

# **Screening for resistance encoding integrons in isolates of Enterococci**

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## Summary

**Background:** Integrons are bacterial genetic elements, working as assembly platforms, by incorporating gene cassettes (GCs) in the bacterial genome. 130 GCs providing antimicrobial resistance to most classes of antibiotics have been described, and most of them are associated with the class 1 integrons. Class 1 integrons are widespread among clinically important Gram-negatives, but are rare in gram positives. Recently, the first detection of integrons in clinical isolates of enterococci was described. Over the last two decades enterococci has emerged as one of the leading causes to nosocomial infections world over, as a result of development of hospital adapted lineages. These lineages tend to possess genes encoding antimicrobial resistance and have increased virulence. It is therefore of great interest to search for integrons in enterococci, as acquisition of integrons in enterococci could lead to an even more critical situation in the treatment of enterococcal infection.

**Material and Methods:** A diverse collection of 274 enterococcal isolates was screened by *IntI1* PCR which is a good marker for presence of class 1 integrons. Additionally, 24 of the isolates were further examined by colony blot hybridization and antibiotic susceptibility testing.

**Results:** All isolates were found to be PCR negative for the *IntI1* integrase. Two possible positive results were detected by colony blot hybridization.

**Conclusion:** Presence of Class 1 integrons was not detected in the enterococci-isolate collection investigated. However, further investigation of two possible positive isolates is required to verify the results. Moreover the whole isolate collection should additionally be investigated by hybridisation techniques before a conclusion of the findings can be made.

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# 1 Introduction

## 1.1 Bacterial evolution and development of antimicrobial resistance

Alexander Fleming discovered the first antimicrobial agent in 1928. He observed inhibition of staphylococci on an agar plate contaminated with a *Penicillium* mold [1]. In 1938 the chemist Ernst Chain and the doctor Howard Florey developed a way to isolate the antibiotic substance, which they called penicillin, from the *Penicillium* fungus and in this way they made use of it to treat bacterial infections [2]. During the World War II, the United States developed mass production of penicillin for treatment of wounded soldiers, and after the war, penicillin was released on the civil market. These events are often described as the founding of the antibiotic era [3], which soon were followed by huge problems with antimicrobial resistance. However already before the beginning of the mass production of penicillin, E. Chain together with the English biochemist Edward P. Abraham suggested that some bacteria harboured an enzyme able to destroy penicillin [4], later verified as beta-lactamase. This suggests that resistance to antimicrobial agents already existed before introduction of antimicrobials in the clinical and agricultural environment. Recent phylogenetic studies verify these assumptions, and even suggests that several antimicrobial resistance genes have existed since a long time before the start of the antimicrobial era [3]. Serine- and metallo-beta-lactamases, for example, probably evolved for more than two billion years ago, and some of them have existed on plasmids for millions of years [5]. Bacteria possess several mechanisms for exchange of genes with other bacteria, collectively known as the process of horizontal gene transfer (see below). Their mutation rates are balanced by constant fine-tuning optimizing their ability to adapt to changing conditions [6]. These properties, together with the short generation times, that for bacteria growing in nature may be as short as 15 minutes, makes bacterial evolution and adaptation very fast [7]. In an environment with high antibiotic pressure, such as in a hospital setting or in the farming industry, resistance genes to antimicrobial agents will spread rapidly, due to selection of the most well adapted bacterial cells. These innate properties of bacteria, together with the extensive use of antimicrobial agents in the clinical setting, since the beginning of the antibiotic era, has created a growing global problem in treating infections. Hospital adapted strains of common commensal bacteria such as *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus faecium* and *Enterococcus faecalis* seem to spread, and

develop new ways of escaping antimicrobial treatment, much faster than the development of new drugs by the pharmaceutical industry. This paints a rather gloomy picture of the treatment of bacterial infections in the future.

## 1.2 Enterococci

Enterococci are Gram-positive bacteria, which belong to the normal flora of the digestive tract of humans, other mammals, birds and reptiles. They are also found in soil, water and food [8]. Enterococci can grow in a wide temperature range from 10°C to 45°C, and are able to survive chemical disinfectants like chlorine and alcohol, which is an important characteristic for their survival and spread in hospitals [8]. Enterococci have for a long time been considered as harmless commensals, but there is growing evidence that some of these bacteria often possess several specific properties that enable them to cause a diversity of infections. Examples are bacteraemia, peritonitis, endocarditis, urinary tract infections, and device related infections [9]. Such infections may occur when the commensal relationship with the host is disrupted, or when patients get infected by hospital adapted lineages (see below). Currently (23.05.2013) there are 48 known species of Enterococci [10]. *Enterococcus faecalis* and *Enterococcus faecium* are the enterococci most commonly found in human faeces [11]. When it comes to clinical infections, *E. faecalis* and *E. faecium* are also here the most important species, and together they account for more than 90% of clinical enterococcal infections [8]. Over the two last decades, the number of hospital-acquired infections caused by enterococci has increased in Norway and other European countries. In particular infections with *E. faecium* have increased. In Norway, *E. faecium* isolates from blood cultures have nearly increased 4-fold over the last 10 years, while the number of blood cultures with *E. faecalis* has doubled, according to NORM- (Norwegian Surveillance System for Antimicrobial Drug Resistance) reports from 2003-2011 [12, 13]. These events are considered to be a result of the development of successful hospital adapted lineages, sometimes called “High Risk Clones”, in particular of *E. faecium* [8, 9]. These lineages tend to possess genes encoding antimicrobial resistance and surface proteins that may mediate interactions between the host and the bacterial cell, as well as genes for biofilm formation [14].

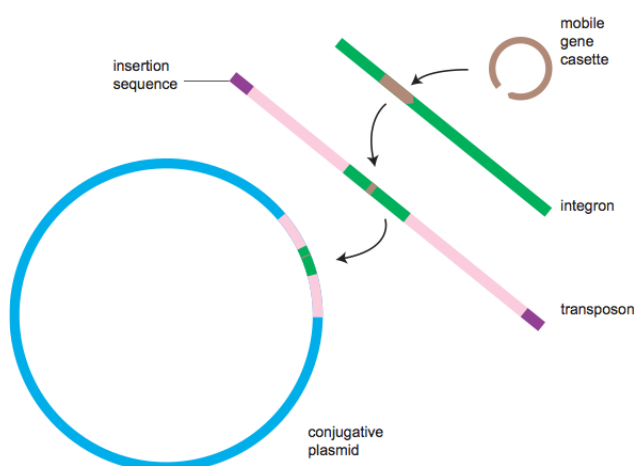
### 1.3 Horizontal gene transfer in enterococci

Of medical importance are also the intrinsic properties of enterococci to participate in horizontal gene-transfer (HGT) [15]. HGT is the process of exchanging genetic material between individual bacteria of the same species or even between different species [7]. This allows bacteria to acquire new features, -and it is one of the major driving forces in bacterial evolution [16]. There are three different major mechanisms that makes HGT possible: **Transduction** by bacteriophage viruses, who may transfer DNA from one bacteria to another, by packing DNA from the infected host-bacteria into their capsid, and transfer it to a new host-bacteria, by infecting it [17]. There is evidence of phage DNA in the genome of enterococci [18, 19], and transduction of both virulence-, and antimicrobial-resistance- genes in enterococci have been demonstrated [20, 21]. There are currently few studies on the role of phages in the genomic flexibility of enterococci. **Transformation** is the process where a cell incorporates exogenous DNA resulting from a direct uptake from its surroundings [22]. This process occurs naturally in some bacteria, but it is not known to play a role in genomic plasticity of enterococci [15]. **Conjugation** is transfer of genetic material between bacteria trough direct cell- to cell contact, in contrast to transduction and transformation [22]. The elements transferred are called conjugative elements (see next paragraph). Conjugation is the most studied process of genetic exchange among enterococci and believed to be the main process responsible for the great plasticity of the enterococcal genome [15].

### 1.4 Mobile genetic elements in enterococci

Mobile genetic elements (MGE) are genetic material with the ability to move within the genome. A basic overview over the major MGEs is listed in Table 1. MGEs can be divided in to two major types: **The conjugative elements** with the ability to move between different bacterial cells and **the transposable elements**, only able to move between different genetic location inside the same bacterial cell. The conjugative elements consist of plasmids and integrative and conjugative elements (ICE) [15], which includes the conjugative transposons [15, 23]. The transposable elements consists of insertion sequence elements (IS), transposons, gene cassettes of bacterial integrons and some other transposable elements. **IS-elements** only code for the functions needed for transposition [15], they are widespread in enterococci and seem to play a role in

hospital adaption of *E. faecium* [18]. **Transposons** consist of genes that code for specific traits in addition to genes encoding their ability to intracellular- or intercellular- (in the case of conjugative transposons) movement. They are mainly classified as composite transposons and complex transposons, the latter including conjugative transposons [23]. Transposons have been crucial in the development of hospital-adapted strains by providing resistance to several antimicrobial agents. Several different genes encoding antimicrobial resistance to agents such as aminoglycosides, glycopeptides, macrolides and tetracyclines are described on different enterococcal transposons [24]. **Integrans** are described below in an own paragraph. **Integrative and conjugative elements (ICE)** (including conjugative transposons) contain genes for integration, excision, regulation and conjugation in addition to one or several genes encoding specific traits [23]. **Plasmids** are extra-chromosomal, self-replicative, and typically circular shaped, double stranded DNA molecules [25]. They contain a set of “backbone genes” encoding their replication, propagation, transfer and mobilisation and stabilisation [25]. In addition they possess several accessory genes that may code for virulence factors, antimicrobial resistance and other specific features. Plasmids are the main vector for horizontal gene transfer in enterococci, and several resistance genes and virulence genes have entered enterococci in this way [15]. Plasmids may also function as a vehicle for transportation of other mobile genetic elements like integrans and transposons (Figure 1) enabling mobile genetic elements that are not self conjugative to be transported into other bacterial cells.



**Figure 1: Showing the hierarchy of mobile genetic elements. Gene cassettes are inserted to integrons, which again may be inserted to transposons, which finally may be inserted into a plasmid. Source: Norman, A., L.H. Hansen, and S.J. Sorensen, *Conjugative plasmids: vessels of the communal gene pool* [25].**

Major group of MGE	Type of mobility	Element	Genetic properties
<b>Bacteriophages</b>	Intercellular mobility	Phage DNA	DNA may be packed in to the capsid of bacteriophage viruses, and hence be transported to a new bacterial cell and incorporated in its DNA.
<b>Conjugative elements</b>	Intercellular mobility	Plasmids	Circular self-replicative DNA-molecules with genes encoding their replication, propagation, transfer, mobilisation and stabilisation. In addition they several accessory genes.
		Integrative and conjugative elements (ICE) (including conjugative transposons).	Contains genes for integration, excision, regulation an conjugation in addition to one or a few genes encoding specific traits
<b>Transposable elements</b>	Intracellular mobility	Insertion Sequence elements (IS)	Only code for the functions needed for transposition.
		Transposons (not conjugative)	Consists of one or a few genes encoding specific traits in addition to genes encoding transposition
		Gene cassettes for integrons	Non-replicative gene which can be found either as a linear form, included in an integron, or as a covalently closed circular free intermediate

**Table 1: Basic overview of the major groups of Mobile Genetic Elements (MGE)**

## 1.5 Genomic evolution of enterococci

Studies have revealed a great accessory genome in enterococci, up to 38% in *E. faecium* [19]. The accessory genome, sometimes called the dispensable genome, consist of genes not present inn all bacteria of the same species, in contrast to the core genome, which is common for all bacteria of the same species [26, 27]. A complete sequencing of the V583 strain, the first vancomycin resistant *E. faecalis* strain isolated in the United States, revealed that more than 25% of the genome consisted of mobile or foreign DNA [18]. Further studies revealed that the genome of V586 holds ~620 kilobases (kb) more than *E. faecalis* OG1RF, a non-clinical strain, used for comparison [28, 29]. Mobile genetic elements accounted for most of the difference found between these strains, including three plasmids, seven phages, and a pathogenicity island. The V583 strain was found to have 639 genes, which were absent in the OG1RF strain. All of these genes, except 45 are associated with mobile genetic elements [28]. It is suggested that clinical enterococcal isolates have an increased ability to exchange and acquire new genetic elements, and hence new traits, compared to the wild type. This suggestion is strengthened by the recent study by Kelli *et. al.* [30], were 48 *E. faecalis* isolates and eight *E. faecium* isolates were analysed for functional CRISPRs and acquired antibiotic resistance. CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) functions as a prokaryotic immune system, and are normally found in most archaeal genomes and in approximately 40% of bacterial genomes [31]. It is believed to provide a degree of protection against foreign genetic elements such as plasmids and phages [31]. Kelli and co-workers found a clearly significant inverse correlation between the presence of CRISPRs and acquired antibiotic resistance in *E. faecalis*, and a similar result for the *E. faecium*. Loss of CRISPRs may in this way have trigged the events that led to acquisition of new traits and hence the development of multiple-resistant hospital adapted strains of *E. faecalis* and *E. faecium*. A big accessory genome put together with the high frequency of mobile genetic elements seen in in hospital adapted- strains points at least in the direction of a genome with high plasticity and great capacity for acquisition of new genes [32].

## 1.6 Integrons

Integrons are bacterial genetic elements, working as assembly platforms that incorporate and excise exogenous gene cassettes (GC) and enable them to work as functional genes [33]. Integrons consist of three major elements; a gene encoding an integrase (*IntI*), a primary recombination site (*attI*) and a promoter ( $P_c$ ) [33]. The integrase, which is a site-specific tyrosine recombinase, catalyses the specific excision and incorporation of the gene cassettes. Gene cassettes are non-replicative minimal functional elements and can be found either in a linear form, included in an integron, or as a covalently closed circular free intermediate [34]. They usually consist of a single open reading frame (ORF) immediately followed by a recombination site (*attC*) [35]. The gene cassettes are inserted at the *attI*-site, and specific recombination occurs at the *attC*-site of the gene cassette (Figure 2). The outward oriented promoter ( $P_c$ ) ensures the expression of the mostly promoter less gene cassettes. Recombination between *attC*-sites usually leads to excision of the gene cassette. In this way the last integrated cassette is expressed at the highest level due to its location closest to the promoter ( $P_c$ ) [34]. Integrons can be divided into two major groups: Chromosomal integrons (CI) and Mobile Integrons (MI). The CIs are located on the chromosome of 17% of sequenced bacterial genomes [35], and they are often described in bacteria from marine or terrestrial ecosystems [34]. They are usually stable, and may contain over 200 genes, many of them with unknown functions. The MIs are not self transposable, but their location on mobile genetic elements such as transposons and plasmids enable them to move [34]. They tend to contain only a few gene cassettes, less than 10 [34]. More than 130 genes involved in antimicrobial resistance and only a few with unknown function are identified on MIs [35]. The MIs are extensively spread among clinical Gram-negative bacteria, but only sporadic found in Gram positives [35]. Five classes of MIs, have been defined based on the sequence of the encoded integrases [33, 35]

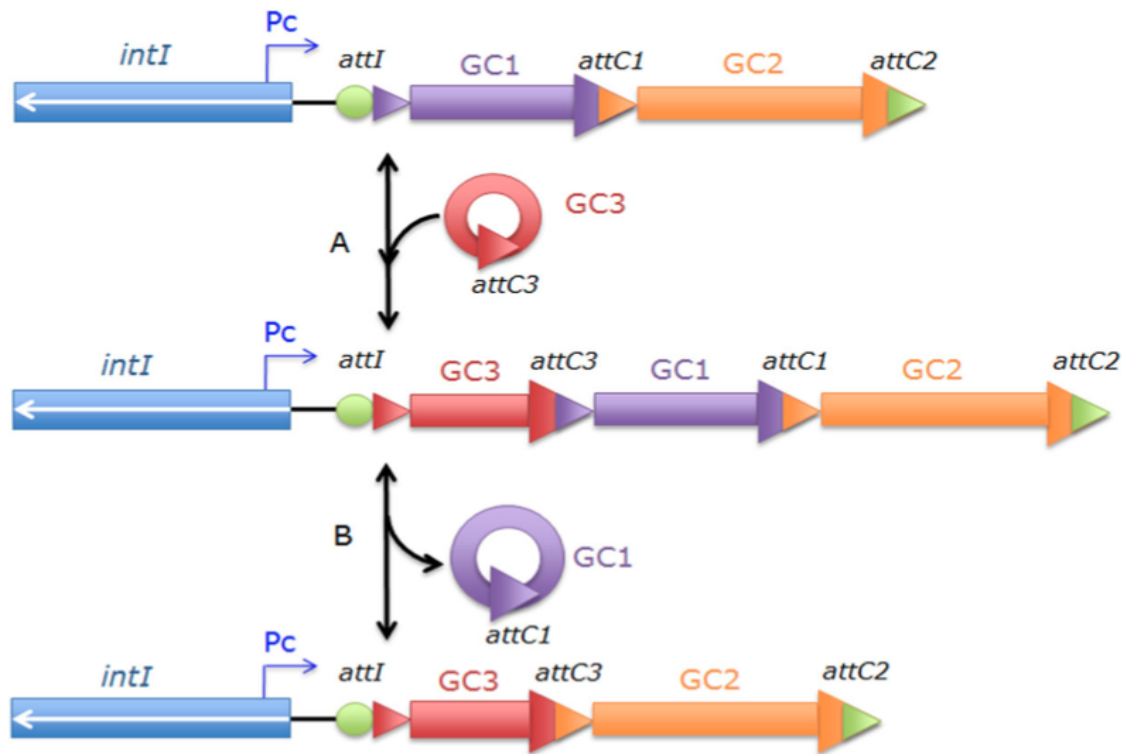


Figure 2: General organization of an integron and gene cassette (GC) recombination mechanism: The *IntI1* protein catalyses the insertion (A) and excision (B) of the GC in the integron, with GC integration occurring at the *attI* recombination site. GC excision preferentially occurs between two *attC* sites. Pc: gene cassette promoter; *attI*: integron recombination site; *attC1*, *attC2*, and *attC3*: *attC* GC recombination sites; *intI*: the integrase gene; GC1, GC2, GC3 are the gene cassettes, and arrows indicate the direction of coding sequences. Source: Stalder T., *et al.*, Integron involvement in environmental spread of antibiotic resistance [34].

