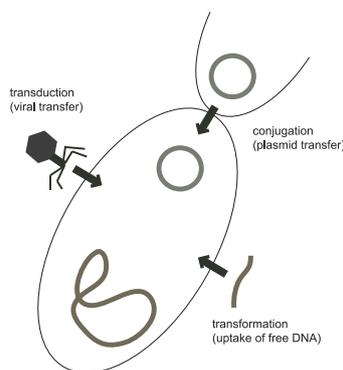
Figure 1. Schematic drawing of mechanisms of horizontal gene transfer between bacteria: transfer of circular plasmids (conjugation), uptake of free DNA (transformation) and transfer by viral delivery (transduction).

Enterococci – clinical importance

Enterococci belong to the normal flora of the gastrointestinal tract of humans and animals. Under normal circumstances they are harmless commensals, and are even believed to have positive effects

on a number of gastrointestinal and systemic conditions (Mitra and Rabbani 1990; Franz et al. 1999; Benyacoub et al. 2003). However, when the commensal relationship with the host is disrupted, enterococci can become opportunistic pathogens and cause invasive diseases (Jett et al. 1994). Though not as virulent as other Gram-positive organisms, they can lead to a variety of clinical syndromes including endocarditis, bacteraemia, meningitis, wound and urinary tract infections, and they are associated with peritonitis and intra-abdominal abscesses (European Antimicrobial Resistance Surveillance System 2007). Outside of a host organism, enterococci are bacteria that are able to survive under unusually wide ranges of temperature, pH, and salinity and that can resist the bactericidal effects of detergents (Shepard and Gilmore 2002).

Over the last two decades, nosocomial infections caused by enterococci have emerged and their incidence has rapidly increased, first in the United States and recently in Europe (Rice 2001; Oteo et al. 2007; Top et al. 2008). The vast majority of clinical enterococcal infections in humans are caused by *Enterococcus faecalis* (around 80% of clinical isolates) and *Enterococcus faecium* (most of the remainder, Huycke et al. 1998). The emergence of *Enterococcus faecalis* and *Enterococcus faecium* was paralleled by increases in glycopeptide and high-level aminoglycoside resistance, both important compounds for the treatment of human infections (Shepard and Gilmore 2002).
The epidemiology of infection with glycopeptide-resistant enterococci (vancomycin-resistant enterococci, VRE) differs between Europe and the United States. In Europe, VRE were frequently isolated from farm animals. This has been associated with the abundant use of avoparcin as a growth promoter in the agricultural industry before 1997 (Bonten et al. 2001). In the United States, avoparcin was never approved for use in agriculture, and neither were any other glycopeptides; consequently, VRE have not been found in animals or healthy persons. However, nosocomial VRE infection and transmission has shown a dramatic increase in the United States and was attributed to the widespread use of vancomycin in US hospitals (Willems et al. 2005).

The emergence of vancomycin-resistant *Enterococcus faecium* (VREF) in the United States in the 1990s was preceded by the emergence of ampicillin-resistant *Enterococcus faecium* (AR-Efm) in the 1980s (Grayson et al. 1991; Jones et al. 1995; Rice 2001; Shepard and Gilmore 2002). In retrospect, it seems likely that the acquisition of ampicillin resistance was an earlier step in hospital adaptation of *Enterococcus faecium*, facilitating the subsequent emergence of VREF (Willems et al. 2005; Leavis et al. 2006). Since 2000, infection rates of VREF were rising in European hospitals (European Antimicrobial Resistance Surveillance System 2007), suggesting that the increase of VREF in Europe follows the American epidemiology with a 10-year delay. During the last three years, however, VREF infection rates have stabilized (ECDC 2010).

Although *Enterococcus faecalis* is the causative agent in most enterococcal infections, a partial replacement of *Enterococcus faecalis* by *Enterococcus faecium* has been noted in the last years, and presently, up to one-third of enterococcal infections in some countries are attributed to this species (Iwen et al. 1997; Damborg et al. 2009). This shift may be explained by changes in the patterns of antimicrobial usage, which may have resulted in the emergence of distinct hospital-associated *Enterococcus faecium* (HA-Efm) strains (Willems et al. 2005, Willems and Schaik 2010). These HA-Efm strains are characterized by ampicillin and quinolone resistance and a putative pathogenicity island (PAI) (Top et al. 2008). It has previously been thought that hospital-associated *E. faecium* from different countries belong to one single clonal lineage, with MLST sequence type 17 as the presumptive founder (Willems et al. 2005). These HA-Efm were thus initially named "clonal complex 17" or CC17. Recently, it appeared that HA-Efm might have evolved from several different ancient clones instead (Willems and Schaik 2010). Still, the hospital-associated *E. faecium* isolates occur to be genotypically distinct from community-derived human isolates and animal strains.

The majority of HA-Efm contain the *esp* gene which codes for one enterococcal surface protein. In *E. faecalis*, *esp* is thought to be an adhesion protein contributing to colonization of urinary tract epithelial cells and biofilm formation (Shankar et al. 2002; Timmers et al. 2002). Although detailed experimental evidence is not yet available, the higher prevalence of the *E. faecium esp* gene in clinical isolates suggests a role of *esp* in the pathogenesis of *E. faecium* infections (Willems et al. 1999; van der Steen et al. 2000; Baldassarri et al. 2001; Eaton and Gasson 2001; Coque et al. 2002; Eaton and Gasson 2002; Leavis et al. 2003). Furthermore, the presence of the *esp* gene in *E. faecium* was also strongly associated with hospital outbreaks of vancomycin-resistant *E. faecium*, suggesting a role for *esp* in nosocomial transmission (van der Steen et al. 2000). The presence of the *esp* gene in isolates from epidemiologically distinct sources seems to differ between *E. faecalis* and *E. faecium*. While the presence of the *esp* gene in *E. faecium* is confined to clinical and epidemic isolates, *esp*-carrying *E. faecalis* are found in isolates from farm animals and food. This could be related to differences in the frequency of horizontal transmission of the *esp* gene in *E. faecalis* and *E. faecium* (Leavis et al. 2004).