### 1.7 Distribution of class 1 integrons in different species including enterococci

Class 1 integrons is the clinically most important class of integrons, as well as the most reported class of integrons in environmental human isolates and animal isolates[34]. They are found in 22-59% of clinical Gram-negative isolates [35, 36], most frequently among members of the *Enterobacteriaceae* family such as *Klebsiella Pneumoniae* and *Escherichia Coli*. For over a decade they have also been known to exist in other Gram-negative genera including *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Burkholderia*, *Campylobacter*, *Citrobacter*, *Enterobacter*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella* and *Vibrio* [37]. They are often associated with non-functional transposons of a class called *Tn402*, and they are frequently incorporated in larger transposons of the *Tn3* family, and plasmids [38]. Since class 1 integrons are naturally occurring in the



environment it has been suggested that these integrons may have evolved from environmental CIs [33, 39], which is supported by findings of class 1 integrons without antimicrobial resistance genes [38, 40]. Evolutionary events may have spread them among pathogen bacteria, which may have led to the accumulation of antimicrobial resistance genes and spread in the clinical setting [34, 35, 40]. Most of the known antibiotic-resistance GCs are linked to class 1 integrons, and among the over 80 different GCs coupled to class 1 integrons, genes that encode resistance to the following antibiotics are found: All known  $\beta$ -lactams, all aminoglycosides, chloramphenicol, trimethoprim, streptothricin, rifampin, erythromycin, fosfomycin, lincomycin and antiseptics of the quaternary-ammonium-compound family (QACs) [33, 37, 39]. Despite the importance of class 1 integrons, little is known about the prevalence of class 1 integron in Gram-positive bacteria. The first evidence of the presence of an integron related gene in Gram-positives, the streptomycin-spectinomycin resistance encoding adenylyltransferase gene (*aadA*) was reported in the plasmid pCG4 from *Corynebacterium glutamicum* in 1998 [41]. One year later the same gene was found in an *E. faecalis* isolate [42]. A screening done in 2001 by Nandi *et. al.* [43] for class 1 integrons in Gram-positive bacteria isolated from poultry litter detected class 1 integrons in several species including *Corynebacterium sp.*, *Aerococcus sp.*, and *Staphylococcus sp.* [43]. This shows that integrons not are limited to Gram-negatives. Class 1 integrons have recently been detected in clinical *E. faecium* and *E. faecalis* isolates from First Affiliated Hospital of Jinan University (FAHJU) in South China [44] where recently also class 1 integrons in clinical isolates of staphylococci were found [45, 46]. The integrons found in enterococci showed a high degree of homology with integrons found in other species isolated in the same hospital. Some of the class 1 integrons from enterococci where by sequencing technologies found to have homology as high as 100% with integrons found in *E. coli* and *Staphylococcus epidermidis*. This suggests inter-generic horizontal transfer of gene cassettes in the hospital setting [44].

## **2 Hypothesis and aim**

Based on previous findings of class 1 integrons in enterococci and due to their great capacity to acquire exogenous DNA, we hypothesise that transfer of integrons to enterococci is possible, and that integrons can be found in a diverse collection of enterococcal isolates.

In this study we aim to explore the prevalence of class 1 integrons in an epidemiologically and geographically diverse strain collection of enterococci.

### 3 Materials and Methods

#### 3.1 Bacterial isolates, positive and negative controls

A collection of 274 different isolates of enterococci was used in this study. The collection had a great diversity, including clinical-, community- and different animal-isolates from different European countries, the U.S. and Canada, collected over the two last decades. A more extensive description of the collection follows under “Results” and in Figure 11-13. All isolates investigated are additionally listed in the appendix. As an integron free negative control for the screening of class 1 integrons, the fully sequenced *E. faecium* strain TX0016 (DO) isolated by Arduino *et. al.* [47] was used. As positive controls three *K. pneumonia* strains known to possess integrons of class 1 were used (Table 2).

Stock number	ID-number	Species	Type of integrase gene present.
K-46-62	FR748151	<i>K. pneumoniae</i>	<i>IntI1</i>
K-45-67	FR748150	<i>K. pneumoniae</i>	<i>IntI1</i>
A 3-45	FR748153	<i>K. pneumoniae</i>	<i>IntI1</i>

Table 2: Integron class 1 positive controls, containing the *IntI1* gene, used in this study.

#### 3.2 Bioinformatics

For investigations of the *IntI1* gene, the primers and the amplicons, before initiation of the laboratory work, bioinformatic-searches were performed, using the BLAST program, found on the web page: <http://blast.ncbi.nlm.nih.gov/>. BLAST, The Basic Local Alignment Search Tool finds regions of similarity between sequences, by comparing nucleotide sequences to sequence databases and calculates the statistical significance of matches [48]. The sequences may be written or pasted in to the query search field in the FASTA format, which is a text-based format used to describe either nucleotide sequences or peptide sequences. The nucleotide-, or amino-acid- sequences are presented as single letters. In the case of DNA sequences, the letters A, T, G and C are used for the nucleotides adenosine, thymidine guanine and cytidine [49]. Different databases for proteins and nucleotides can be chosen, based on species. In this study BLAST searches were performed for the primes used, for the sequenced amplicons of

the *Int11* gene of the positive controls, as well as the *Int11* gene found in *E. faecalis* by Xu and co-workers [44].

### 3.2.1 BLAST searches performed with the primers as query

To verify that the primers intended to use for PCR would bind to the *Int11* gene, a BLAST search was performed on the forward and the reverse primers from the two primer pairs IntI1 and IntM1 (Table 6) Properly designed primers would match with nucleotide sequences on the *Int11* gene on bacterial sequences in the database. The primer sequences were pasted singly in to the “Query Sequence” field (Figure 3). Database were set to “Nucleotide collection (nr/nt)”, organism to “bacteria (taxid:2)”, expect threshold to “1000” and “blastn” algorithm, which is search for “somewhat similar sequences” was chosen. The rest of the parameters were used as the default set up. One search was performed for each primer with the settings listed above, and one search for each primer with organism set to “Enterococcus (taxid:1350)”.

The screenshot displays the NCBI BLAST Standard Nucleotide BLAST interface. At the top, it says "NCBI/BLAST/blastn suite" and "Standard Nucleotide BLAST". Below this, there are tabs for "blastn", "blastp", "blastx", "tblastn", and "tblastx", with "blastn" selected. The main section is titled "Enter Query Sequence" and contains a text input field with the sequence "CCTCCCGCACGATGATC". To the right of this field are "Clear" and "Query subrange" options. Below the input field, there are options for "Or, upload file" and "Job Title" (set to "Int11-F #CCTCCCGCACGATGATC"). There is also a checkbox for "Align two or more sequences". The "Choose Search Set" section includes "Database" (set to "Nucleotide collection (nr/nt)"), "Organism" (set to "bacteria (taxid:2)"), and "Exclude" options. The "Program Selection" section has "Optimize for" options, with "Somewhat similar sequences (blastn)" selected.

Figure 3: Screenshot of the BLAST program. In this run, the sequence of the primer IntI1-F was used as query. Source: <http://blast.ncbi.nlm.nih.gov/>

### **3.2.2 Verification of PCR amplicons by BLAST search of sequenced DNA.**

Nucleotide BLAST was performed on the 12 sequenced samples of the amplicons (described later, listed in Table 16) This was done to verify the amplicons as fragments of the *IntI1* gene. The 12 different FASTA files received from the sequencing lab was pasted singly in to the “Query Sequence” field. The parameters “Nucleotide collection (nr/nt)”, “bacteria (taxid:2)”, “threshold 1000” and “megablast” algorithm were chosen.

### **3.2.3 Homology analyses of the *E. faecalis* *IntI1* gene by BLAST search**

To get an impression of how conserved the *IntI1* gene is, a BLAST search of the gene was carried out. The sequence of the *IntI1* gene from the first integron discovered in *E. faecalis* by Xu and co-workers [44] is available in “GeneBank”, under the GenBank number: FJ753285.1 and the name “*Enterococcus faecalis* strain XJ-SHZ-111 class I integron integrase-like (*intI1*) gene, partial sequence”. A BLAST search was performed using the GeneBank number as query (Figure 4) The parameters “Nucleotide collection (nr/nt)”, “bacteria (taxid:2)”, threshold “1000” and “megablast” algorithm, was chosen. The *IntI1* gene from the *E. faecalis* strain is only a partial sequence. Therefore it was chosen to use the sequence of the *IntI1* gene from the top hit, “*Escherichia coli* 1540 plasmid pIP1206 complete genome”, which showed a max identity of 100% and a query cover of 98% whit the partial sequenced *IntI1* gene from *E. faecalis* (Figure 5.) This *E. coli* plasmid has GenBank number AM886293.1, and the *IntI1* gene is found in the region from base 109735 to 110748 and consists of 1014 bp. This 1014 bp sequence was then used as query in a new BLAST search with the same parameters.

Enter Query Sequence BLAST N programs search nucleotide databases using a nucleotide query.

Enter accession number(s), gi(s), or FASTA sequence(s) Clear Query subrange

FJ753285.1 From

To

Or, upload file Velg fil ingen fil er markert

Job Title Enterococcus faecalis strain XJ-SHZ-111

Enter a descriptive title for your BLAST search

Align two or more sequences

---

Choose Search Set

Database  Human genomic + transcript  Mouse genomic + transcript  Others (nr etc.):

Nucleotide collection (nr/nt) +

Organism bacteria (taxid:2)  Exclude +

Optional Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown.

Exclude  Models (XM/XP)  Uncultured/environmental sample sequences

Optional

Entrez Query

Optional Enter an Entrez query to limit search

---

Program Selection

Optimize for  Highly similar sequences (megablast)

More dissimilar sequences (discontiguous megablast)

Somewhat similar sequences (blastn)

Choose a BLAST algorithm

---

**BLAST** Search database Nucleotide collection (nr/nt) using Megablast (Optimize for highly similar sequences)

Show results in a new window

Figure 4: Screenshot of the BLAST program. In this case, the GenBank number FJ753285.1 for the *IntI1* gene in “*Enterococcus faecalis* strain XJ-SHZ-111 class I integron integrase-like (*intI1*) gene, partial sequence” was used as the search query. Source: <http://blast.ncbi.nlm.nih.gov/>

### Sequences producing significant alignments:

Select: [All](#) [None](#) Selected:0


	Description	Max score	Total score	Query cover	E value	Max ident	Accession
<input type="checkbox"/>	<a href="#">Enterococcus faecalis strain XJ-SHZ-111 class I integron integrase-like (intI1) gene, partial sequ</a>	1631	1631	100%	0.0	100%	<a href="#">FJ753285.1</a>
<input type="checkbox"/>	<a href="#">Escherichia coli 1540 plasmid pIP1206 complete genome</a> 	1354	2476	98%	0.0	100%	<a href="#">AM886293.1</a>
<input type="checkbox"/>	<a href="#">Escherichia coli SMS-3-5 plasmid pSMS35_130, complete sequence</a>	1354	1939	98%	0.0	100%	<a href="#">CP000971.1</a>
<input type="checkbox"/>	<a href="#">Klebsiella pneumoniae subsp. pneumoniae HS11286 plasmid pKPHS3, complete sequence</a>	1343	1403	98%	0.0	100%	<a href="#">CP003225.1</a>
<input type="checkbox"/>	<a href="#">Escherichia coli plasmid pCQP15 insertion sequence IS26, partial sequence; transposon Tn3, coi</a>	1072	1132	69%	0.0	100%	<a href="#">FJ744121.1</a>
<input type="checkbox"/>	<a href="#">Enterobacter aerogenes plasmid pYS10 beta-lactamase (blaLAP-1) gene, partial cds; putative Orf</a>	1072	1132	69%	0.0	100%	<a href="#">FJ167861.1</a>
<input type="checkbox"/>	<a href="#">Escherichia coli strain 3A11 plasmid pHN3A11, complete sequence</a>	1061	1121	69%	0.0	100%	<a href="#">JX997935.1</a>
<input type="checkbox"/>	<a href="#">Escherichia coli plasmid pIP1206 transposase, plasmid efflux fluoroquinolone (pef), and class 1 i</a>	1061	1121	69%	0.0	100%	<a href="#">EF150886.1</a>
<input type="checkbox"/>	<a href="#">Escherichia coli plasmid pHPA tnpA, tnpR, blaTEM-1, rmtB, qepA, tnpA genes for transposase, r</a>	1055	1115	69%	0.0	100%	<a href="#">AB263754.2</a>
<input type="checkbox"/>	<a href="#">Escherichia coli plasmid pVOG1 insertion sequence IS26, partial sequence; transposon Tn3, coi</a>	1050	1110	69%	0.0	100%	<a href="#">FJ183463.1</a>
<input type="checkbox"/>	<a href="#">Aeromonas hydrophila strain C34 class 1 integron, partial sequence</a>	806	806	49%	0.0	100%	<a href="#">JQ837992.1</a>
<input type="checkbox"/>	<a href="#">Aeromonas sp. C37 class 1 integron, partial sequence</a>	806	806	49%	0.0	100%	<a href="#">JQ837985.1</a>

Figure 5: Screenshot of the BLAST program showing the list of hits based on the search using the partial sequenced *IntI1* gene from *E. faecalis* with GenBank number FJ753285.1 as query. The top hit "*Escherichia coli* 1540 plasmid pIP1206 complete genome" (red arrow) was then used for further BLAST searches. Source: <http://blast.ncbi.nlm.nih.gov/>

### 3.3 Screening for the integrase gene *IntI1* by PCR

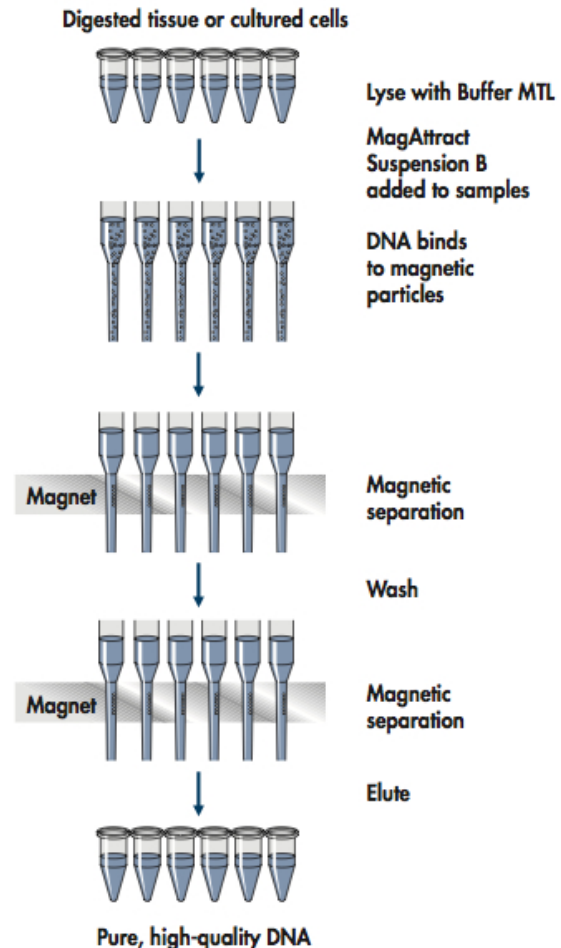
To be able to screen for the *IntI1* gene in the 274 selected strains all stored as frozen bacterial stocks at -80°C in 2 ml tubes, several steps had to be performed. First the defrosting and cultivation of the bacteria, followed by extraction and isolation of the bacterial DNA. Then setup and run of the PCR reactions, and finally detection of DNA by gel-electrophoresis and trans-illumination UV-photographing. A more extensive description of these steps follows below.

#### 3.3.1 Cultivation of the bacteria

All isolates used, were stored as frozen bacterial stocks at -80°C in 2 ml tubes. The frozen bacteria were spread on standard blood agar plates with inoculating loops. The tubes with the bacterial stocks were rapidly put back in the freezer to prevent them from defrosting. The blood agar plates were incubated over the night (16-20 h) at 37°C. On the next day, colonies on the agar plates were inspected to be homogenous and with appearance consistent with enterococci.

### 3.3.2 DNA Extraction and purification

The principle for DNA extraction and purification is to get DNA fragments out of the cell, and remove organelles, proteins and fragments of the cell membrane, leaving relatively pure DNA. In this study silica coated magnetic particles and robot extraction of the DNA was used (Figure 6) The principle for this type of DNA extraction is lysis of the cells in the presence of a chaotropic solution, followed by addition of silica-coated magnetic particles [50]. The DNA binds to these silica-coated magnetic particles, which are collected by magnetic force. Meanwhile several wash steps remove non-bound matter and salts. The purified DNA is at last eluted in water [50]. To perform this robot extraction, single colonies were first picked from the blood agar plates with cotton tip swabs and dissolved in 0,85% NaCl in distilled water. The amount of bacteria added were adjusted to reach a density of 1,0 McFarland. The density was measured



with DEN-1, McFarland Densitometer. 2 ml of the solution were then added to 2 ml microcentrifuge tubes by the use of a pipette. The tubes were all pre-marked with number corresponding to the bacterial colonies. Another set of microcentrifuge tubes were also marked with colony numbers. The tubes were all placed in the DNA-extraction robot: Qiagen BioRobotM48. This robot lyses the bacteria and extracts the DNA from up to 48 bacterial inoculates per cycle. The protocol "Bact 200 µl." was followed and all reagents used, were found in the kit "MagAttract® DNA Mini M48 Kit" described in the protocol "MagAttract® DNA Mini M48 Handbook". The isolated DNA was stored in microcentrifuge tubes at 4°C.

Figure 6: Principle for the DNA extraction and purification. Source: MagAttract DNA Mini M48 Handbook 04/2012. Downloaded from <http://www.qiagen.com/Knowledge-and-Support/Resource-Center/>



### **3.3.3 PCR**

Two PCR reactions were performed on all DNA isolates: SodA PCR and IntI1 PCR. In addition IntM1 PCR were performed on some isolates. For the IntI1, and the IntM1 no pre made "Stock solution" of primers were available. Freeze dried primers from "Eurogentec" were therefore used. The primers, delivered in microcentrifuge tubes were dissolved to form a "stock solution" by adding a variable volume of TE Buffer specified in the data sheet from the manufacturer to the tubes to get a final concentration of 100 nM. For the SodA PCR, frozen "stock solutions" were available. The "stock solutions" were diluted with distilled water to form the "working solutions", which were used as reagents in the master mixes. The sequences of the primers and the reagents used in the master mixes are listed under the more specific description of each PCR reaction. For each PCR reaction 22,5 µl master mix and 2,5 µl of isolated DNA were mixed in 8-tubed PCR-strips, or in 96-well PCR-plates. They were then spun briefly in a centrifuge before the PCR program was run in the thermal block cyclor: "GeneAmp PCR System 9700" from Applied Biosystems. After completed PCR the PCR-products were stored at +4°C.

#### **3.3.3.1 SodA PCR**

To verify that the isolation of the DNA had been success full, detection of the house keeping gene, superoxide dismutase (*sodA*) by PCR was performed on all extracted samples. Detection of the *sodA* gene from Gram-positive cocci show up as a sharp band of 400-500 bp on this PCR [51]. DNA from other species do not form a band on the gel. For enterococci this band is 438 bp [52]. Negative control is according to the protocol *E. coli* ATCC 25922 but this tended to become false positive, so *K. penumonia* FR748151 and *K. penumonia* FR748150 were used as negative controls. Positive control was *E. faecalis* ATCC 29212. The primer sequences and reagents used to make the SodA master mix are listed in Table 3 and 4, and the PCR cycle program is shown in Table 5.

Primer names	Sequences 5'-3'	[ Stock-]	[ Work-]
<b>SodA d1</b>	CCI TAY ICI TAY GAY GCI YTI GAR CC	250 $\mu$ M	50 $\mu$ M
<b>SodA d2</b>	ARR TAR TAI GCR TGY TCC CAI ACR TC	250 $\mu$ M	50 $\mu$ M

**Table 3: Sequences of the SodA Primers**

Reagent	40 reactions $\mu$ l	Final concentration
Accugene grade water (distilled water)	<b>384</b>	-
JumpStart™ REDTaq®	<b>500</b>	
<b>sodA d1</b> (50 $\mu$ M)	<b>8</b>	<b>400 nM</b>
<b>sodA d2</b> (50 $\mu$ M)	<b>8</b>	<b>400 nM</b>
Volume of master mix	<b>900</b>	-
Template DNA to be added	<b>100</b>	-
Total Reaction volume	<b>1000</b>	-

**Table 4: Reagents used to make sodA master mix . This table show volumes needed for 40 PCR reactions. For each sample, 22,5  $\mu$ l master mix and 2,5  $\mu$ l DNA-extract were added**

95°C 150 seconds

95°C 30 seconds ]

37°C 60 seconds ] 30 cycles

72°C 60 seconds ]

72°C 7 minutes

4°C  $\infty$

**Table 5: SodA PCR program**

### **3.3.3.2 *IntI1* integrase PCR**

To screen for class 1 integrons, two primer pairs which bind to conserved regions of the *IntI1* integrase gene were tested. The BLAST search and the methodical testing in the lab revealed no significant differences between the two primer pairs, and for the screening the *IntI1* primer pair described by Goldstein *et. al.* [53] was chosen over the *IntM1* primer pair described by Su *et. al.*[54]. The binding sites on the *IntI1* gene for both primer pairs are shown in Figure 7. The primer sequences and the reagents used to make the *IntI1* and *IntM1* master mixes are listed in Table 6 and Table 7. The cycle conditions are listed in Table 8. A combined *IntI1/IntM1* PCR was also carried out. This was done under further investigation of two isolates positive for the *IntI1* gene on DIG-Hybridizaion, based on the theory that a possible point mutation in the primer-binding site in the *IntI1* gene of the two isolates could yield negative *IntI1* PCR. Two master mixes were made after the same protocol as showed in Table 7 and Table 8, but using the reverse primer from one of the primer pairs, and the forward primer from the other. “*IntI1/IntM1* Short” consists of *IntM1-U* and *IntI1-F* and “*IntI1/IntM1* Long” consists of *IntM1-D* and *IntI1-R*

Primer/probes	Sequense 5'-3'	Amplicon size (bp)	Reffe rance	[ Stock-solution]	[ Working-solution]
<b>IntI1-F</b>	CCTCCCGCACGATGATC	280	[53]	100 µM	10 µM
<b>IntI1-R</b>	TCCACGCATCGTCAGGC				
<b>IntM1-D</b>	GAAAGGTCTGGTCATACATG	565	[54]	100 µM	10 µM
<b>IntM1-U</b>	ACGAGCGCAAGGTTTCGGT				

Table 6: Sequences of the IntI1 and the IntM1 primer pairs.

Reagent	40 reactions µl	Final concentration
Accugene grade water	<b>350</b>	-
JumpStart REDTaq	<b>500</b>	
<b>IntI1-F / IntM1-D (10 µM)</b>	<b>25</b>	<b>250 nM</b>
<b>IntI1-R / IntM1-U (10 µM)</b>	<b>25</b>	<b>250 nM</b>
Volume of Mastermix	<b>900</b>	-
Template DNA to be added	<b>100</b>	-
Total Reaction volume	<b>1000</b>	-

Table 7: Reagents used to make IntI1 and IntM1 master mix . This tabelle show volumes needed for 40 PCR reactions. For each sample, 22,5 µl master mix and 2,5 µl DNA-extract were added.

96°C 60 seconds	
96°C 30 seconds ]	
55°C 30 seconds ]	30 cycles
72°C 30 seconds ]	
72°C 7 minutes	
4°C ∞	

Table 8: PCR cycle conditions used for IntI1 and IntM1 PCR reaction

Table 6 Table 7 Table 8

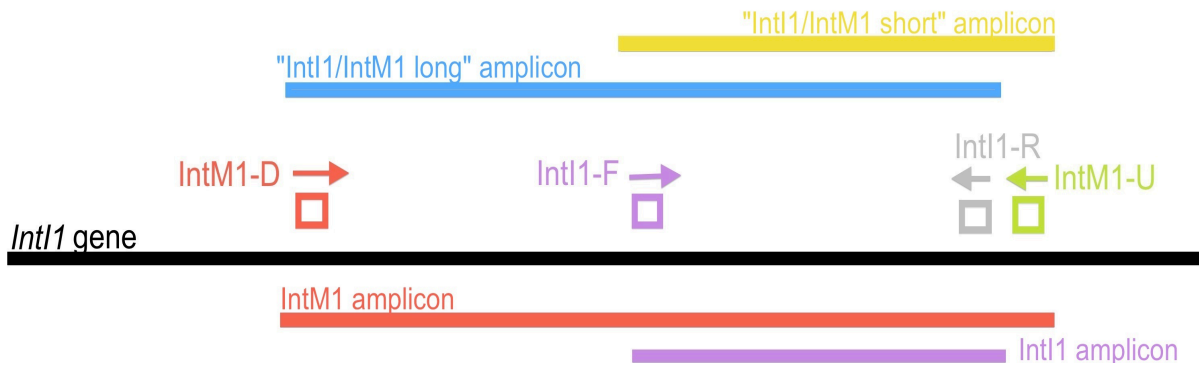


Figure 7: The binding sites for the primer pairs are shown by the location of the coloured boxes on the *IntI1* gene indicated by the black line. The arrows indicate primer direction. The amplicons formed in the different PCR reaction are shown as lines aligned along the gene. Adapted from Figure 18.

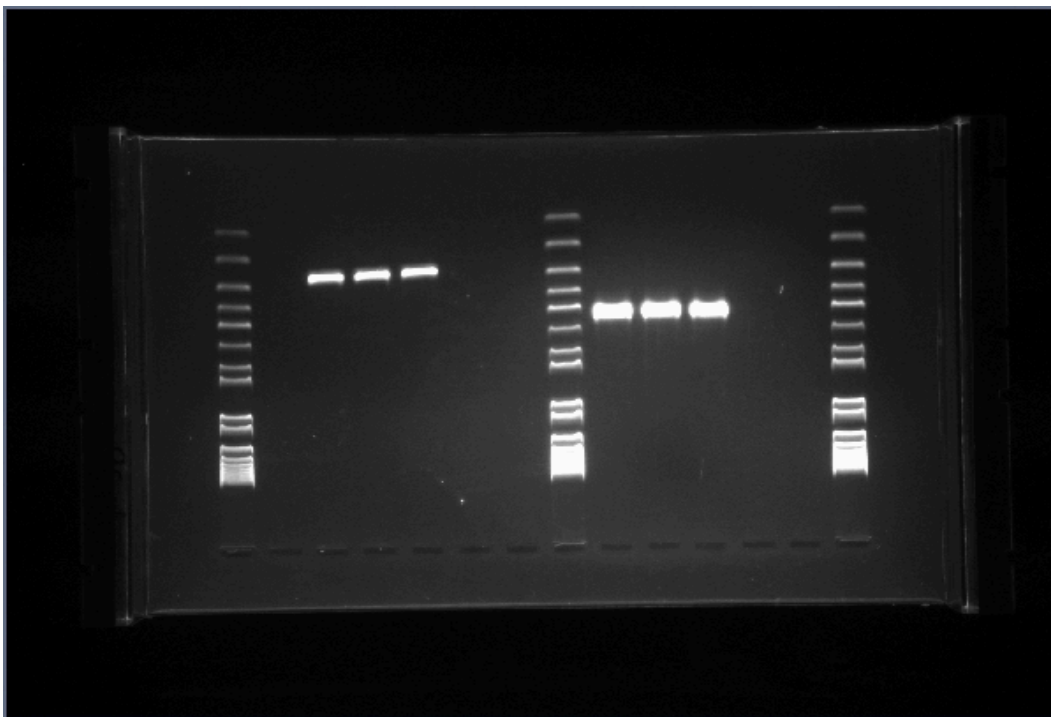


Figure 8: Testing of the IntM1 and IntI1 primers. Self poured 1% Agarose gel was run with the PCR products of the three different *IntI1* positive controls listed in Table 1. IntM1 PCR products are separated in the 5 wells to the left, and the IntI1 PCR products are separated in the 5 wells to the right. The wells with no bands contain the negative control *E. faecium* strain TX0016 (DO) and negative water control. The amplicons from IntI1 PCR formed bands of 280 bp for the positive controls, whereas IntM1 amplicons formed bands of 565 bp

### **3.3.4 Gel-Electrophoresis**

Separation of PCR products, were done by gel electrophoresis, followed by UV-visualization. Both 2% agarose pre-cast E-gels and 1% agarose gels poured in the laboratory were used.

#### **3.3.4.1 1% Agarose gel poured in the laboratory**

For a typical 90 ml gel, 0,90 grams of dry agarose was poured into an Erlenmeyer flask, and mixed with 95 ml of 0,75% TBE Buffer. The mixture was brought to boiling in the microwave, and boiled for approximately 30-60 seconds. In this way the volume was reduced to 90 ml. The Erlenmeyer flask was then cooled under running cold water to approximately 70°C. 0,5 µl GelRed™ pr. 10 ml solution was then added. In the case of a 90 ml gel, 4,5 µL GelRed™ was added. The solution was poured in to a mould with comb to form a rectangular gel with wells. The gel was left to solidify for minimum 30 min. The gel was then put in a gel tank with a positive and negative electrode. The tank was filled with 0,75% TBE Buffer. 5 µl of PCR product were added to the wells in the gel. As a marker, 2,5 µl of 100 bp ladder were added. The gels were run for 90 min at 120 Volts.

#### **3.3.4.2 2% Agarose 96-well pre cast e-gels.**

Pre-cast E-gels of the type “Invitrogen 2% E-Gel® 96 Agarose” were used. The manual “E-Gel 96 High-Throughput Agarose Electrophoresis System” was followed. These gels are buffer less, and designed for fast, high-throughput DNA electrophoresis. Each gel contains 96 sample lanes and 8 marker lanes. 5 µl of PCR product was mixed with 15 µl of sterilized water to form a total loading sample of 20 µl. The samples were loaded into the wells by pipette. 5 µl of 100 bp ladder were loaded in to the maker wells. The gel was put in an E-Base™ Electrophoresis Device and run for 15 minutes.

### **3.3.5 Detection of results by UV-trans illuminator camera**

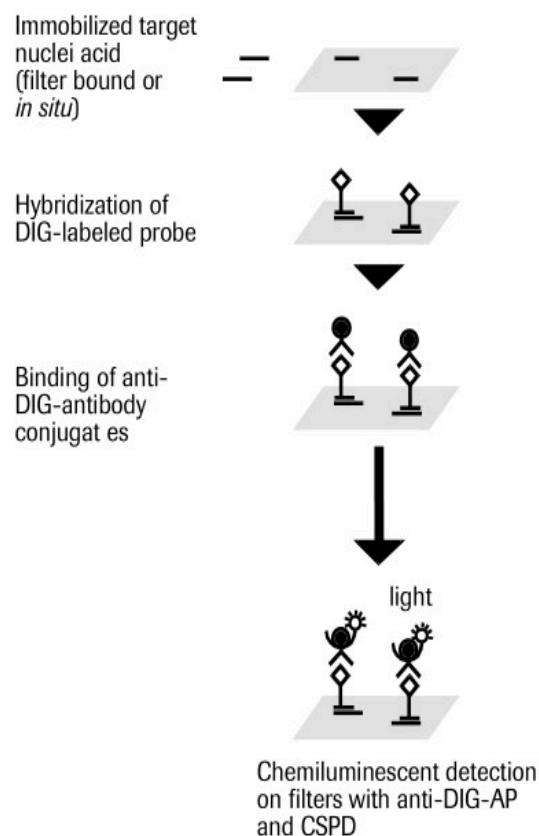
The UV-trans illuminator with camera, “GelDoc XR” from BioRad, and the Software “Quantity One” was used to detect the bands on the gel. The gel was placed in the camera box and trans-illumination UV-light was turned on. The function for automatic focusing was used, and the pictures were analysed on the computer in “Quantity One”.

### 3.4 Colony Blot hybridization with DIG-dUTP labelling

As a different approach in the search for integrons, colony blot hybridization was done on 24 representative selected enterococcal isolates. A complete list is found in the appendix. Two hybridization reactions were done; one using a SodA probe, and one using an IntI1 probe. The strains *K. pneumoniae* FR748151 and *K. pneumoniae* FR748150 were used as positive controls for the IntI1 hybridization and as negative controls for the SodA hybridization. Positive SodA and negative IntI1 controls were not used. The labelling of the probes were done with “PCR DIG-Probe Synthesis Kit” from Roche. With this kit DIG-dUTP (Digoxigenin-uridine-triphosphate) is incorporated in the probe for DNA labelling, and the probe is hybridized with DNA on a filter (Figure 9) Under the detection procedure, *Anti-digoxigenin-alkaline-phosphatase* binds to the DIG-probes. Finally CSPD, which is a chemiluminescent substrate for alkaline phosphatase, is added and produces visible light upon binding to the *Anti-digoxigenin-alkaline-phosphatase*.