Research objectives

Aim of this study is to investigate the occurrence of hospital-associated bacteria in the water chain. It was hypothesized that hospital waste water and municipal waste water systems may lead to an input of resistant bacteria into the environment. Given that ampicillin-resistant and *esp*-positive *E. faecium* have nearly exclusively been found in hospitalized humans, we suggest to use these bacteria as an indicator for hospital-associated resistant bacteria in general. The first research objective was thus to quantify hospital-associated *E. faecium* in hospital sewage, STP influent and STP effluent. By studying surface water used for drinking water production, we sought to identify possible public health risks of an increased presence of resistant bacteria in the environment. Technically, the detection of hospital-associated *E. faecium* was based on enumeration of enterococci with ampicillin resistance and the *esp* gene as specific markers and on a molecular characterisation of the HA-Efm strains.

Materials and methods

Sampling points and filtration

Five-liter samples from hospital sewage, STP's influent and effluent and surface water samples were collected from eleven locations in alcohol cleaned containers and sterile bottles. Eight random sewage samples were collected from December to May 2009. Samples were taken from two sewers that collected sewage from, inter alia, the hematology and geriatric ward. These two wards had the highest point prevalences (34.6 and 34.8%, respectively) of HA-Efm in a previous study (Top et al. 2007). Six 24h proportional influent and effluent samples from January until May 2009 were also collected from the STP in Utrecht (STPU), where UMCU's sewage is treated and discharged in the surface water network. Samples were kindly provided from the laboratory of the STPU. Additional five random surface water samples during the same period were collected from Nieuwegein used as a drinking water preparation source and surface water from the Dutch Rhine in Lobith. Single random samples from another hospital sewage (Sophia), influent and effluent of the STP in Someren (STPS), sewage from an elderly house and surface water samples from Dommel before and after the STPS (only enrichment method) in Someren were also kindly send to our lab from the RIVM in Bilthoven.

Isolation, identification and quantification

Samples were transported to the lab in cool conditions and processed within six hours of collection. A series of 3-fold dilutions of the samples were then prepared in peptone physiological salt solution (Oxoid, CM0733). A membrane filtration method was used for the detection and enumeration of all intestinal enterococci (ISO 7899) and a parallel method but with the addition of 16mg/L (CLSI guidelines) ampicillin (Sigma, A0166) in both media for the detection and enumeration of all ampicillin-resistant enterococci (ARE). In short, 0.22μm, 47mm cellulose nitrate filters (Whatman) were used and then aseptically placed on Slanetz and Bartley agar (Oxoid, CM0377) petri dishes, incubated at 36°C for 44 hours and then confirmed on Enterococcosel agar (BD, 212207) at 44°C for 2h. All typical colonies that showed a tan to black color in the surrounding medium were counted as total intestinal enterococci and ARE. All well isolated ARE colonies from all filters were stored. Data collected from all 11 locations were checked for dilution plausibility, and counts were derived from filters containing between 10 and 100 colonies. In addition to the membrane filtration an enrichment method was also used for ARE isolation. Triplicate double strength 5ml enterococcosel broths with 75mg/L (CLSI guidelines) aztreonam (Sigma, A6848) for gram-negative bacteria inhibition, were inoculated with 5ml raw sample and incubated at 37°C for 48h. Using a 10μl sterile loop, enterococcosel agar petri dishes with 16mg/L ampicillin were streaked and incubated at 44°C for another 48h. Roughly 40 well isolated colonies from all three plates were stored. A total of 1960 ARE isolates were collected from both membrane filtration and enrichment method and stored in 22% glycerol (Merck) in -80°C until further use. Ampicillin-sensitive (ampS) *Enterococcus faecalis* strain (ATCC29212) was used as a positive and negative control, an E.coli strain (ATCC25922) as a negative control and an E.faecium CC17 (E1162) an ampicillin-resistant (ampR) and *esp* positive, as a positive control. E1162 and E0980 strains were kindly given to us by Dr. Rob Willems (UMCU).

DNA extraction and multiplex single colour qPCR

From the total 1960 ARE isolates stored from both methods, a smaller portion of approximate 10 isolates of each sampling date, location and method were again cultured on enterococcosel agar with

16mg/L ampicillin at 44°C for 48h. A total of 707 (304 filtration, 403 enrichment) isolates were successfully recovered from most locations and dates. Six well isolated colonies from each isolate were picked and suspended in 20µl lysis buffer 0.25% SDS, 0.05 N NaOH (Merck). After incubation at 95°C for 5 min a short centrifugation was performed. Samples were then diluted with 180µl (10 mM Tris-HCl, pH 8.5, Merck), thoroughly mixed, and another centrifugation for 5 min at 14,000rpm was performed to remove cell debris. Supernatants from 10x dilutions were frozen at -20°C until further use. During this study an experimental multiplex assay with a single colour qPCR machine was designed. In brief, two suitable primer pairs were selected for the identification of *Enterococcus faecium* based on the amplification of a fragment internal to the *ddl* gene encoding a D-Ala–D-Ala ligase, and of the *esp* virulence gene of *Enterococcus faecium* associated with epidemicity in hospitals. Primers were primarily chosen based on the large difference between them in product size, so that the *ddl* amplicon would melt at a much higher temperature than the *esp* amplicon (Leavis et al. 2003; Depardieu et al. 2004) (Table.1). Figure 2 demonstrates that the melting curve for the *esp* PCR product (red curve) is well separated from the melting curve for the *ddl* PCR product size was confirmed with electrophoresis revealing only the expected 510 bp *esp* product and 1091bp dll product (Figure 2). qPCR assays were performed in duplicate in a total of 20µl containing 10µl of iQ SYBR Green Supermix (Biorad) 5µl bacterial DNA, 200nM for each esp primer, 600nM for each *ddl* primer and 1.8µl sterile milliQ water. An initial denaturation step for activation of the hot-start iTaq™ DNA polymerase enzyme at 95°C for 3 min was followed by 32 cycles of denaturation at 95°C for 15sec, annealing at 56°C for 15sec, extension at 70°C for 1min with signal acquisition and another 15sec at 83.5°C for signal acquisition. The melt curve was measured for 81 cycles at 55°C for 15sec. For each 96-well plate E1162 was used as a positive control for both *esp* and *ddl* genes, E0980 for *ddl* and ATCC 29212 as a negative control. After amplification, ~15% of randomly selected qPCR products from various 96-well plate sets were subjected to agarose gel electrophoresis. 5µl of the qPCR products selected were mixed with 2µl of gel loading buffer (5X Go Tag Green Mastermix, Promega) and electrophoresed in a 1.5% agarose gel (Invitrogen) for 1.5h at 90V (<0.1A) in 1X TAE (Tris-acetate-EDTA). A 100bp Benchtop DNA ladder (Promega) was used as a molecular size marker.