**Figure 9: Illustration of the main principle for DIG-Probe hybridization: First Immobilized target DNA from the colonies are bound to the nitro-cellulose filter. Hybridization binds the DIG-labelled probe to matching DNA sequences on the filter. Under the detection step *Anti-digoxigenin-alkaline-phosphatase* binds to the DIG-probes. Finally CSPD, which is a chemiluminescent substrate for the alkaline phosphatase, is added and produces visible light upon binding to the *Anti-digoxigenin-alkaline-phosphatase*. The light is detected by exposure on conventional film.**

Source: “DIG Luminescent Detection Kit –Protocol” Downloaded from <https://cssportal.roche.com/cssp>



### 3.4.1 Cultivation of colonies on filter covered BHI Agars

The colonies were first cultured over night on standard blood agars as described previously. A positive charged nitro-cellulose filter (Roche Diagnostics) were cut to fit a standard petri dish. This was placed on a petri dish with Brain Heart Infusion Agar (BHI Agar). Sterile toothpicks were used to transfer colonies of enterococci from the blood agars over to the BHI agar covered with nitro cellulose filter, as 2-3 mm strips, one for each colony (Figure 10). To such BHI agars with nitrocellulose filters were made; one for the SodA hybridization and one for the IntI1 hybridization. The BHI agars were then incubated over night, 16-20 hours at 37°C.

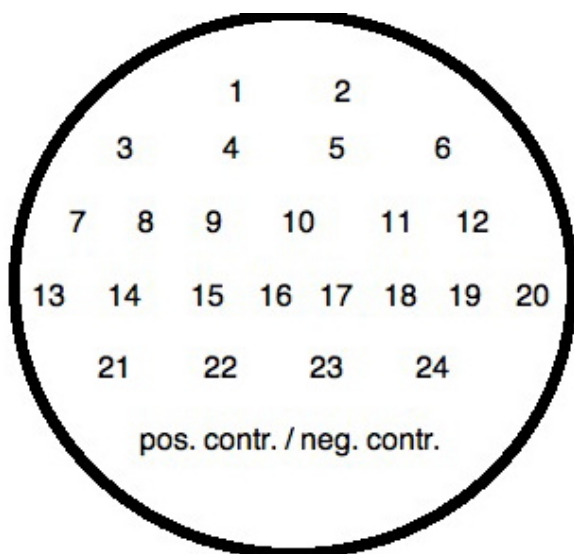


Figure 10: Colonies were applied to the BHI agar plates covered with nitro-cellulose filters in this pattern.

### 3.4.2 Transfer of the colonies to the nitrocellulose filter.

On the next day four “Whatmann 3MM” filters were cut to fit big petri dishes. Each of the four Whatmann filters was put in single big petri dishes. The filters were then covered with separate solutions in moderate amounts, just enough to keep the filters wet (Table 9). The nitro-cellulose filters were put in the solutions with the colony side up and incubated for a given time in each solution (Table 9). A pair of tweezers was used to move the nitro-cellulose filter with the bacterial colonies between the four petri dishes. After incubation in the 4<sup>th</sup> solution, the nitro-cellulose filters was incubated in UV-light for 1 minute to crosslink the nucleic acids. The nitro-cellulose filter was then stored wrapped in aluminium foil to protect it from light at + 4°C.



Dish number	Incubation	Solution name	Ingredients
1	60 minutes at 37°C	<b>Lysis solution</b>	10mM Tris-HCl, 1mM EDTA 25% 10 mg/ml lysozyme, 0,30 U/ul Mucolysine
2	5 minutes at room temperature	<b>Denaturation solution</b>	0,5 M NaOH, 1,5M NaCl
3	5 minutes at room temperature	<b>Neutralizing solution</b>	1,5M NaCl, 1M TrisCl pH 7,5
4	5 minutes at room temperature	<b>Transfer solution</b>	2 x SCC

**Table 9: The four different solutions used to transfer the colonies to the nitro-cellulose filter.**

### 3.4.3 Purification of PCR-products for production of the DIG-labelled probe

PCR products from previous run PCR reactions, which were positive on gel electrophoresis, were used. Amplicons from three different template DNAs (Table 10) were used for the production of each of the two probes.

PCR reaction name	Isolate number	Alternate number	Species	Amount of PCR product purified
SodA	K 60-02	908301850	<i>E. faecium</i>	17 µL
SodA	K 60-04	651578	<i>E. faecium</i>	17 µL
SodA	K 58-37	41996	<i>E. faecalis</i>	17 µL
Int11	K 46-62	FR748151	<i>K. pneumoniae</i>	17 µL
Int11	K 45-67	FR748150	<i>K. pneumoniae</i>	17 µL
Int11	A3-45	FR748153	<i>K. pneumoniae</i>	17 µL

**Table 10: PCR products used for production of the SodA probe and the Int11 probe.**

The purification was done with E.Z.N.A.<sup>™</sup> Gel Extraction Kit according to the protocol for this kit. PCR products were transferred into 1.5 ml microcentrifuge tubes and an equal volume of Binding Buffer (XP2) was added. The tubes were vortexed. “HiBind<sup>™</sup> DNA columns” were placed in 2 ml collection tubes and the samples were applied to the DNA columns. The columns were centrifuged at 10 000 rpm for 60 seconds. The liquid in the tube was discarded and the DNA column was placed back into the collection tube. 300 µl of Binding Buffer (XP2) was then added into the column, which were centrifuged, and the flow-through were discarded as in the previous step. Tubes were reused and 700 µl SPW Wash Buffer was added to the columns, which were again centrifuged and liquids were discarded as above. The empty DNA columns were then centrifuged for 2 min at maximum speed. In the final step, the DNA columns were placed into clean microcentrifuge tubes. 30 µl of Elution Buffer (10mM Tris, pH 8,5) were added and the tube centrifuged to elute the DNA.

#### ***3.4.4 Preparation of DIG labelled probe for hybridisation.***

“PCR DIG Probe Synthesis Kit” from Roche was used and the protocol for this kit was followed. Two DIG-labelling reaction mixes and two control mixes were made; as shown in Table 11 and Table 12, respectively; one for the SodA reaction and one for the Int11 reaction. The reactions were set up in PCR-tubes and PCR was performed in a thermal block cycler (GeneAmp PCR System 9700 from Applied Biosystems). The PCR reactions were performed at the same cycle conditions as used for the first amplifications making the PCR product.

<b>DIG probe</b>			
<b>Reagent</b>	<b>SodA</b>	<b>IntI1</b>	<b>Volume</b>
PCR buffer with MgCl <sub>2</sub> 10× concentration			5 µl
PCR DIG Labeling Mix			5 µl
Primer 1 10mM	SodA d1	IntI1-F	5 µl
Primer 2 10mM	SodA d2	IntI1-R	5 µl
Template DNA	2 µl K 60-02 2 µl K 60 -04 2 µl K 58-37	2 µl K 46-62 2 µl K 45 -67 2 µl A3-45	6 µl
Enzyme mix			1 µl
ddH <sub>2</sub> O			23 µl
<b>Total volume of DIG-probe</b>			<b>50 µl</b>

**Table 11: Ingredients and volumes used to make 50 µl DIG-probes. Differences in ingredients between the SodA and the IntI1 probes are listed.**

<b>Control</b>			
<b>Reagent</b>	<b>SodA</b>	<b>IntI1</b>	<b>Volume</b>
Red Taq Jump Start			39 µl
Primer 1 10mM	SodA d1	IntI1-F	5 µl
Primer 2 10mM	SodA d2	IntI1-R	5 µl
Template DNA	2 µl K 60-02 2 µl K 60 -04 2 µl K 58-37	2 µl K 46-62 2 µl K 45 -67 2 µl A3-45	6 µl
ddH <sub>2</sub> O			23 µl
<b>Total volume of control</b>			<b>78 µl</b>

**Table 12: Ingredients and volumes for 78 µl controls. Differences in ingredients between the SodA and the IntI1 controls are listed.**

### ***3.4.5 Verification of the probe and controls by agarose gel electrophoresis***

To verify presence of amplicons in the two DIG-reactions, and to compare the size of the DIG-amplicons and the control-amplicons, separation and visualisation by gel electrophoresis and trans-UV light was performed. The control amplicons consisted of the same template DNA and the same primers as the DIG-probes (Table 12) but no DIG-molecules were incorporated. A 1% agarose gel was poured as previously described. 2,5 µl of probe and control were used, and 2,5 µl of 100bp ladder was used. The gel was run according to the protocol, at 70 V for 60 minutes, followed by 120 V in 60 additional minutes for further separation of the bands. The gel was photographed and analysed as described previously.

### ***3.4.6 Estimating the probe concentration***

The “DIG Luminescent Detection Kit” from Roche and protocol for this kit was used for this procedure. The SodA- and IntI1-probe described previously were diluted to form solutions of  $10^{-1}$  to  $10^{-5}$ . The first dilution was made by adding 1 µl probe to 9 µl Tris-HCl, pH 8,5 in a 2 ml tube. The following dilutions were made of 1 µl of the previous made solution and 9 µl of ddH<sub>2</sub>O. The tubes were vortexed between every dilution step. Standard DNA of known concentration (1000 pg/µl) from the Kit was diluted in the same way. 1 µl of each dilution were spotted in rows on a positively charged nylon membrane, and cross-linked by UV-light. A standard detection procedure “Immunological detection of DIG nucleotides” was then carried out. After development of the film, the probe DNA and the control DNA were compared to estimate the probe concentrations (Figure 16)

### ***3.4.7 Immunological detection of DIG nucleotides***

This procedure is described in protocol “DIG Luminescent Detection Kit” from Roche. The nylon membrane was incubated in different solutions in a five-step procedure. The membrane was moved between the solutions with forceps. All steps, which were performed during slow shaking, are listed in Table 13

Container	Incubation	Solution name	Volume	Ingredients
1	5 minutes at room temperature	<b>Wash buffer</b>		0,1 M maleic acid, 0,15 M NaCl pH7,5, Tween 20 ((v/v) 0,3%)
2	30 minutes at room temperature	<b>Blocking solution</b>	100 ml	0,1 M maleic acid, 0,15 M NaCl pH7,5
3	30 minutes at room temperature	<b>Anti-digoxigenin-alkaline-phosphatase. Fab fragments</b>	20 ml	2 µl anti-DIG in 20 ml blocking solution
4	2 x 15 minutes at room temperature	<b>Wash buffer</b>	100 ml	0,1 M maleic acid, 0,15 M NaCl pH7,5, Tween 20 ((v/v) 0,3%)
5	5 minutes at room temperature	<b>Detection buffer</b>	20 ml	0,1 M Tris-HCl, 0,1 M NaCl ph 9,5

**Table 13: Immunological detection of DIG nucleotides. The nylon membrane was incubated in these 5 different solutions, each for a given time.**

After performing the described 5 steps, the nylon membrane was transferred in to a reaction bag in transparent plastic. 1 ml of CSPD, was added to the membrane. CSPD is a chemiluminescent substrate for alkaline phosphatase, which produces visible light upon binding to the *Anti-digoxigenin-alkaline-phosphatase*. The solution was distributed evenly and the bag was sealed with a heat sealer. The bag was then incubated at 37°C for 15 minutes to induce the luminescence reaction. The exposure was preformed in the dark room. The reaction bag was placed in an exposure cassette (Agfa CR MD4.0) together with conventional radiographic film and exposed for 15-90 minutes and developed in an automatic film processor (Agfa, Curix 60).

### **3.4.8 Hybridization**

The two nitrocellulose filters with the colonies were incubated in a pre-hybridization solution in a hybridization tube for 1 hour at 68°C in a hybridization oven. In the meantime the DIG-labelled probes were diluted in pre-hybridization solution 1:1. They were then denatured at 99°C for 15 minutes in the thermal block cycler, and then cooled on ice immediately after. The denatured probes were then transferred to 8 ml pre-warmed hybridization solution and vortexed well to make the probe solution. The pre-hybridisation solution in the hybridisation tube was then replaced with the probe-solution. The nitrocellulose filters were then incubated in the probe solution in hybridisation tubes for 68°C overnight (16-20 h). On the next day the nitrocellulose filters were taken out of the tubes and washed with 100 ml 2x Stringency buffer in 2 x 5 minutes in containers during slow shaking. Afterwards, the standard detection procedure “Immunological detection of DIG nucleotides” as previously described was carried out.

### **3.5 Sequencing of the positive controls**

For verification of the PCR amplicons, sequencing of the PCR products from the positive controls used in the IntI1 PCR and IntM1 PCR were done. All samples are listed in Table 16. The IntI1 and the IntM1 PCR products of the three integron class 1 positive strains; K46-62, K45-67, A3-45 were purified with ExoSAP-IT® kit according to the ExoSAP-IT® protocol. 5 µl of PCR-product and 2 µl of ExoSAP-IT® were mixed. It was then incubated for 15 min in 37°C to degrade primers and nucleotides, and then incubated at 80°C in 15 minutes for inactivation of the ExoSAP-IT®. The sequencing reaction was then set up according to the “BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol” for termination reaction. The reaction was set up as (Table 15) shows, in 12 marked 0,2 ml PCR-tubes. The tubes were then placed in the thermal block cycler for PCR reaction, and the reaction was jump-started. The program used is shown in (Table 14) After the cycle sequencing reaction was finished, the PCR-tubes were delivered to the Sequencing lab at Room L6.202A in the MH-building.

96°C 60 seconds	
96°C 10 seconds (denaturation)	
50°C 5 seconds (annealing)	25
cycles	
60°C 4 minutes (primer extension)	
4°C ∞	

**Table 14: PCR cycle program used to prepare the samples for the sequencing reaction.**

<b>Reaction setup for the 12 sequenced samples</b>	
<b>Reagent</b>	<b>Volume</b>
BigDye® 3.1	1 µl
Template DNA (5 µl PCR-product + 2 µl ExoSAP-IT®)	7 µl
BigDye® Terminator 5x Sequencing buffer	2,5 µl
Primer (2 mM)	1,6 µl
ddH <sub>2</sub> O	8 µl
<b>Total reaction volume</b>	<b>20,1 µl</b>

**Table 15: Reaction setup for the 12 sequenced samples.**

<b>The 12 amplicons which were sequenced</b>				
<b>Sample number</b>	<b>Isolate number</b>	<b>Alternate isolate number</b>	<b>Primer (2 mM)</b>	<b>Species</b>
101	K-46-62	FR748151	IntI1-F	<i>K. pneumoniae</i>
102	K-46-62	FR748151	IntI1-R	<i>K. pneumoniae</i>
103	K-45-67	FR748150	IntI1-F	<i>K. pneumoniae</i>
104	K-45-67	FR748150	IntI1-R	<i>K. pneumoniae</i>
105	A3-45	FR748153	IntI1-F	<i>K. pneumoniae</i>
106	A3-45	FR748153	IntI1-R	<i>K. pneumoniae</i>
201	K-46-62	FR748151	IntM1-D	<i>K. pneumoniae</i>
202	K-46-62	FR748151	IntM1-U	<i>K. pneumoniae</i>
203	K-45-67	FR748150	IntM1-D	<i>K. pneumoniae</i>
204	K-45-67	FR748150	IntM1-U	<i>K. pneumoniae</i>
205	A3-45	FR748153	IntM1-D	<i>K. pneumoniae</i>
206	A3-45	FR748153	IntM1-U	<i>K. pneumoniae</i>

**Table 16: List of the 12 sequenced samples.**

### 3.6 Antibiotic susceptibility testing.

The 24 representative isolates chosen for hybridization (listed in the appendix) were also tested for antibiotic susceptibility by agar diffusion tests, and screened for vancomycin resistance on vancomycin agar plates. The colonies were cultivated on standard blood agars over night as previously described. Single colonies were picked with cotton tip swabs and dissolved in a solution of 0,85% NaCl in distilled water. The amount of bacteria added were adjusted to reach a density of 0,5 McFarland. The density was measured with DEN-1, McFarland Densitometer. For the disc diffusion test, the solution was swabbed on Mueller-Hinton agar plates by making a cross on the agar. The agar was then spun on a rotating disc, while swabbing to make a uniform distribution of colonies. The solution was also swabbed on blood agars for cultivation of the controls. Filter-paper discs with antibiotics listed in Table 17 were stamped on to the discs. The Mueller-Hinton agars and the blood agars were cultivated at 37°C over night (16-20 h). The next day, the growth zones were measured and antibiotic susceptibility determined by comparing measured values to the break points set in the Norwegian guidelines “AFAs kliniske brytningspunkter v. 2.1.1, 2011-03-28” [55]

Abbreviation	Generic name	Class of antibiotic	Concentration
AMP	Ampicillin	β-Lactam antibiotic	2
CN	Gentamicin	Aminoglycoside	30
LZD	Linezolid	Oxazolidone	10
RL	Sulfasalazine	Sulphonamides	100
W	Trimethoprim	Dihydrofolate reductase inhibitor	5
SXT	Trimethoprim- Sulfasalazine	Combination	25

Table 17: Filter- paper discs with antibiotics, which were used for the susceptibility testing.

For the screening for vancomycin resistance, BHI agars with 6 mg/L vancomycin and plain BHI-agars were used. The petri dishes were split in six and marked with the isolate numbers of six isolates. One BHI agar with vancomycin and one plain BHI agar were used parallel. A 10 ml droplet of each 0,85% NaCl bacterial solution was applied first to the BHI agar and then to the BHI with vancomycin. The dishes were left to air dry for



some minutes before they were incubated at 35°C for 24 hours. A positive and a negative control (Table 18) were also cultivated after the same procedure. The next day the colonies on the to different agars were inspected parallel. Growth of bacteria on BHI with 6mg/L vancomycin was interpreted as resistance to vancomycin.

<b>Stem</b>	<b>MIC</b>	<b>BHI</b>	<b>BHI with 6mg/L Vancomycin</b>
<i>E. faecalis</i> ATCC 51299 (vanB)	8 mg/ml	Growth	Growth
<i>E. faecalis</i> ATCC 29212	3 mg/ml	Growth	No growth

**Table 18: Postive and negative control used in the sceening for vancomycin resistance.**

## 4 Results

### 4.1 Introduction of results and the collection of the enterococci

A diverse collection of 274 isolates of enterococci was screened for the presence of class 1 integrons. The distribution of the different sample sources, different species and different countries of origin are showed in Figure 11-13. A complete list of all 274 isolates is found in the appendix. The majority of the isolates were blood culture-isolates from Norwegian patients from year 2008, from 20 different hospitals. These were included in the annual report from NORM (Norwegian Surveillance System for Antimicrobial Drug Resistance) [56]. The majority of the isolates were *E. faecium* followed by *E. faecalis*. The pattern of antimicrobial resistance among the isolates had a great variety including resistance to penicillins, aminoglycosides, glycopeptides, trimethoprim and linezolid. Isolates from animals such as poultry, swine and bovine were also included. It was expected to find integrons class 1 in such a diverse collection.

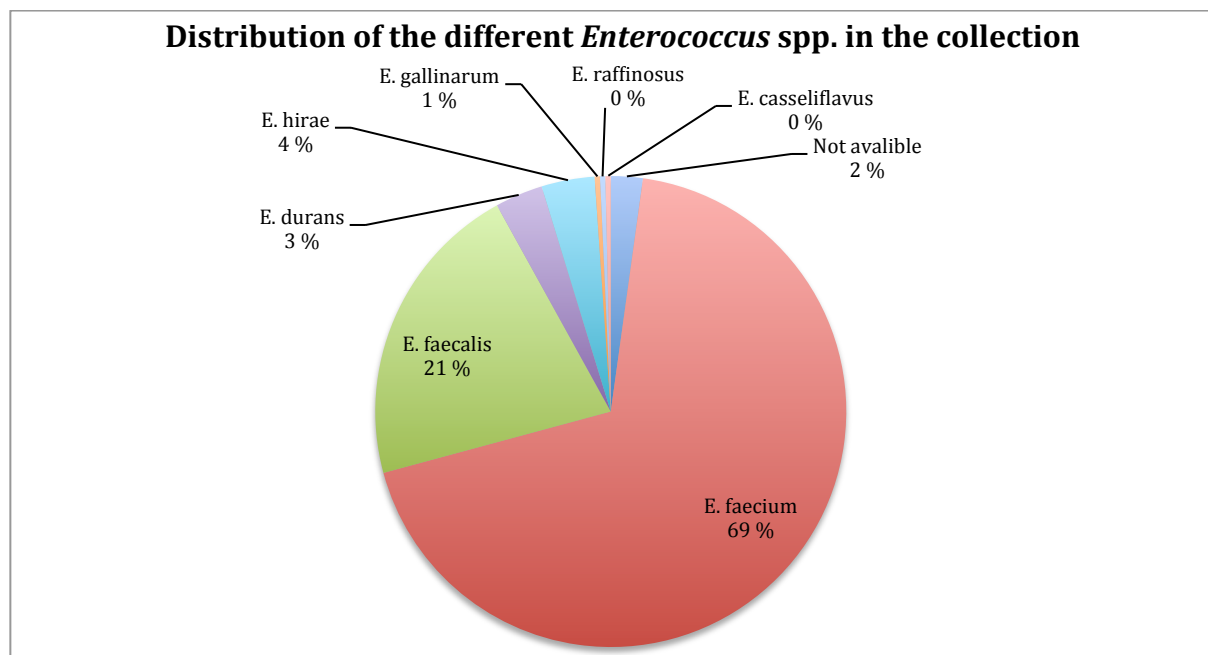
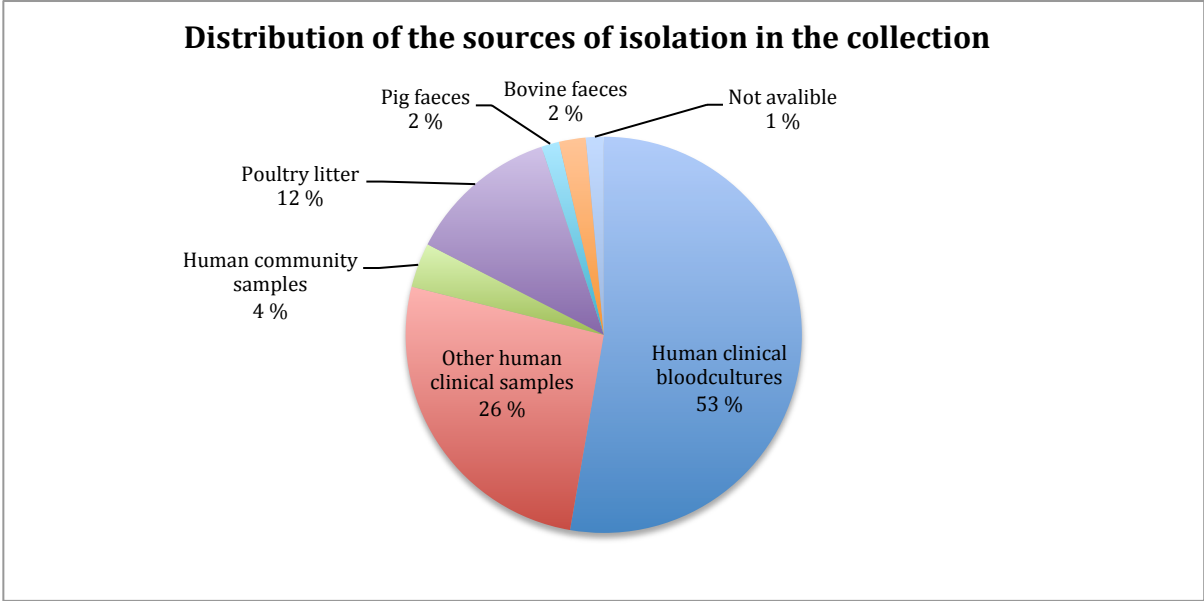
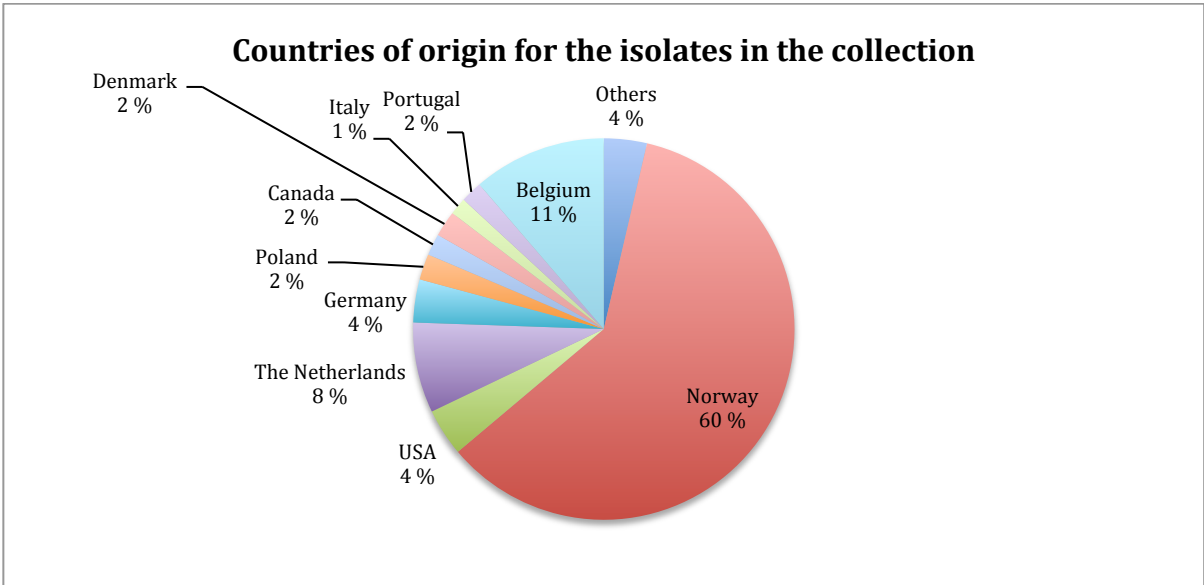


Figure 11: Distribution of the different *Enterococcus* spp. for the 274 *Enterococci* isolates used in this study.



**Figure 12: Distribution of the sources of isolation for the 274 *Enterococci* isolates used in this study.**



**Figure 13: Distribution of the countries of origin in the collection of 274 *Enterococci* isolates used in this study.**

## 4.2 No integrons were found using *IntI1* PCR

All 274 DNA-isolates were screened for the presence of the integrase gene *IntI1*, which is a good marker for presence of class 1 integrons in the DNA. BLAST analyses were performed, before the PCR reactions were carried out, to investigate the homology of the *IntI1* gene and the primer binding-sites (see bellow). SodA PCRs confirming successful isolation of DNA were also carried out (see bellow) before the *IntI1* PCRs were run. None of the enterococci isolates run by *IntI1* PCR turned out to be *IntI1* positive (Figure 14) The three *K. pneumonia* isolates listed in Table 2 known to possess class 1 integrons, formed all clear bands as expected of 250-300 bp on the agarose gels. The positive controls were confirmed to possess *IntI1* genes by DNA sequencing of the amplicons, as described bellow.

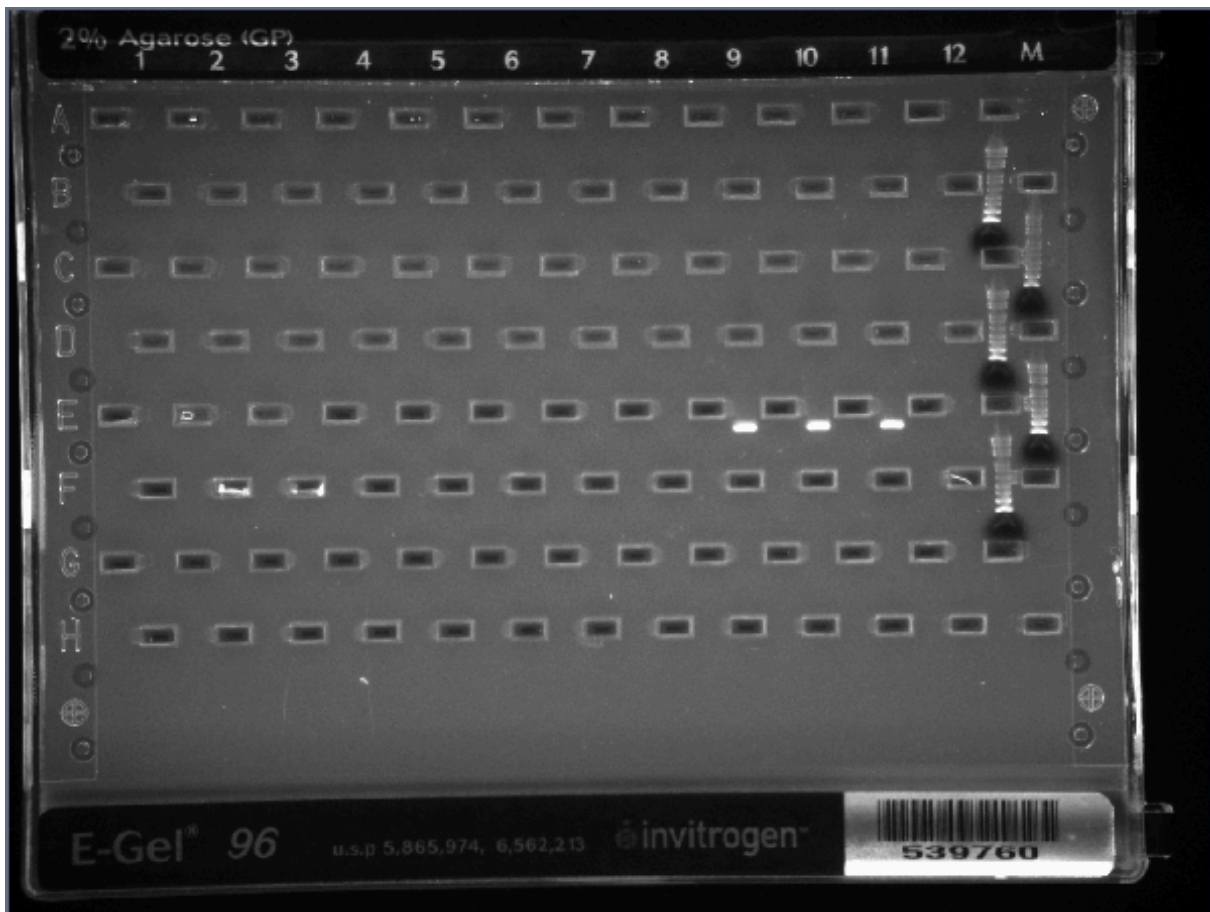
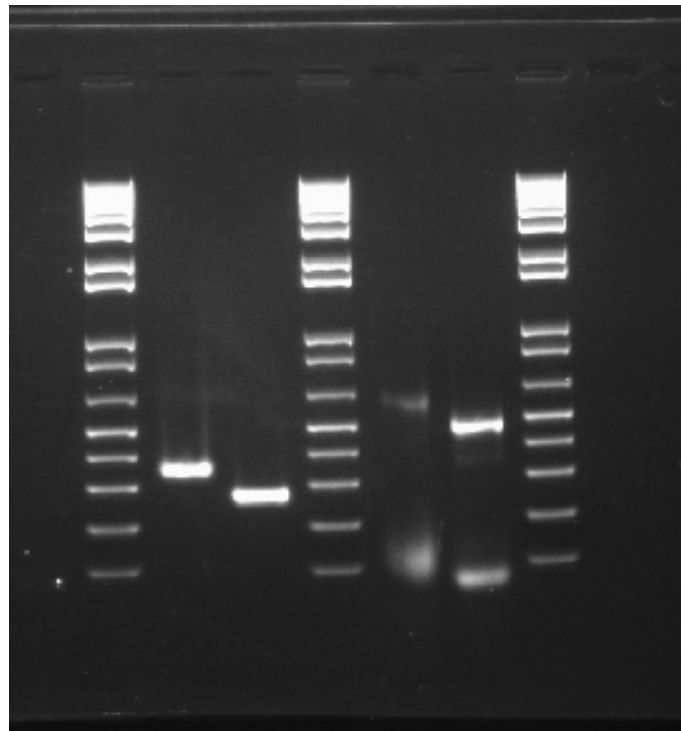


Figure 14: One of several precast E-Gels run in this study. On this gel 91 samples and 3 positive- and 2 negative controls were run. Only the positive controls from well 9-D, 10-D and 11-D form bands on the gel.

### 4.3 DIG-label hybridization yielded to possible positive results

DIG-label hybridization was performed on 24 representative isolates in the search for integrons by a different approach and to possibly verify the PCR-results. A probe based on the *SodA* amplicon, and a probe based on the *IntI1* amplicon was used. Two control amplicons consisting of the same template DNA and the same primers as the corresponding DIG-probes were used to verify presence of the amplicon in the DIG-probe solution, and to compare amplicon size between the probe and the control. Figure 15 show successful incorporation of DIG-nucleotides in the two probes, and an expected increase in the molecular size compared to the control.



**Figure 15: Verification of the probe and controls by agarose gel electrophoresis. From left: *IntI1* probe, *IntI1* control, ladder, *SodA* probe and *SodA* control. The amplicons are visibly present in the probes, and the DIG-probe molecules are as expected slightly bigger than the controls. Some contamination of short fragments, are visible in the *SodA* probe and control.**

The probes were spotted to reveal the DNA concentration of the probes: An estimation based on the film from the probe spotting (Figure 16) yielded a probe concentration of 100pg/  $\mu$ l for the *sodA*- probe, and 500-1000 pg/ $\mu$ l for the *IntI1*- probe.

The exposure of the DIG-probe hybridization of the 24 investigated isolates gave no signals on the film after 15 minutes of exposure. For the next exposure, the time was set to 1h 20 minutes. This yielded 24 positive signals on the *sodA* filter, and both negative controls gave no signal. On the *IntI1* filter, two positive signals showed up in addition to the two positive controls (Figure 17) These two signals are consistent with isolate number 3 and 7 on the nitrocellulose filter, which corresponds to the isolates TUH 32-79 and TUH 41-76 which is both *E. faecium*.