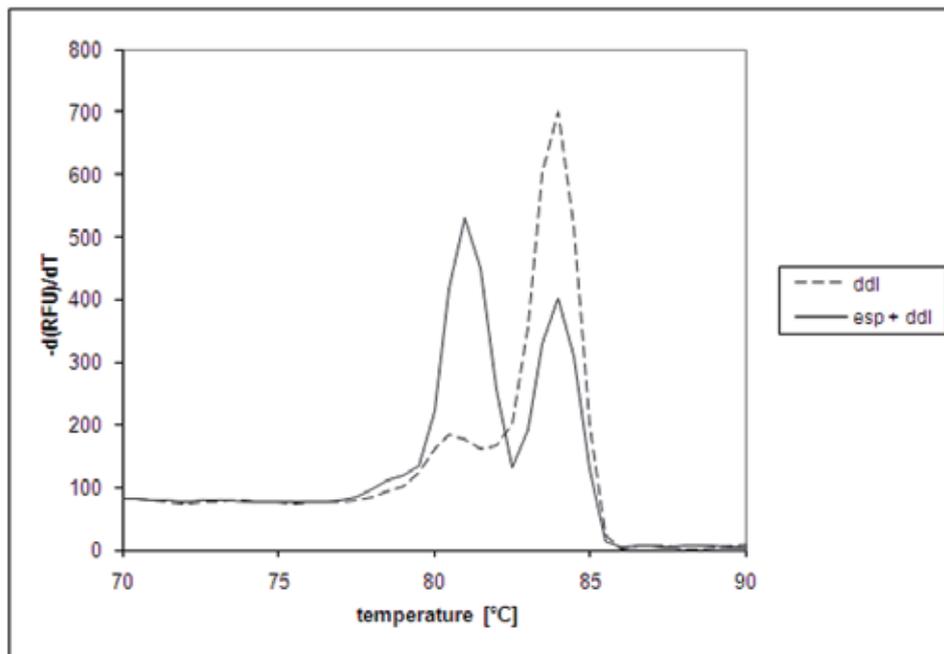| Table 1. Primers used in this study					
Primer	Sequence	Gene	Encoding	bp PCR product	Reference
ddlFD-fw	GAGTAAATCACTGAACGA	*ddl*	D-Ala-D-Ala ligase of E. faecium	1094	Depardieu et al., 2004
ddlFD-rv	CGCTGATGGTATCGATTCAT				
espHL-fw	AGATTTCATCTTTGATTCTTGG	*esp*	enterococcal surface protein	510	Leavis et al., 2003
espHL-rv	AATTGATTCTTTAGCATCTGG				

Figure 2. Simultaneous analysis of esp- and ddl carriage in Enterococcal isolates: meltcurve analysis of a multiplex PCR of a strain carrying both esp and ddl (left, solid line) and a strain carrying only ddl (right, broken line).

Bacterial counts (prevalence) were expressed as counts / 100 ml and grouped according to the sample origin. While total enterococci and ARE were determined directly, AR-Efm and HA-Efm prevalence were calculated from ARE total counts by correction with the proportion of AR-Efm and HA-Efm amongst selected ARE isolates (Figure 3). Further, resistance percentages were calculated amongst total enterococci for ARE (figure 3). For enterococci derived from enrichments, resistance percentages for AR-Efm and HA-Efm were calculated amongst total ARE (figure 4).

Statistical analysis
The difference between averaged log transformed counts at different sampling locations was tested by a statistical test procedure for comparison of multiple means of samples with different and non-normal distributions in unbalanced designs (Herberich et al. 2010). Statistical testing was performed in R (R development core team 2010).

Multiple-locus variable-number tandem repeat analysis (MLVA)
The same AR-Efm DNA supernatants derived from the filtration method from all six locations (UMCU-North and South, STPU influent and effluent, Lobith and Nieuwegein) previously extracted and screened for the *esp* and *ddl* genes were also used to further assess the genetic relatedness of *E. faecium* isolates, based on differences in numbers of tandem repeats in multiple loci on the chromosome of *E. faecium*, as previously described with minor modifications (Top et al. 2004). An MLVA profile was created from the number of repeats for each of the VNTR loci and for each MLVA profile, an MLVA type (MT) was assigned (http://www.mlva.umcutrecht.nl).

Multilocus sequence typing (MLST)
The genetic relatedness of MLVA types was confirmed with MLST on a subset of representative isolates. MLST was carried out with a standard set of primers that amplify the 7 genes included in the *E. faecium* MLST scheme (Homan et al. 2002). Information on these loci, the latest set of primers, amplification conditions and details of all isolates are available on the MLST Web site (http://

efaecium.mlst.net). Sequence results were read, evaluated, aligned, compared to the reference set of alleles and for each MLST profile a sequence type (ST) was assigned using BioNumerics sequencing software (version 5.0; Applied Maths). Profile clustering was performed with the goeBURST algorithm (Francisco et al. 2009). This algorithm is implemented as a Java applet at http://goeburst.phyloviz.net/.

Results

Hospital-associated *E. faecium* among ampicillin-resistant enterococci, and enumeration of total Enterococci and hospital-associated *E. faecium*

The numbers of total enterococci and ampicillin-resistant enterococci were determined by culture methods. The proportion of *E. faecium* and hospital-associated *E. faecium* among the ampicillin-resistant enterococci was determined by amplification of the *esp* and *ddl* gene in a subset of the isolates (if possible, 10 per location and date of sampling, totalling 304). An overview on the results of the determination of *E. faecium* and *esp* among the ampicillin-resistant isolates is shown in table 2. In general, a very large proportion of the ampicillin-resistant enterococci were shown to be hospital-associated *E. faecium*.