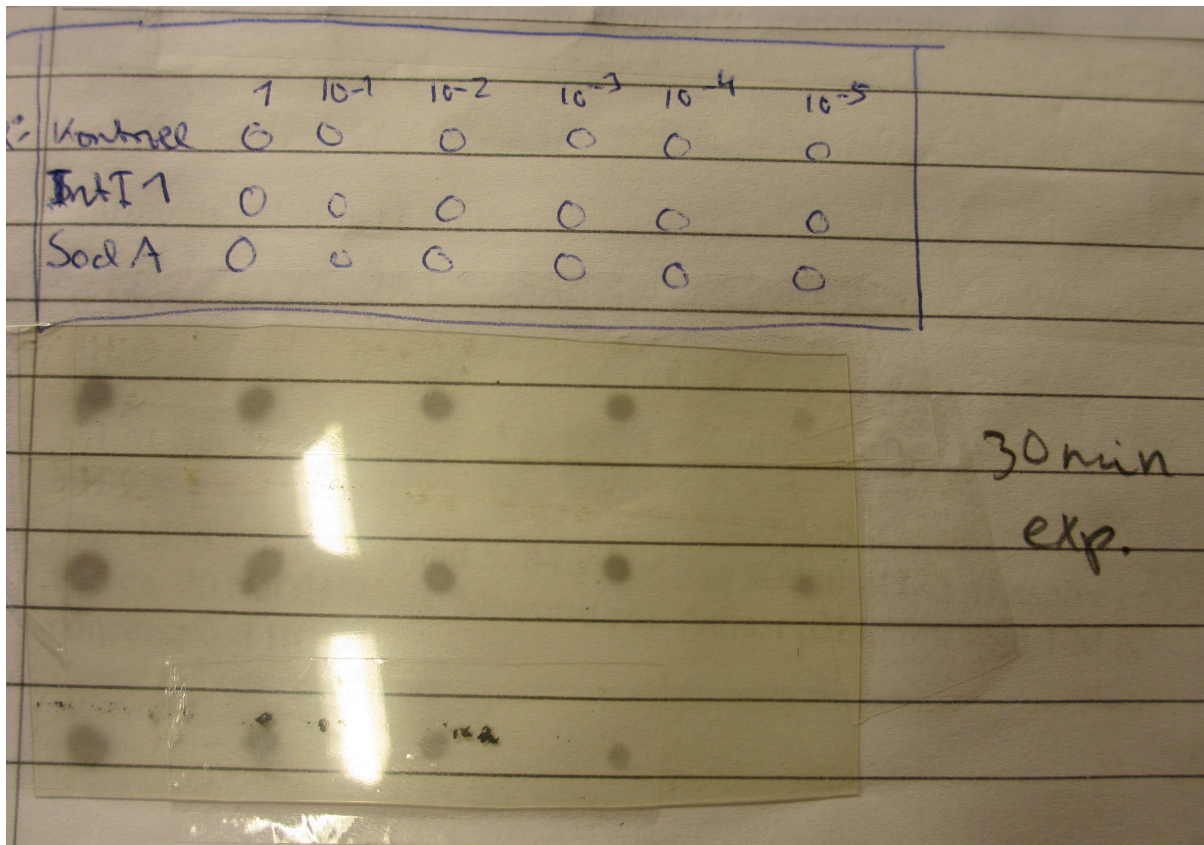


Figure 16: Drawing showing how the probes were spotted on the filter, and the result of the exposure on the film after 30 min. The upper line of spots is the control DNA with a concentration of 1000 pg/ $\mu$ l

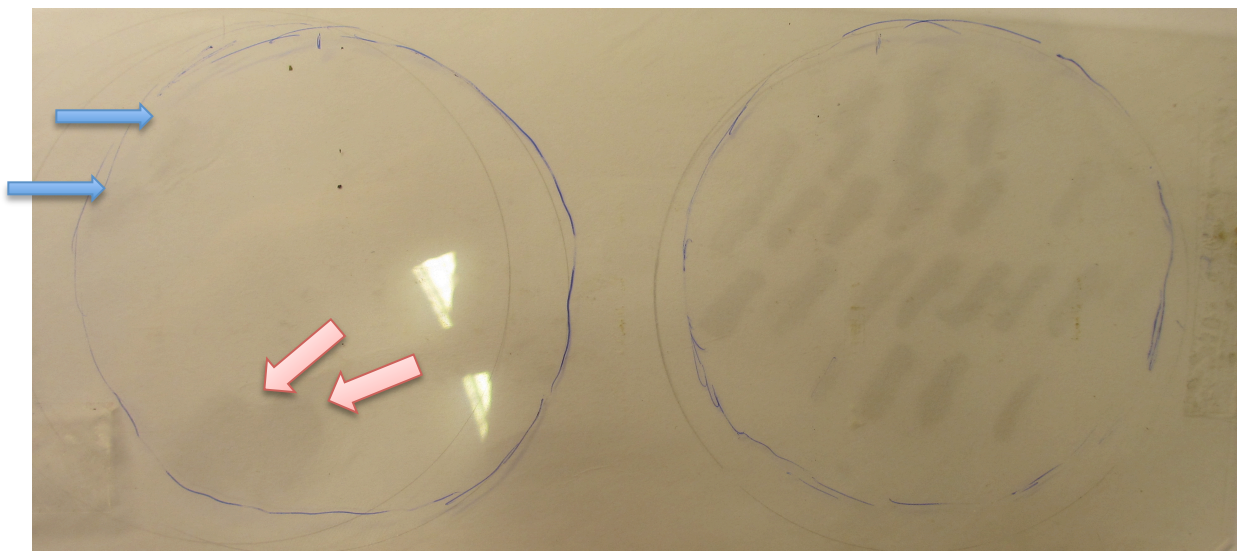


Figure 17: The film exposure of the DIG-probe hybridization of the 24 investigated isolates after 1h 20 minutes. This yielded two weak positive signals in the upper left corner (blue arrows) of *Int1* filter (left) and two positive controls at the bottom (red arrows). 24 positive signals are seen on the *sodA* filter (right) and the two negative controls are negative

#### **4.4 Combined *IntI1/IntM1* –PCR did not confirm the two possible positive results.**

For further investigation of to *IntI1* positive isolates, TUH 32-79 and TUH 41-76, from the DIG-probe hybridization, it was chosen to set up to new PCR-reactions (described previously). The amplicons from the positive controls formed a band in the order 250-300 bp on the gel after “IntI1/IntM1 Short” PCR was preformed. The tested isolates and the negative control turned all out negative. For the setup “IntI1/IntM1 Long” the positive controls formed a band in the order 400-500 bp on the gel, whereas also here the tested isolates and the negative controls turned out negative, and leaving the finding in the isolates, TUH 32-79 and TUH 41-76 unconfirmed.

#### **4.5 Sequencing and Bioinformatics results**

##### **4.5.1 BLAST search of the primers revealed homology with sequences in the *IntI1* gene for both primer pairs.**

To verify that the primers used for PCR would bind to the *IntI1* gene, a BLAST search was performed with the sequences of the two primer pairs IntI1 and IntM1 (Table 6) as query. All of the four searches gave over 1000 hits, and all 50 top hits showed 100% homology with the sequences in sequenced strains possessing the *IntI1* gene. All of the four primers also “found” the “*Enterococcus faecalis* strain XJ-SHZ-111 class I integron integrase-like (*intI1*) gene, partial sequence” in GeneBank, described by Xu *et. al.*[44]. The function “Graphics” was used to revile the binding sites of both primer pairs in the *IntI1* gene (Figure 18) which again was used to calculate the expected amplicon sizes for all PCR reactions run in search for the *IntI1* gene.

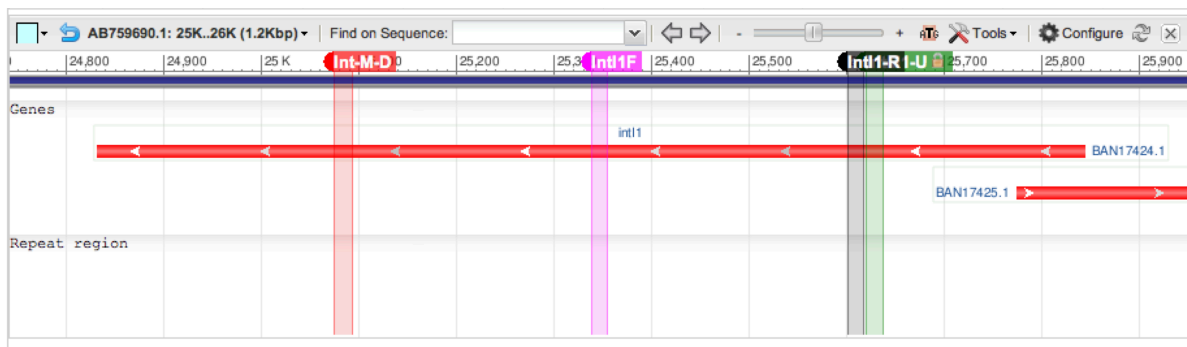


Figure 18: Screenshot from “Graphic view” in the BLAST program, showing the binding sites of the IntM1 and IntI1 primer pairs as red, purple, black and green vertical lines on the 1014 bp long *IntI1* gene, illustrated by the red horizontal line. Source: <http://blast.ncbi.nlm.nih.gov/>

#### 4.5.2 All sequenced amplicons from the positive controls matched sequenced *IntI1* genes in GenBank

To verify that the amplicons formed in the PCR reactions, where fragments of the *IntI1* gene, PCR products from the three integron class 1 positive strains; K46-62, K45-67, A3-45 used in the *IntI1* PCR and *IntM1* PCR were sequenced. The data from the 12 sequenced samples, listed in Table 16 were used as query for 12 separate BLAST searches. All of the 12 searches gave over 800 hits with the top 50 hits showing a Query cover of 97% or more, and a Max identity of 98% or more. All the 50 top hits were in sequenced strains possessing the *IntI1* gene (Figure 19). All the 12 amplicons all showed a high degree of homology with the “*Enterococcus faecalis* strain XJ-SHZ-111 class I integron integrase-like (*intI1*) gene, partial sequence” in GeneBank, described by Xu *et al.*[44]. Query covers were 93% or higher and Max identity were 96% or higher for all the matches between the 12 amplicons and the *E. faecalis* strain.



**Sequences producing significant alignments:**

Select: [All](#) [None](#) Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max score	Total score	Query cover	E value	Max ident	Accession
<input type="checkbox"/>	<a href="#">Salmonella enterica subsp. enterica serovar Typhimurium plasmid pYT4 DNA, antibiotic resistance</a>	433	433	98%	1e-118	99%	<a href="#">AB723628.1</a>
<input type="checkbox"/>	<a href="#">Pseudomonas aeruginosa plasmid pOZ176, complete sequence</a>	433	866	98%	1e-118	99%	<a href="#">KC543497.1</a>
<input type="checkbox"/>	<a href="#">Klebsiella pneumoniae subsp. pneumoniae KPX plasmid pKPX-2 DNA, complete sequence</a>	433	433	98%	1e-118	99%	<a href="#">AP012056.1</a>
<input type="checkbox"/>	<a href="#">Klebsiella pneumoniae subsp. pneumoniae KPX plasmid pKPX-1 DNA, complete sequence</a>	433	866	98%	1e-118	99%	<a href="#">AP012055.1</a>
<input type="checkbox"/>	<a href="#">Klebsiella pneumoniae class I integron integrase gene, partial cds</a>	433	433	98%	1e-118	99%	<a href="#">KC543110.1</a>
<input type="checkbox"/>	<a href="#">Klebsiella pneumoniae plasmid pNDM-1saitama01 DNA, complete sequence, strain: NDM-1saitar</a>	433	866	98%	1e-118	99%	<a href="#">AB759690.1</a>
<input type="checkbox"/>	<a href="#">Acinetobacter baumannii strain D30 genomic resistance island Abar26 genomic sequence</a>	433	433	98%	1e-118	99%	<a href="#">KC665626.1</a>
<input type="checkbox"/>	<a href="#">Escherichia coli strain KR5.12 int11 integrase (int11) gene, partial cds</a>	433	433	98%	1e-118	99%	<a href="#">JQ912639.1</a>
<input type="checkbox"/>	<a href="#">Proteus sp. KR5.6 int11 integrase (int11) gene, partial cds</a>	433	433	98%	1e-118	99%	<a href="#">JQ912638.1</a>
<input type="checkbox"/>	<a href="#">Escherichia coli strain KR4.11 int11 integrase (int11) gene, partial cds</a>	433	433	98%	1e-118	99%	<a href="#">JQ912637.1</a>
<input type="checkbox"/>	<a href="#">Aeromonas sp. KR4.4 int11 integrase (int11) gene, partial cds</a>	433	433	98%	1e-118	99%	<a href="#">JQ912636.1</a>
<input type="checkbox"/>	<a href="#">Bacterium KR2.4 int11 integrase (int11) gene, partial cds</a>	433	433	98%	1e-118	99%	<a href="#">JQ912635.1</a>

**Figure 19: Screenshot from BLAST showing the top hits for a search using the sequence of the amplicon from the Int11-F PCR reaction for *K. Pneumoniae* strain K46-62. Source: <http://blast.ncbi.nlm.nih.gov/>**

#### **4.5.3 BLAST search of *Int11* gene revealed a high degree of homology between the *Int11* genes found in different species.**

To get an impression of how conserved the *Int11* gene is, a BLAST search with the *Int11* gene from the *E. faecalis* strain mentioned above was carried out. The sequence of the *Int11* gene with GeneBank number FJ753285.1 was used as Query for the search. The *Int11* gene from the *E. faecalis* strain is only a partial sequence, in GenBank described as “partial start” and “partial stop” and the length is 883 bp. Investigation of 10 randomly selected *Int11* genes in GenBank which were completely sequenced, revealed a length of the of 1014 pb for all of the genes. It was therefore chosen to use the complete *Int11* gene with the highest homology to the partial sequenced gene FJ753285.1 to perform a new BLAST search. This BLAST search yielded over 1000 hits. The 100 first hits showed identity of 99% or more, whereas all 1000 hits showed an identity of 94% or more. The function “Taxonomy Report” revealed that the hits were spread among more than 20 different genera, and *Pseudomonas*, *Escherichia* and *Klebsiella* being the genera with largest number of hits.

#### 4.6 All isolates were *sodA* positive, confirming successful DNA-isolation.

To confirm that DNA from Gram-positive bacteria had been successfully isolated, we performed a *SodA* PCR on all isolates. A band in the order 400-500 bp, on the agarose gels, where as expected found for all isolates (Figure 20). This confirmed that DNA had been successfully isolated in all cases.

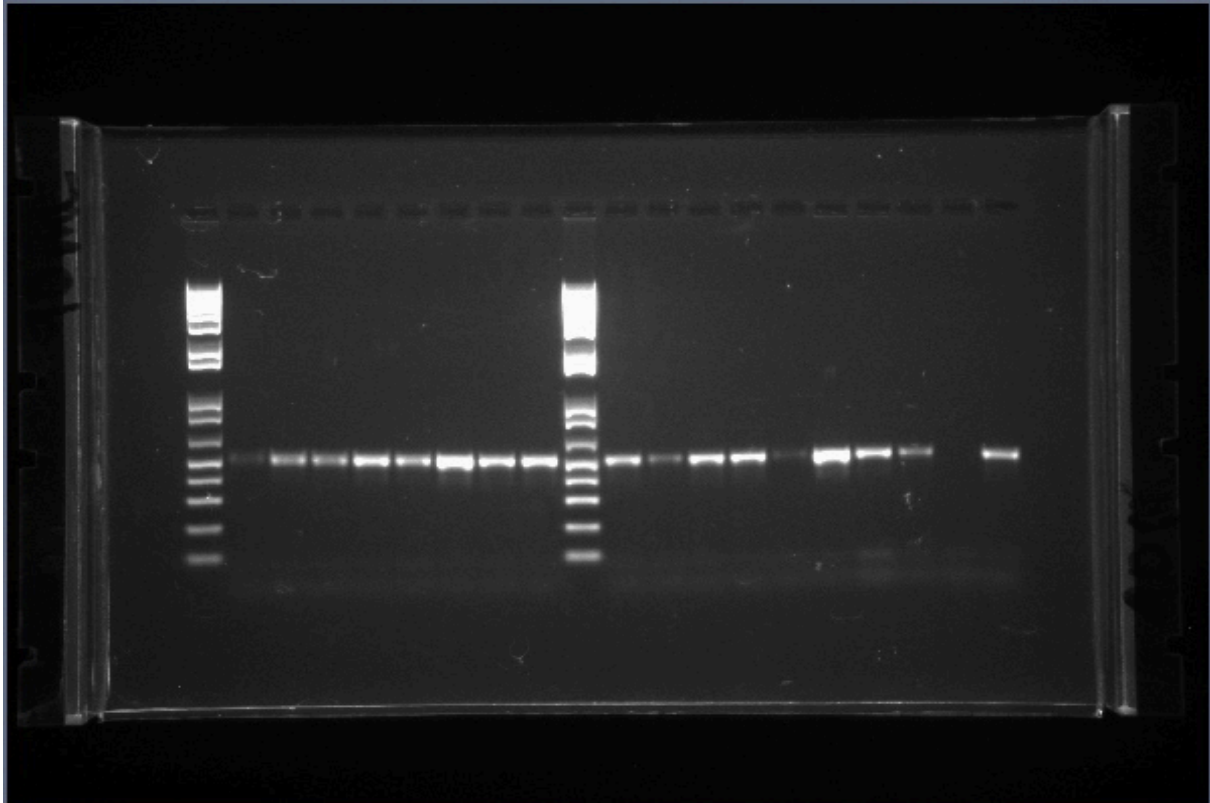
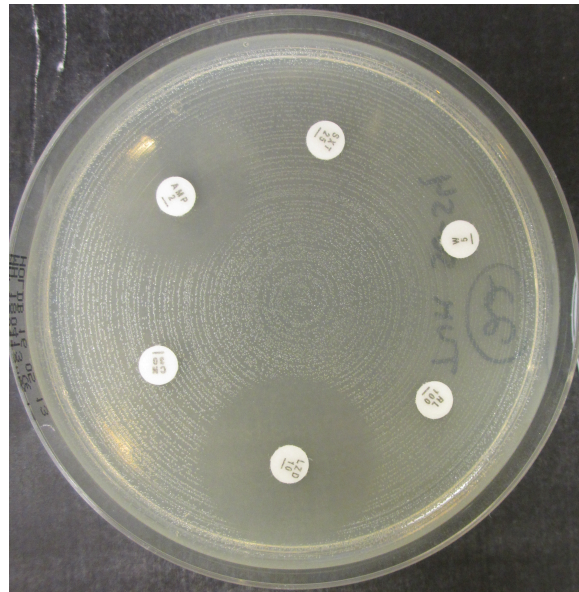


Figure 20: Self poured 1% Agarose Gel, with *sodA* positive PCR-products, forming bands in the order 400-500 bp, This fits with the 438 bp amplicon the PCR is supposed to yield.

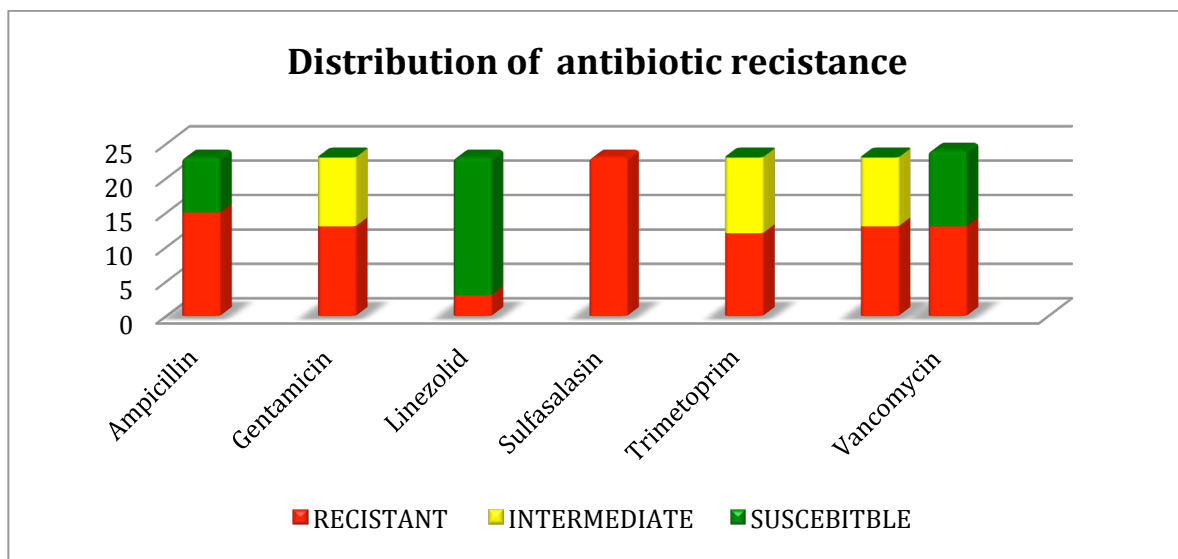
#### 4.7 Antibiotic susceptibility testing revealed a variety of resistance profiles.

To get an impression of the pattern of antimicrobial resistance in the collection, 24 representative selected isolates (all listed in the appendix) were tested for antibiotic susceptibility by standard agar diffusion tests (Figure 21) and screened for vancomycin resistance on vancomycin agar plates. The test panel for agar diffusion tests included Ampicillin, Gentamicin, Linezolid, Sulfasalazine, Trimethoprim and Trimethoprim-Sulpha (Table 17) Enterococci are intrinsic resistant to sulphonamides such as sulfasalazine, and intermediate resistance for aminoglycosides such as gentamicin. The activity of trimethoprim and trimethoprim-sulpha on enterococci are uncertain, and the breakpoints set by EUCAST (The European Committee on Antimicrobial Susceptibility

Testing), which relate only to urinary tract infections, categorizes the wild type population as intermediate.[57] Hence all isolates tested showed resistance to sulfasalazine, and intermediate resistance to gentamicin, trimethoprim and trimethoprim-sulpha. One of the isolates, K120a, a Belgian poultry isolate, grew poorly, and reading the resistance profile was therefore not possible. Figure 22 shows the distribution of resistance against different antimicrobials in the 24 tested isolates.



**Figure 21: Example of standard diffusion test on one agar gel. This isolate shows resistance to SXT (Trimethoprim-Sulfa), W (Trimethoprim), RL (Sulfasalazine), and CN (Gentamicin), and susceptibility to LZD (Linezolid) and AMP(Ampicillin)**



**Figure 22: Distribution of antibiotic resistance against the 7 different antimicrobials in the 24 tested isolates**

## 5 Discussion

Class 1 integrons, which are widespread among clinically important Gram-negatives, have recently been detected in clinical *E. faecium* and *E. faecalis* isolates [44]. It was therefore of great interest to search for integrons in enterococci, and it was expected to find integron positive isolates in a diverse collection of enterococci. We screened 274 enterococcal isolates by running *IntI1* PCR, which is a good marker for presence of class 1 integrons.

### 5.1 No class 1 integrons were found by PCR-screening

A diverse collection of 274 enterococcal isolates was investigated in this study. If class 1 integrons were widespread in enterococci, it would be expected to find integrons in at least some isolates. However, the presence of integrons or integron related genes in enterococci are only described twice: First time in an *E. faecalis* strain isolated in Wisconsin, USA, which harboured the integron related *aad* gene [42] and second in 13 *E. faecalis* and 2 *E. faecium* strains isolated in South China in 2001-2004, where the *IntI1* gene was found in addition to several integron related gene cassettes [44]. In the latter study, transfer of the integrons from gram negatives to enterococci in the hospital setting is suggested. This theory is supported by the facts that enterococci are known for their vast capacity to acquire as well as disseminate genes [32] and horizontal gene transfer among enterococci are described to occur in the digestive tract [58, 59]. This makes the intestine in a hospitalized patient to a perfect place for transfer of antibiotic resistance genes, because of the presence of both antimicrobials and microbes. Nandi *et al.* [43] described findings of several integron class 1 harbouring Gram-positives including staphylococci in poultry litter, which also is an environment where antimicrobials and microbes are abundant in high levels. Integrons might therefore spread to Gram-positives including enterococci in the right environment, but they might not disseminate that quickly. However, if transfer of integrons from Gram-negatives to gram-positives is possible, it might just be a question of time, before integrons also will be found in enterococci in Europe as well.

## 5.2 Two possible positive results by Colony Blot Hybridisation

The *IntI1* hybridization of 24 representative selected isolates gave two possible integrase positive isolates: TUH 32-79 and TUH 41-76. One was isolated from human faeces in a Dutch hospital in year 2000 and the other from human faeces in an Italian hospital in 2002. To investigate these isolates two further PCR reactions were set up, however both, turned out negative. Two possible explanations for this are considered. 1) The *IntI1* gene found by hybridisation in the TUH 32-79 and TUH 41-76 is a true positive and 2) The *IntI1* gene found is a false positive. If the first explanation is true, the *IntI1* gene found in the two isolates does not have a high degree of homology with the *IntI1* genes described in literature and submitted to GenBank. In this hybridization experiment, a probe of 280 bp was used, whereas the primers only were 17-19 bp long. This means that the probability for the probe to hybridize to the gene searched for still is high, even if some mutations and rearrangements have taken place in the gene. For PCR on the other hand: If one of the short primer-binding sites is disrupted, it will result in a negative PCR. This could be a possible explanation for the negative PCR on the TUH 32-79 and TUH 41-76 isolates, even if integron class 1 is present. If the second alternative is true, and the hybridization results are false, the DIG-probes must have bound to other DNA fragments during the hybridization. This is possible, because hybridization techniques are less specific than PCR. During hybridization, some probes will bind to all kinds of DNA present in the sample and it is just the amount of bound probes that show the differences between a positive and a negative result, and a longer exposure time would have made all results look positive, due to more light on the film. There might be some DNA sequences in the TUH 32-79 and TUH 41-76 isolates, that are not the *IntI1* gene, but have similarities to structures in the *IntI1* probe, and therefore induce a higher degree of probe binding than the rest of the isolates. There might be that the exposure time were just right for those two isolates to become positive, and some minutes longer exposure time would have yielded several false positives. The signals from TUH 32-79 and TUH 41-76 were not as clearly positive as the two *K. pneumonia* strains that were used as positive controls (Figure 17) One mistake in the performance of colony blot hybridization was the lack of an *IntI1*-negative control strain e.g. *E. faecium* strain TUH 32-56/ TX0016 (DO). If we had included this negative control in the hybridization, we could have prolonged the exposure time, until the TUH 32-56 strain also would produce a signal on the film. At

that point we would have been sure that other positive results could be false negative. Results on the film at a shorter exposure time, whit the negative control not giving a signal, would more probable be true positives.

### **5.3 Possible methodical errors leading to false negative *IntI1* PCR**

There are a lot of possible methodical reasons for a PCR reaction to turn out negative even when the sequence searched for is present in the sample. These include, no presence of DNA in the DNA extract, wrong composition or loss of ingredient in the master mix, not suitable cycle sequencing conditions, and failure in the production of or the run of the agarose gel. In this case, all of these mentioned possible reasons are excluded: Soda PCR confirmed presence of Gram-positive DNA in the DNA isolates. The presence of positive controls, which came out as positives in all 12 *IntI1* PCR runs that were done, confirms that the master mix, the cycle sequencing conditions and the agarose gel all functioned properly. One other possibility is the presence of the integrase gene *IntI1*, but mismatch between the primers and the primer-binding sequences in the *IntI1* gene, due to changes in the gene e.g. by mutations. This is however considered as unlikely as the *IntI1* gene seems to be very conserved, with a high degree of homology[44]. In the BLAST searches run in this study, the 100 first hits showed an identity of 99% or more, whereas all 1000 hits showed an identity of 94% or more. The 1000 hits were spread among more than 20 different genera, which confirms a conserved structure of the *IntI1* gene. A set of primers that bind the *IntI1* positive *K. pneumonia* controls used in this study should hence also bind theoretically present *IntI1* genes in enterococci. The BLAST search of the *IntI1* primers showed 100% identical matches with regions in the *IntI1* gene in a vast number of different *IntI1* harbouring strains of different genera including the *E. faecalis* strain added by Xu.et al[44]. Based on these observations, one can say that a false negative *IntI1* PCR reaction in this study is unlikely.

#### **5.4 Possible consequences of spread of integrons to enterococci**

The findings by Xu et. al [44] point in the direction of spread of class 1 integrons from Gram-negatives to enterococci in a hospital setting. In this study however, we did not do any reliable findings of integrons of class 1 among the 274 isolates screened. It is possible that integrons might be acquired by enterococci e.g. by conjugation from Gram-negatives. However they might not possess the promoter apparatus to make use of the integrons and express their gene cassettes. If the enterococci can't make use of the integrons, bacteria possessing the integrons will not be favoured by evolutionary selection over other bacteria in e.g. an antimicrobial environment. If this is the case, integrons who has entered enterococci will not disseminate. If the opposite is the case, a much gloomier picture is painted. Hospital adapted strains of enterococci is already a worldwide issue[8, 9, 15] and resistance genes to all clinical important antibiotics have been described[24, 60]. Enterococci are also known to have transferred several antimicrobial resistance genes to hospital-adapted bacteria of other genera [61-63]. As there are known over 130 different gene cassettes linked to class 1 integrons, associated with antimicrobial resistance[34], an inclusion of this apparatus in enterococci could be catastrophic. Up to eight different gene-cassettes are described present at once in one Mobile integron such as class 1 integrons[35]. Such integrons could be transferred to conjugative transposons ore plasmids, which would yield vessels of multi-resistance[25], transferable to other bacteria, and even other genera.

## 6 Proposed further investigations

The two possible positive isolates, TUH 32-79 and TUH 41-76, should be further investigated to clarify if they possess integrons or not. A new colony blot hybridization of the two strains, with the presence of with an *IntI1*-negative control should at least be performed. It is also possible to do a hybridization based on the *IntM1* primer pair. This primer pair produces a longer amplicon, of 565 bp, and hence a probe of the same size. This in contrast to the 280 bp *IntI1* probe. The longer *IntM1* probe would have an increased sensitivity, and a positive signal from the TUH 32-79 and TUH 41-76 isolates would point in the direction of the presence of an *IntI1* gene. It would also be favourable to investigate all the 274 isolates in the collection by hybridization techniques, where the possibility to reduce the specificity is present, and in this way one may be able to detect genes with a lower degree of homology.

## 7 Concluding remarks

Class 1 integrons have been observed in enterococcal isolates from South China in one study. In this study we investigated 274 isolates from different western countries and from different epidemiological origin by PCR, and all of the isolates were found to be negative. 24 of the isolates were further examined by colony blot hybridization, and two possible positives were detected. Further investigation of the two possible positive isolates is required to verify the results. Moreover the whole isolate collection should additionally be investigated by hybridisation techniques before a conclusion of the findings can be made.



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## 9 Appendix

### 9.1 List of all 274 enterococcal isolates used in this study

Number	ID / Stock number	Alternative ID	Species	Isolate source	Year	Origin	Reference or source
<b>Paper: Rosvoll et. al 2010</b>							
1	TUH 44-39	A0249	<i>E. faecium</i>	Poultry litter	1999	Norway	(Johnsen <i>et al.</i> , 2005)
2	TUH 44-29	A0248	<i>E. faecium</i>	Human community sample	1999	Norway	(Johnsen <i>et al.</i> , 2005)
3	TUH 44-34		<i>E. faecium</i>	Poultry litter	1998	Norway	(Johnsen <i>et al.</i> , 2005)
4	TUH 41-64	U37	<i>E. faecium</i>	Other human clinical samples	1996	USA	(Grady and Hayes, 2003)
5	TUH 12-01	C68	<i>E. faecium</i>	Other human clinical samples	1996	USA	(Carias <i>et al.</i> , 1998)
6	TUH 12-04	A0885	<i>E. faecium</i>	Other human clinical samples	1996	USA	(Carias <i>et al.</i> , 1998)
7	TUH 2-18		<i>E. faecium</i>	Other human clinical samples	1996	Norway	(Dahl <i>et al.</i> , 2003)
8	TUH 2-08		<i>E. faecium</i>	Other human clinical samples	1995	Sweden	(Torell <i>et al.</i> , 1997)
9	TUH 32-56	TX0016 (DO)	<i>E. faecium</i>	Other human clinical samples	1992	USA	Arduino <i>et. Al.</i> , 1994: Intl negative control
10	TUH 32-62	E0510	<i>E. faecium</i>	Other human clinical samples	1998	Australia	Willems, R. J. L. 2005
11	TUH 32-64	E0013	<i>E. faecium</i>	Other human clinical samples	1992	UK	Willems, R. J. L. 2005
12	TUH 32-65	E1272	<i>E. faecium</i>	Other human clinical samples	NA	France	Willems, R. J. L. 2005
13	TUH 32-66	E1279	<i>E. faecium</i>	Other human clinical samples	NA	Germany	Willems, R. J. L. 2005
14	TUH 32-67	E0403	<i>E. faecium</i>	Human community sample	1997	The Netherlands	Willems, R. J. L. 2005
15	TUH 32-68	E0996	<i>E. faecium</i>	Human community sample	1998	The Netherlands	Willems, R. J. L. 2005
16	TUH 32-69	E0470	<i>E. faecium</i>	Other human clinical samples	1999	The Netherlands	Willems, R. J. L. 2005
17	TUH 32-70	E0734	<i>E. faecium</i>	NA	2000	The Netherlands	Willems, R. J. L. 2005
18	TUH 32-71	E0745	<i>E. faecium</i>	Other human clinical samples	2000	The Netherlands	Willems, R. J. L. 2005
19	TUH 32-72	E0155	<i>E. faecium</i>	Other human clinical samples	1995	USA	Willems, R. J. L. 2005
20	TUH 32-73	E1652	<i>E. faecium</i>	Other human clinical samples	2002	The Netherlands	Willems, R. J. L. 2005
21	TUH 32-74	E0300	<i>E. faecium</i>	Other human clinical samples	1994	USA	Willems, R. J. L. 2005
22	TUH 32-75	E0073	<i>E. faecium</i>	Other human clinical samples	1995	The Netherlands	Willems, R. J. L. 2005
23	TUH 32-76	E1304	<i>E. faecium</i>	Other human clinical samples	NA	Portugal	Willems, R. J. L. 2005
24	TUH 32-77	E1173	<i>E. faecium</i>	Other human clinical samples	NA	Portugal	Willems, R. J. L. 2005
25	TUH 32-78	E0125	<i>E. faecium</i>	Other human clinical samples	1995	The Netherlands	Willems, R. J. L. 2005
26	TUH 32-79	E0729	<i>E. faecium</i>	Other human clinical samples	2000	The Netherlands	Willems, R. J. L. 2005
27	TUH 32-80	E0092	<i>E. faecium</i>	Human community sample	1997	The Netherlands	Willems, R. J. L. 2005
28	TUH 4-21		<i>E. faecium</i>	Poultry litter	1995	Norway	(Simonsen <i>et al.</i> , 1998)
29	TUH 4-22		<i>E. faecium</i>	Poultry litter	1995	Norway	(Simonsen <i>et al.</i> , 1998)
30	TUH 4-65	A0884	<i>E. faecium</i>	Other human clinical samples	NA	USA	(Dahl <i>et al.</i> , 1999)
31	TUH 44-23		<i>E. faecium</i>	Human community sample	1998	Norway	(Johnsen <i>et al.</i> , 2005)
32	TUH 44-31	A0888	<i>E. faecium</i>	Poultry litter	1998	Norway	(Johnsen <i>et al.</i> , 2005)
33	TUH 44-40	A0887	<i>E. faecium</i>	Poultry litter	1999	Norway	(Johnsen <i>et al.</i> , 2005)
34	TUH 45-12	A0886	<i>E. faecium</i>	Poultry litter	1999	Norway	(Johnsen <i>et al.</i> , 2005)
35	TUH 45-22		<i>E. faecium</i>	Poultry litter	1999	Norway	(Johnsen <i>et al.</i> , 2005)
36	TUH 45-03	A0889	<i>E. faecium</i>	Human community sample	1998	Norway	(Johnsen <i>et al.</i> , 2005)
37	TUH 45-05		<i>E. faecium</i>	Human community sample	1999	Norway	(Johnsen <i>et al.</i> , 2005)
38	TUH 44-52		<i>E. faecium</i>	Poultry litter	1999	Norway	(Johnsen <i>et al.</i> , 2005)
39	TUH 44-50	A0251	<i>E. faecium</i>	Poultry litter	1998	Norway	(Johnsen <i>et al.</i> , 2005)
40	TUH 44-42		<i>E. faecium</i>	Human community sample	1998	Norway	(Johnsen <i>et al.</i> , 2005)
41	TUH 44-47		<i>E. faecium</i>	Human community sample	1999	Norway	(Johnsen <i>et al.</i> , 2005)
42	TUH 45-35		<i>E. faecium</i>	Poultry litter	1999	Norway	(Johnsen <i>et al.</i> , 2005)
43	TUH 45-25		<i>E. faecium</i>	Human community sample	1998	Norway	(Johnsen <i>et al.</i> , 2005)