Table 2. The number of ampicillin-resistant enterococci that were tested and found positive for species identity (E. faecium, AR-Efm) and hospital association (esp carriage, HA-Efm), and their percentages, in isolates retrieved from the filtration method

|	ARE n	AR-Efm n	HA-Efm n	AR-Efm / ARE %	HA-Efm / ARE %
UMCU north	83	80	71	96	86
UMCU south	60	59	58	98	97
STP influent	40	39	34	98	85
STP effluent	43	43	35	100	81
Lobith	40	40	23	100	58
Nieuwegein	17	15	15	88	88
Sophia hospital	18	18	12	100	67
Someren elderly home	3	3	0	100	0

The counts of total enterococci and hospital-associated *E. faecium* (determined as the number of ampicillin-resistant *E. faecium* that carried the *esp* gene) determined in the different samples are shown in table 3 and are visualized in figure 3.

Table 3. Overview of counts of total enterococci, ampicillin-resistant enterococci, and esp-carrying E. faecium in different water samples. The average and standard deviation over all sampling dates are given. Locations with a statistically different count are denoted by a different letter in column "group", while counts in two groups sharing a letter are not statistically significant. Last, the average percentage of ampicillin resistance and hospital association (esp-carrying E. faecium) among total enterococci is shown

	Total Enterococci [log CFU / 100 ml] Average	Std. dev.	group	Ampicillin-resistant Enterococci [log CFU /100 ml] Average	Std. dev.	group	Resistance %	*esp*-carrying *E. faecium* [log CFU/ 100 ml] Average	Std. dev.	group	% of total Enterococci
UMCU north	7.15	0.26	a	5.77	0.74	a	11	5.65	0.79	a	10
UMCU south	6.36	0.99	ab	4.42	1.63	abc	6	4.41	1.64	abc	6
STP influent	5.90	0.18	b	4.22	0.45	b	3	4.38	0.37	b	3
STP effluent	3.89	0.25	cd	2.67	0.81	ce	9	2.82	0.28	c	8
Lobith	2.30	1.14	de	1.36	1.12	cd	16	1.60	1.02	cd	13
Nieuwegein	1.46	0.67	e	1.17	1.21	de	13	1.12	1.22	d	23

Concentrations of enterococci were highest in hospital sewage (around $10^6 - 10^7$ CFU / 100 ml). Concentrations in sewage treatment plant influent were smaller than 10^6 CFU / 100 ml, and numbers of enterococci were reduced during sewage treatment by about two log units (effluent: around 10^4 CFU / 100 ml). In surface water, concentrations were between 6 and 3200 CFU / 100 ml. Concentrations of ampicillin-resistant enterococci and *esp*-carrying *E. faecium* showed the same trends of reduction from hospital waste water to surface water. However, the reduction during sewage treatment was slightly smaller for *esp*-carrying *E. faecium*, and these hospital-associated *E. faecium* were only detectable in 5 out of 8 surface water samplings. For all total enterococci, ampicillin-resistant enterococci and hospital-associated *E. faecium*, STP effluent counts were statistically different from hospital waste water counts (UMC North) and STP influent counts. Surface water counts were significantly smaller than STP effluents only for the total enterococci and HA-Efm counts in Nieuwegein. However, the statistical significance of these differences is largely influenced by the variance between counts on different sampling dates. These variances are lower for STP counts, which is in accordance with the method of sampling: in the STP, samples are taken time-proportionally, whereas single batch samples were taken at all other sampling locations, leading to greater variability in counts between samples. The percentages of ampicillin-resistant enterococci and *esp*-carrying *E. faecium* among total enterococci are also shown in table 3. However, these are expected to have a large variation: while counts are generally compared on a log scale, resistance percentages are given on a normal scale and can be greatly changed by small deviations in total or resistant counts.

Figure 3. Box- and whisker plot of the counts of total enterococci and of esp-carrying E. faecium (hospital-associated E. faecium) in the different sampling locations. Averages of the different sampling dates are taken.
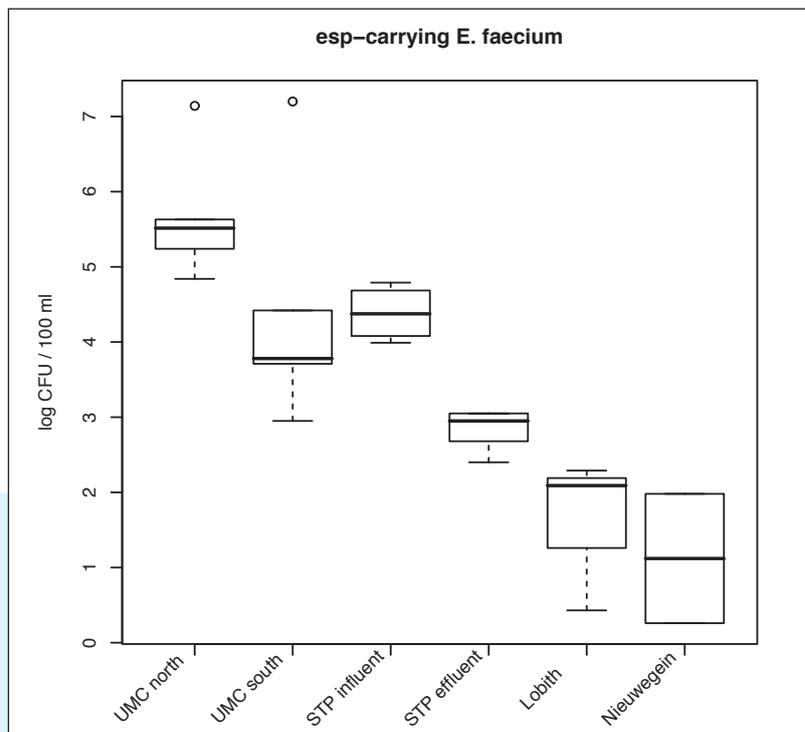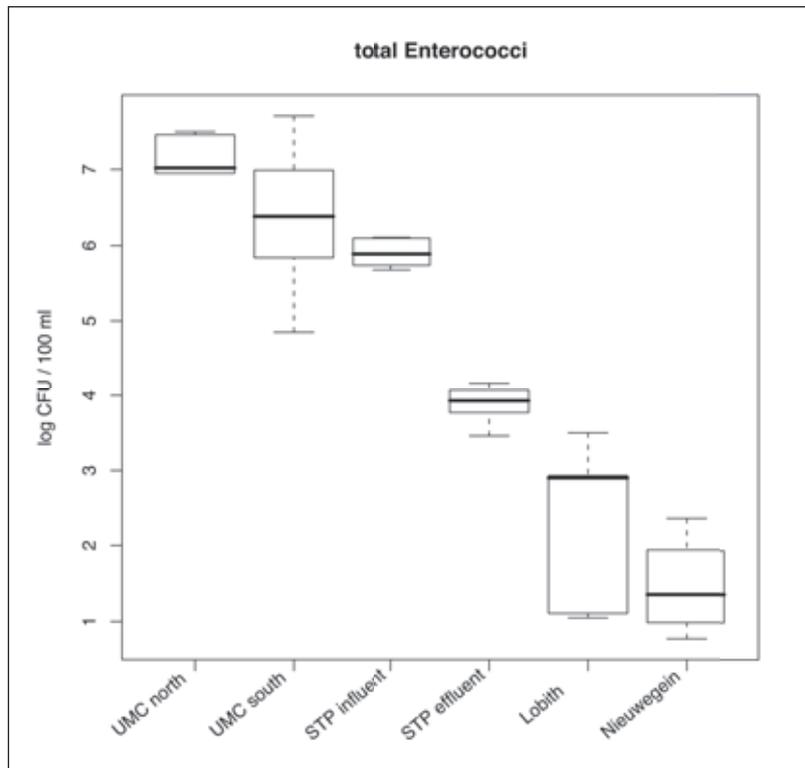
Enrichment results

The remaining 403/667 ARE isolates from the enrichment method were also PCR-tested for presence of the *esp* and *ddl* gene. We observed lower HA-Efm prevalence in STPU influent and effluent, and in surface water samples from Lobith and Nieuwegein. Additional data from Dommel surface water samples were also obtained with 65% AR-Efm (11/17) and 0% HA-Efm (0/17) from Dommel-before, and 75% AR-Efm (12/16) and 0% (0/16) HA-Efm from Dommel-after (Figure 4). Since the enrichment method can be biased by the preferential growth of some strains, these results were not further used.

Figure 4. Percentages of E. faecium and esp-carrying E. faecium among ampicillin-resistant enterococcal isolates obtained from enrichments.
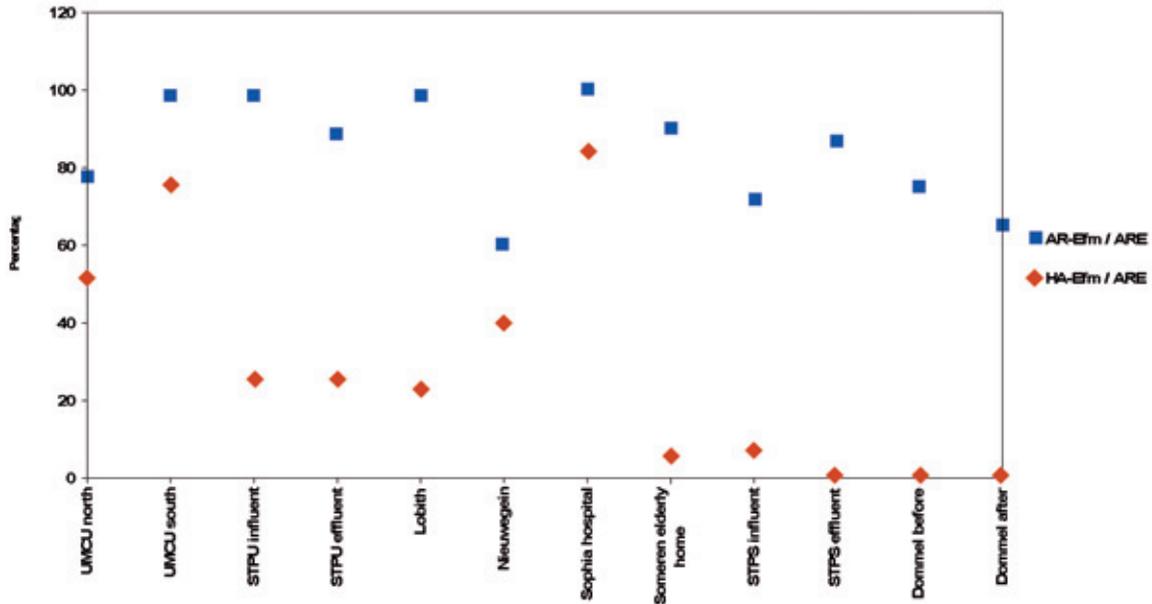
Multiple-locus variable-number tandem repeat analysis results

MLVA typing of 214/276 AR-Efm strains from six locations derived from the filtration method (UMCU-North n=46, UMCU-South n=46, SPTU-Influent n=36, STPU-Effluent n=38, Lobith n=37, Nieuwegein n=11) revealed 13 different MTs (Figure 5), including two MTs not previously described (MT-333 and MT-334). MT-159 (71%) was found to be the most predominant genotype from all six locations and MT-12 (14%) was the second most frequent MT found in most locations (Figure 6). Average frequency of the *esp* gene from all six locations was 99% for MT-159 and 100% for MT-12, in contrast with the MT-1 isolates that carried the *esp* gene in only 1 out of 8 isolates, and with nine MT-139 isolates collected the same day from Lobith with complete *esp* absence. Low *esp* frequencies were also found in nine other MT's found from various locations in smaller numbers (MT-10 n=5, MT-7 n=4, MT-205 n=2, MT-148 n=1, MT-25 n=1, MT-31 n=1, MT-39 n=1, MT-333 n=2, and MT-334 n=1).

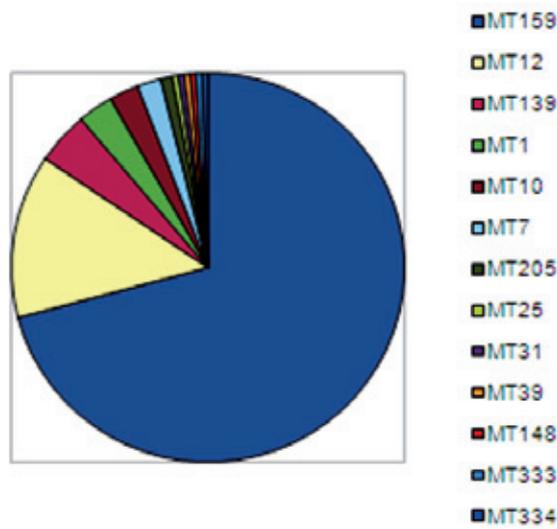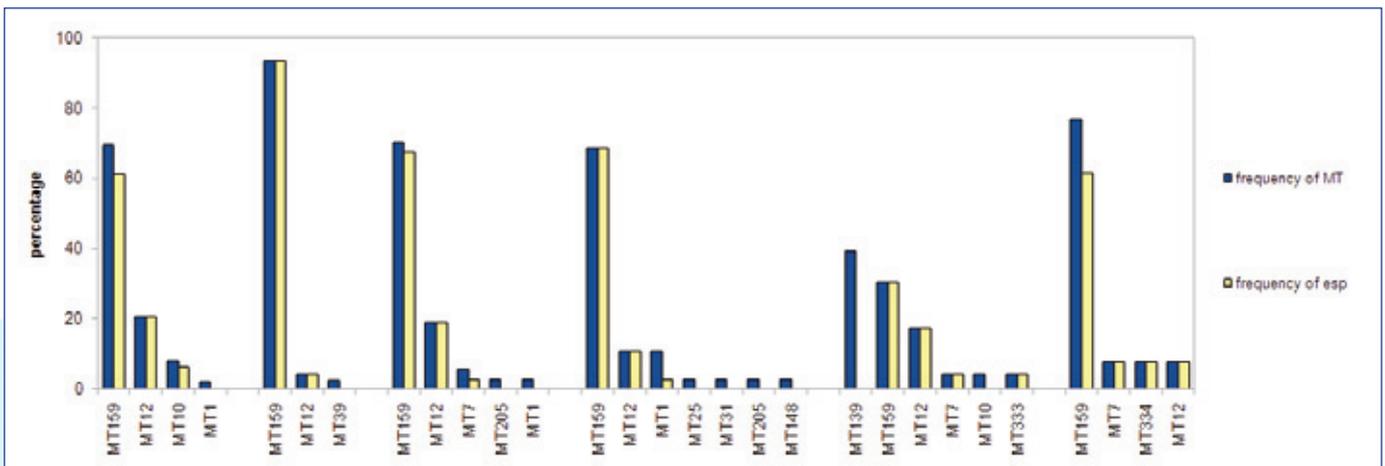*Figure 5. Percentages of MLVA types among isolates obtained from all sampling locations.*

Figure 6. Frequency of MLVA types per location

Multi-locus sequence typing results

To confirm hospital association of the MT types found, MLST typing was performed on MT-159 (n=6) and MT12 (n=6), for which one representative strain from each cluster and from all six locations was randomly selected. Single strains from MT-139 and MT-333 from Lobith, MT-205 from STPU-influent and MT-334 from Nieuwegein were also typed by MLST (Table 4). All six MT-12 isolates revealed a single sequence type ST-117, in contrast with MT-159 isolates which showed ST-78 (4/6) and two single-locus variants (SLVs) of ST-78 (ST-192, from STPU effluent, and ST-341 from Lobith). MT-139 revealed the ST-266, a triple-locus variant (TLV) from ST-78. MT-205 and MT-333 revealed the same ST-18, a double-locus variant (DLV) from the predicted founder ST-17. MT-334 was not previously described from the *E. faecium* MLST allelic profile, the nearest match was ST-192 with a SLV. Figure 7 shows a population snapshot of the genetic relatedness of all *E. faecium* isolates contained in the MLST database, with ST identified in this study explicitly shown.

While figure 7 suggests that many *E. faecium* sequence types originated from a common ancestor (ST17, after which the hospital-derived isolates had primarily been termed clonal complex 17), newer clustering methods suggest that there might actually be several clonal complexes (originating from e.g. ST17, ST18, ST78 and ST192), of which some are predominantly found in hospitals (Willems and Schaik 2010). Some other clonal complexes do not exclusively exist of hospital-related isolates, but also comprise community- or animal derived strains (Willems and Schaik 2010).

Table 4. Multilocus sequence types (ST) of selected isolates with representative MLVA types (MT)

MT	Location	ST	*esp*
MT159	UMCU south	ST78	+
MT159	UMCU north	ST78	+
MT159	STPU influent	ST78	+
MT159	STPU effluent	ST341	+
MT159	Nieuwegein	ST78	+
MT159	Lobith	ST192	+
MT12	UMCU south	ST117	+
MT12	UMCU north	ST117	+
MT12	STPU influent	ST117	+
MT12	STPU effluent	ST117	+
MT12	Nieuwegein	ST117	+
MT12	Lobith	ST117	+
MT139	Lobith	ST266	–
MT205	STPU influent	ST18	–
MT333	Lobith	ST18	–
MT334	Nieuwegein	NF (nearest match with ST192)	+

Figure 7. Population snapshot of E. faecium isolates according to their MLST sequence types generated with goeBURST. Sequence types are indicated by numbered boxes. The size of the ST box is relative to the number of isolates with a particular ST in the database. Circles indicate ST groups that have often been associated with hospital outbreaks (Willems and Schaik 2010). ST found in this study are indicated together with their origin. goeBURST algorithm as described in Francisco et al, 2009
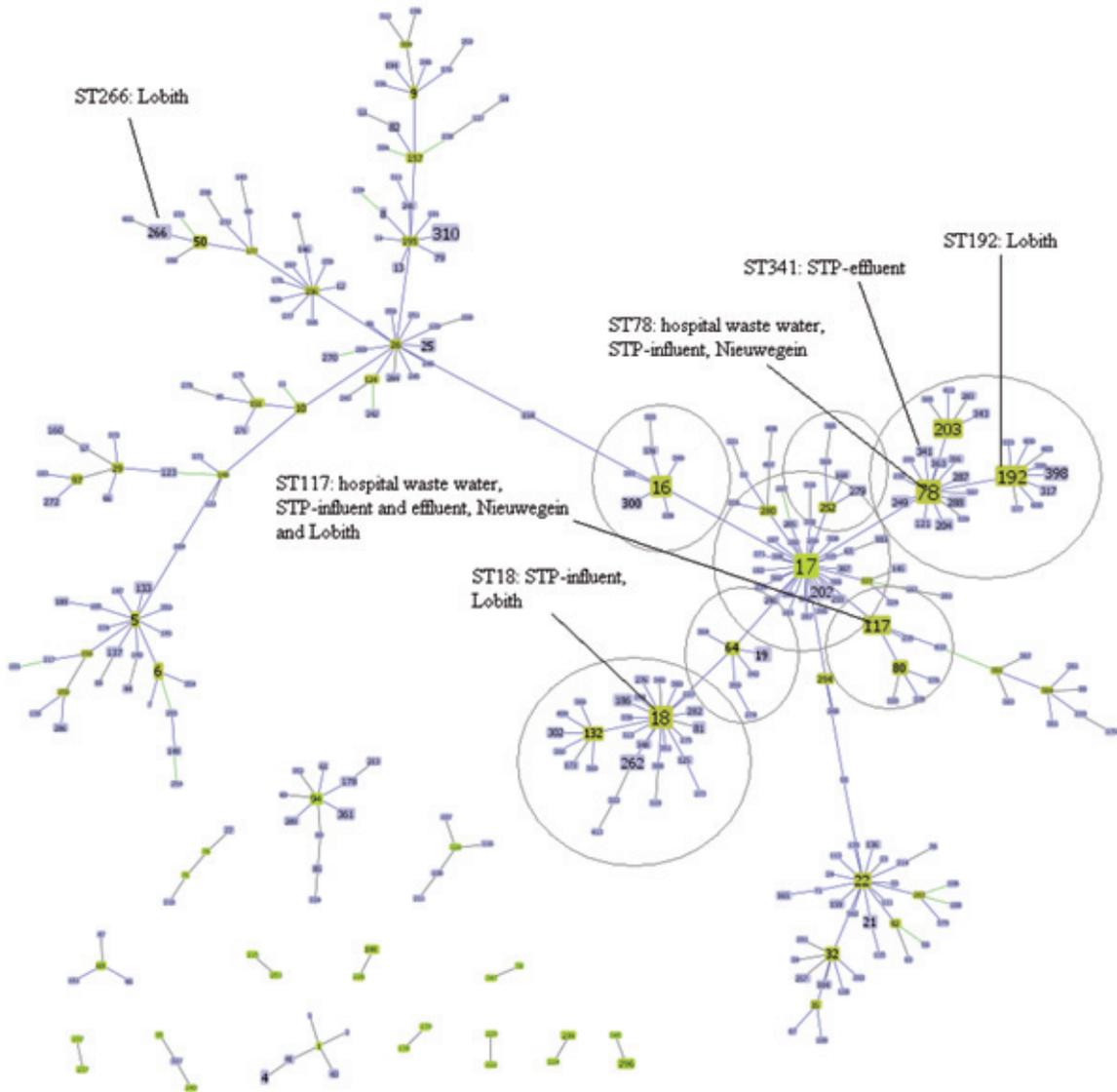
Discussion

This study aimed at estimating the flow of hospital-associated *Enterococcus faecium* (HA-Efm) strains from hospital sewage to STPs and surface water, using ampicillin resistance and the *esp* gene as specific markers.

As expected, we found the highest concentrations of HA-Efm in hospital sewage of wards selected for their high prevalence of hospital-associated *E. faecium*, a clear indication of its nosocomial origin. Recent studies indicated that the epidemiology of HA-Efm within the haematology and gastroenterology / nephrology wards of this hospital (UMCU) is characterized by high admission, high acquisition and high environmental contamination rates, resulting from cross-transmission, readmission and antibiotic pressure (de Regt et al. 2008).

The two sampling locations showed different absolute concentrations, which is most likely due to differences in the contribution of the wards with high HA-Efm prevalence to the waste water. In addition, the sample from UMCU south had been diluted with effluent from other wards from close-by drainage pipes at several sampling events. Random samples from both hospital sides had larger variations in bacterial counts than samples from the sewage treatment plant, which can be assigned to the 24h proportional sampling at the STP.

We found lower HA-Efm concentrations in the STPU influent compared to the UMCU effluent, due to dilution with municipal sewage. Previous studies indicated that dilution of hospital effluent by municipal sewage is normally more than 100-fold (Kümmerer and Henninger 2003) and the number of multi-drug resistant bacteria in sewage correlated with the size and the number of hospitals connected to an STP (Kümmerer 2004).

In Utrecht, the total yearly volume the STPU received for 2007 and 2008 was $2.6*10^7$ m³, with a contribution of the UMCU of about $1.98*10^5$ m³ in 2008 (0.76%). The STPU also treats effluent from a number of other smaller hospitals in Utrecht. Still, if it is assumed that hospital effluent represents 0.76% of STPU inflow and that HA-Efm are not present in municipal waste, HA-Efm counts in sewage influent would amount to roughly $8*10^2$ HA-Efm / 100 ml influent. Concentrations found in influent were about thirty times as high ($2.4*10^4$/100 ml), resulting in a hospital contribution of about 3.3%. Based on these estimates, the majority of the total amount of HA-Efm would be emitted from municipal sewage. Recent studies found that AR-Efm prevalence in the general community is small (0%-6%), but study designs were limited in sample size and material specificity and results may present an underestimation (Aarestrup et al. 2000; Duh et al. 2001; Novais et al. 2006; Biavasco et al. 2007). In addition, dogs have been shown to be frequent carriers of human hospital-associated HA-Efm (Damborg et al. 2009). Total HA-Efm produced and discharged from 1-6% of the general community (420 000 inhabitant equivalents, which is an overestimation of the number of people of that area) will exceed hospital discharge by far, as high-risk wards have about 50 beds, and the total hospital has about 1000 beds. Thus, though community carriage is relatively low, it might dominate absolute discharge due to the relatively low number of hospitalized patients amongst the whole community. Similarly, in Germany, only one-quarter of the total consumption of antibiotics can be attributed to hospitals (Kümmerer and Henninger 2003). Still, more studies are necessary to confirm the major role of the community in HA-Efm/AR-Efm discharge.

Sewage treatment led to a further reduction in total enterococci (2.01 log units on average, n=6) and HA-Efm (1.45 log units on average, n=4). The Utrecht RWZI consists of a primary settler, two aerated activated sludge treatments and settling tanks and has no tertiary treatment. The overall reduction is slightly lower than expected for a relatively large treatment plant (Hoogenboezem 2007; Kistemann

et al. 2008). Secondary treatment seems to be slightly (but statistically significantly) less efficient for hospital-associated bacteria than for other enterococci. Previous studies on antibiotic resistance levels in STPs have obtained contradictory results on whether or not wastewater treatment can increase the proportion of antibiotic resistant bacteria (Guardabassi et al. 1998; Goni-Urriza et al. 2000). More recent studies found that the biological treatment process in a conventional wastewater treatment plant may result in a selective increase of the antibiotic resistant bacteria population and the increased occurrence of multidrug resistant bacteria (Zhang et al. 2009). Although the mechanisms that contribute to a selective increase of antibiotic resistant bacteria in STPs remain undefined, a number of studies have shown that the conditions in STP favor antibiotic resistant bacteria (Goni-Urriza et al. 2000; Iwane et al. 2001; Schwartz et al. 2003). Some studies also indicated that the environmental conditions in STPs may increase the likelihood of gene transfer: Kruse and Sorum (1994) reported that a high percentage (89%) of multi-resistant coliforms isolated from the STP could partially or completely transfer their resistance patterns to the recipient strain. All these studies suggested that STP might provide an environment in favor of gene transfer, resulting in the selective increase of antibiotic resistant bacteria in STP.

We observed further dilution of enterococci concentrations in surface water samples. The concentrations of total enterococci at the two surface water locations were between 6 and 3200 CFU / 100 ml. There is little legal guidance on the bacterial loads of surface waters. However, assuming that surface waters might incidentally be used as bathing waters, the European Bathing water directive (2006/7/EC) can be taken for comparison. While the 95% percentile of enterococci at the Rhine by Lobith exceeded the limits set for a qualification as 'sufficient' according to the Bathing water directive, enterococci at Nieuwegein were low enough to classify as 'excellent'. With respect to resistant enterococci, at Nieuwegein, AR-Efm and HA-Efm were only observed two times, as the prevalence of AR-Efm was below the concentration that could be detected in the volume of water that could be filtered until the filter was clogged with debris. Likewise, in Lobith, AR-Efm and HA-Efm could not be detected at each sampling event.

To confirm that the *esp*-carrying *E. faecium* isolates identified in our study were indeed hospital-related, MLVA and MLST typing was performed. With respect to the sequence types of HA-Efm found in our study, we found two types to be dominating: MT-159 and MT-12. MT-1 was found in most locations, but at a much lower frequency. Until 2005, MT-1/ST-17 was the dominant clone found in hospitals, but it has since then partially been replaced by MT-159 and MT-12 (Top et al. 2008). Although MT-159 and MT12 were only documented in the late 90's from hospital outbreaks in Korea, Italy and Germany, both MT's have shown increasing tendencies. Specific adaptations to the hospital environment, like the acquisition of ampicillin resistance and a putative PAI that facilitate efficient spread could be the reasons for the current success of MT-159 and MT-12. The MT found in our study are thus similar to types associated with hospital outbreaks. Although MT-1 is a type often connected with hospital outbreaks, MT-1 was not found in our study in any of the UMCU-South samples.

The results of MLVA typing were confirmed with MLST typing. The MT-159 isolates tested showed sequence type ST-78 and two other highly genetically related genotypes (ST-192 and ST-341). All six MT-12 samples tested were of ST-117. Both ST-78 and ST-117 are typically hospital-associated *E. faecium* (Willems and van Schaik 2009). 240 out of 243 isolates in the MLST database that represent the sequence types ST-18, ST-78, ST-117, ST-266 and ST-192 come from hospital-derived isolates, and in a recent study on human and animal *E. faecium* MLST types, neither pigs nor poultry isolates showed one of these types (de Leener et al. 2005). ST-18 has only very occasionally been found in the community (2 times, Freitas et al. 2009), and in hospital waste water (Freitas et al. 2010, Caplin et al. 2008). There is one report on ST-78 in a pig (Biavasco et al. 2007).

However, recent studies in search for community reservoirs also found the genotypes ST-78, ST-192 and ST-266 in dogs and cats and suggested that these animals might be part of the community HA-Efm reservoir. Interestingly, the putative virulence gene content of canine isolates differed considerably

from that usually observed in ST-78 isolates from human infections. Notably, animal-derived isolates lacked the *esp* gene (Damborg et al. 2009). As most of our ampicillin-resistant isolates carried the *esp* gene (table 2), it is less likely that these originated from dogs or cats. An exception was an isolate with ST-266, which was one out of nine *esp*-negative isolates of MT 139 collected at Lobith the same day. The ST-266 is found more frequently in dogs and cats than in human isolates and also lacks the *esp* gene. Larger decentralized studies should be conducted in communities, residential animals, food industry, municipal sewage and surface water to characterize community AREF and HA-Efm epidemiology. With respect to the public health risks of the presence of hospital-associated *E. faecium* isolates in sewage effluent and surface waters, we conclude that a possible pathway for uptake of these bacteria consists of swimming in surface waters in the proximity of sewage effluent (see also Gezondheidsraad 2001). As concentrations of HA-Efm in water intended for drinking water production were lower than 100 CFU / 100 ml in all instances, and as drinking water production from the Nieuwegein site includes sand filtration (with a pathogen removal efficiency of 2-3 log10 reductions (Hijnen et al. 2004)) and subsequent treatment steps with an even higher removal efficiency, the presence of HA-Efm in drinking water is highly unlikely. Still, it might be advisable to include resistant bacteria in the parameters that are regularly controlled in surface water intended for drinking water production.

Conclusion 6

We found hospital sewage effluent to be a significant environmental input source of hospital-associated resistant bacteria. Still, input via hospitals does only explain a minor part of the total load in the sewage treatment plant, highlighting the role of AR-Efm and HA-Efm reservoirs in the community. Further, there were indications that these bacteria might survive sewage treatment slightly better than non-resistant enterococci. Surface water may thus serve as a vector for the dissemination of antibiotic resistance to the environment.

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