44	TUH 45-30	<i>E. faecium</i>	Poultry litter	1999	Norway	(Johnsen <i>et al.</i> , 2005)
45	TUH 7-15	<i>E. faecium</i>	Other human clinical samples	NA	USA	(Dahl <i>et al.</i> , 1999)
46	TUH 7-55	<i>E. faecium</i>	Other human clinical samples	NA	Germany	(Dahl <i>et al.</i> , 1999)
47	K8-42	<i>E. faecium</i>	Other human clinical samples	2003	Norway	Rosvoll <i>et al.</i> 2010
48	K8-43	<i>E. faecium</i>	Other human clinical samples	2003	Norway	Rosvoll <i>et al.</i> 2010
49	K8-45	<i>E. faecium</i>	Other human clinical samples	2003	Norway	Rosvoll <i>et al.</i> 2010
50	K9-26	<i>E. faecium</i>	Other human clinical samples	2003	Norway	Rosvoll <i>et al.</i> 2010
51	K9-27	<i>E. faecium</i>	Other human clinical samples	2003	Norway	Rosvoll <i>et al.</i> 2010
52	K9-28	<i>E. faecium</i>	Other human clinical samples	2003	Norway	Rosvoll <i>et al.</i> 2010
53	K9-72	<i>E. faecium</i>	Other human clinical samples	2004	Norway	Rosvoll <i>et al.</i> 2010
54	K8-50	<i>E. faecium</i>	Other human clinical samples	2001	Norway	Rosvoll <i>et al.</i> 2010
55	TUH 2-40	<i>E. faecium</i>	Other human clinical samples	1995	Norway	(Torfoss <i>et al.</i> , 1999)
56	TUH 2-13	<i>E. faecium</i>	Other human clinical samples	1996	Norway	(Haarr <i>et al.</i> , 1998)
57	TUH 7-56	<i>E. faecium</i>	Other human clinical samples	1995	Norway	(Torfoss <i>et al.</i> , 1999)
58	K8-62	<i>E. faecium</i>	Other human clinical samples	1999	Norway	Rosvoll <i>et al.</i> 2010
59	K8-64	<i>E. faecium</i>	Other human clinical samples	2001	Norway	Rosvoll <i>et al.</i> 2010
60	K8-74	<i>E. faecium</i>	Other human clinical samples	2002	Norway	Rosvoll <i>et al.</i> 2010
61	K8-69	<i>E. faecium</i>	Other human clinical samples	2001	Norway	Rosvoll <i>et al.</i> 2010
62	K8-68	<i>E. faecium</i>	Other human clinical samples	2001	Norway	Rosvoll <i>et al.</i> 2010
63	K9-10	<i>E. faecium</i>	Other human clinical samples	2002	Norway	Rosvoll <i>et al.</i> 2010
64	K18-79	<i>E. faecium</i>	Other human clinical samples	2004	Norway	Rosvoll <i>et al.</i> 2010
65	TUH 41-73	<i>E. faecium</i>	Poultry litter	2003	Italy	(Biavasco <i>et al.</i> , 2007)
66	TUH 41-76	<i>E. faecium</i>	Other human clinical samples	2002	Italy	(Biavasco <i>et al.</i> , 2007)
67	TUH 41-67	<i>E. faecium</i>	Other human clinical samples	2002	Italy	(Biavasco <i>et al.</i> , 2007)

#### Belgian animal isolates

	K17a	<i>E. faecium</i>		2005 and earlier	Belgium	P. Butaye
68			Poultry litter			
69	K40b	<i>E. faecium</i>	Poultry litter	"	Belgium	P. Butaye
70	V63b	<i>E. faecium</i>	Pig faeces	"	Belgium	P. Butaye
71	V128	<i>E. faecium</i>	Pig faeces	"	Belgium	P. Butaye
72	K101b	<i>E. durans</i>	Poultry litter	"	Belgium	P. Butaye
73	K4a	<i>E. durans</i>	Poultry litter	"	Belgium	P. Butaye
74	K21b	<i>E. durans</i>	Poultry litter	"	"Belgium	P. Butaye
75	K70	<i>E. durans</i>	Poultry litter	"	Belgium	P. Butaye
76	K116a	<i>E. durans</i>	Poultry litter	"	Belgium	P. Butaye
77	K118c	<i>E. durans</i>	Poultry litter	"	Belgium	P. Butaye
78	K120a	<i>E. durans</i>	Poultry litter	"	Belgium	P. Butaye
79	K121	<i>E. durans</i>	Poultry litter	"	Belgium	P. Butaye
80	K51b	<i>E. hirae</i>	Poultry litter	"	Belgium	P. Butaye
81	K56b	<i>E. hirae</i>	Poultry litter	"	Belgium	P. Butaye
82	K66a	<i>E. hirae</i>	Poultry litter	"	Belgium	P. Butaye
83	K73a	<i>E. hirae</i>	Poultry litter	"	Belgium	P. Butaye
84	K74b	<i>E. hirae</i>	Poultry litter	"	Belgium	P. Butaye
85	K79b	<i>E. hirae</i>	Poultry litter	"	Belgium	P. Butaye
86	K115b	<i>E. hirae</i>	Poultry litter	"	Belgium	P. Butaye
87	K141	<i>E. hirae</i>	Poultry litter	"	Belgium	P. Butaye
88	V70b	<i>E. hirae</i>	Pig faeces	"	Belgium	P. Butaye
89	V106c	<i>E. hirae</i>	Pig faeces	"	Belgium	P. Butaye
90	B137	NA	Bovine faeces	"	Belgium	P. Butaye
91	B136b	NA	Bovine faeces	"	Belgium	P. Butaye
92	B134c	NA	Bovine faeces	"	Belgium	P. Butaye
93	B113b	NA	Bovine faeces	"	Belgium	P. Butaye
94	B111b	NA	Bovine faeces	"	Belgium	P. Butaye
95	B107b	NA	Bovine faeces	"	Belgium	P. Butaye
96	K29a	<i>E. faecium</i>	Poultry litter	"	Belgium	P. Butaye
97	K26b	<i>E. faecalis</i>	Poultry litter	"	Belgium	P. Butaye
98	K25b	<i>E. faecium</i>	Poultry litter	"	Belgium	P. Butaye

#### Norm-2008



99	K58-37	41996	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
100	K58-39	89332	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
101	K58-41	5028	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
102	K58-42	8569	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
103	K58-44	et2	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
104	K58-45	et7	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
105	K58-46	et8	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
106	K58-48	et25	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
107	K58-49	10024691	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
108	K58-53	11045431	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
109	K58-54	11290601	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
110	K58-55	92774	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
111	K58-57	62883	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
112	K58-60	8290	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
113	K58-62	50095526 50131627	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
114	K58-63	C	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
115	K58-64	50135485	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
116	K58-71	908301544	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
117	K58-72	908302487	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
118	K58-73	651937	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
119	K58-77	656406	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
120	K58-78	658545	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
121	K58-79	12507	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
122	K59-01	49964	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
123	K59-02	75753	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
124	K59-03	85710	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
125	K59-04	691	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
126	K59-05	3347	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
127	K59-06	16301	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
128	K59-08	34425	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
129	K59-09	35015	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
130	K59-10	141080041 2	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
131	K59-12	141080727 5	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
132	K59-13	141081343 5	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
133	K59-15	141082094 3	<i>E. faecalis</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al 2013
134	K59-16	89332	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
135	K59-17	18317	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
136	K59-18	21864	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
137	K59-19	36488	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
138	K59-20	5977	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
139	K59-21	20641	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
140	K59-22	29966	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
141	K59-23	19156	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
142	K59-25	418541	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
143	K59-26	et10	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
144	K59-27	et20	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
145	K59-28	20575	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
146	K59-29	20972	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
147	K59-30	22135	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
148	K59-31	22857	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
149	K59-32	54271	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
150	K59-33	78135	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
151	K59-34	104499	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
152	K59-35	89364	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
153	K59-36	5282	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
154	K59-37	14944	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012



155	K59-40	30697	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
156	K59-41	30817	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
157	K59-42	36783	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
158	K59-43	37471	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
159	K59-44	43134	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
160	K59-46	64750	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
161	K59-48	115920	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
162	K59-49	129766	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
163	K59-50	908900774	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
164	K59-51	10146163	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
165	K59-52	10181971	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
166	K59-54	10403171	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
167	K59-55	10454581	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
168	K59-56	10487521	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
169	K59-57	10527341	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
170	K59-58	10852801	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
171	K59-62	81163	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
172	K59-63	79932	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
173	K59-64	69202	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
174	K59-65	33027	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
175	K59-66	71328	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
176	K59-67	50103605	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
177	K59-68	50108366	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
178	K59-69	50108430	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
179	K59-70	50116045	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
180	K59-71	50117412	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
181	K59-73	50150755	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
182	K59-74	50159291	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
183	K59-76	50183388	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
184	K59-77	50154026	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
185	K59-78	7101	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
186	K60-02	908301850	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
187	K60-04	651578	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
188	K60-05	653294	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
189	K60-06	655387	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
190	K60-07	656443	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
191	K60-08	657214	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
192	K60-09	506120	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
193	K60-10	508404	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
194	K60-12	9339	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
195	K60-13	20167	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
196	K60-14	38459	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
197	K60-15	40648	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
198	K60-20	73323	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
199	K60-21	87205	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
200	K60-22	14473	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
201	K60-23	15722	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
202	K60-24	17147	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
203	K60-25	18580	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
204	K60-26	26495	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
205	K60-27	27719	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
206	K60-29	31229	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
207	K60-30	39254	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
208	K60-31	141080193 7	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
209	K60-32	141080268 2	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
210	K60-33	141080351 7	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012
211	K60-35	141080371	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway	Rosvoll et al, 2012

		9						
212	K60-36	141080450						Rosvoll et al, 2012
		0	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway		
213	K60-38	141081024						Rosvoll et al, 2012
		3	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway		
214	K60-39	141081068						Rosvoll et al, 2012
		1	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway		
215	K60-40	141081126						Rosvoll et al, 2012
		8	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway		
216	K60-42	141082046						Rosvoll et al, 2012
		2	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway		
217	K60-43	141082511						Rosvoll et al, 2012
		4	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway		
218	K60-44	141082510						Rosvoll et al, 2012
		4	<i>E. faecium</i>	Human clinical bloodcultures	2008	Norway		

#### ACE-Project

								T. Mikalsen
219	TUH 50-45	U0229/O0 160/50661 9	<i>E. faecium</i>	Human clinical bloodcultures	1995	The Netherlands		T. Mikalsen
220	TUH 50-46	U0218/Q1 825/77040 2 I	<i>E. faecium</i>	Human clinical bloodcultures	1997	The Netherlands		T. Mikalsen
221	TUH 51-67	E1463/A5F LR(vre)	<i>E. faecium</i>	Human clinical bloodcultures	1998	Spain		T. Mikalsen
222	K45-11	O2T878	<i>E. faecium</i>	Human clinical bloodcultures	1998	Sweden		T. Mikalsen
223	TUH 50-50	NIZP292/O 2	<i>E. faecium</i>	Other human clinical samples	2002	Poland		T. Mikalsen
224	TUH 50-47	U0106/04- 535869, X0958	<i>E. faecium</i>	Human clinical bloodcultures	2002	The Netherlands		T. Mikalsen
225	TUH 50-65	VRE-10	<i>E. faecium</i>	Human clinical bloodcultures	2004	Denmark		T. Mikalsen
226	TUH 50-68	UW6900	<i>E. faecium</i>	Human clinical bloodcultures	2005	Germany		T. Mikalsen
227	TUH 50-63	VRE-84	<i>E. faecium</i>	Other human clinical samples	2008	Denmark		T. Mikalsen
228	TUH 50-09	E1644	<i>E. faecium</i>	Other human clinical samples	2002	Germany		T. Mikalsen
229	TUH 50-48	U0262/X22 09/04- 575201-01	<i>E. faecium</i>	Human clinical bloodcultures	2002	The Netherlands		T. Mikalsen
230	TUH 50-49	U0367/Y13 32, 05- 557653-01	<i>E. faecium</i>	Human clinical bloodcultures	2004	The Netherlands		T. Mikalsen
231	TUH 50-41	E2603/B05 006589	<i>E. faecium</i>	Human clinical bloodcultures	2004	The Netherlands		T. Mikalsen
232	TUH 50-43	E4076/000 919 0	<i>E. faecium</i>	Human clinical bloodcultures	2005	The Netherlands		T. Mikalsen
233	TUH 50-67	UW6847	<i>E. faecium</i>	Human clinical bloodcultures	2006	Germany		T. Mikalsen
234	TUH 51-72	UW6880	<i>E. faecium</i>	Human clinical bloodcultures	2006	Germany		T. Mikalsen
235	TUH 51-65	hospital outbreaksp italized patient H3	<i>E. faecium</i>	Other human clinical samples	2006	Portugal		T. Mikalsen
236	TUH 50-62	VRE-106	<i>E. faecium</i>	Other human clinical samples	2007	Denmark		T. Mikalsen
237	K55-27	K55-27	<i>E. faecium</i> <i>E. faecium</i>	NA	2008	Sweden The Netherlands		T. Mikalsen
238	TUH 32-63	E1626		Other human clinical samples	1965	Netherlands		T. Mikalsen
239	TUH 51-66	229710	<i>E. faecalis</i>	Other human clinical samples	1992	Portugal		T. Mikalsen
240	TUH 51-68	2724	<i>E. faecalis</i>	Human clinical bloodcultures	NA	Italy		T. Mikalsen
241	TUH 51-69	217691	<i>E. faecalis</i>	NA	NA	Portugal		T. Mikalsen
242	TUH 51-61	E1828/H10	<i>E. faecalis</i>	Human clinical bloodcultures	2001	Spain		T. Mikalsen
243	TUH 50-42	E3450/605 785	<i>E. faecalis</i>	Human clinical bloodcultures	2006	The Netherlands		T. Mikalsen
244	TUH 50-69	UW7001	<i>E. faecalis</i>	Human clinical bloodcultures	2006	Germany		T. Mikalsen
245	TUH 50-54	340/07	<i>E. faecalis</i>	Human clinical bloodcultures	2006	Poland		T. Mikalsen
246	TUH 50-55	1665/07	<i>E. faecalis</i>	Human clinical bloodcultures	2007	Denmark		T. Mikalsen
247	TUH 50-61	VRE-115	<i>E. faecalis</i>	Human clinical bloodcultures	2007	Denmark		T. Mikalsen
248	TUH 50-52	3962/09	<i>E. faecalis</i>	Human clinical bloodcultures	2008	The Netherlands		T. Mikalsen
249	TUH 2-6	V583	<i>E. faecalis</i>	Human clinical bloodcultures	1981	USA		T. Mikalsen

		UW7782/"					T. Mikalsen
250	TUH 50-74	435/96"	<i>E. faecalis</i>	Other human clinical samples	1996	Poland	
251	TUH 50-66	UW1833	<i>E. faecalis</i>	Other human clinical samples	1998	Germany	T. Mikalsen
		UW7784/"					T. Mikalsen
252	TUH 50-71	7239/99"	<i>E. faecalis</i>	Other human clinical samples	1999	Poland	
		UW7753/"					T. Mikalsen
253	TUH 50-70	7239/99"	<i>E. faecalis</i>	Human clinical bloodcultures	NA	Spain	
254	TUH 50-75	UW7789	<i>E. faecalis</i>	Other human clinical samples	2004	Poland	T. Mikalsen
255	TUH 50-77	UW5744	<i>E. faecalis</i>	Other human clinical samples	2004	Germany	T. Mikalsen
256	TUH 50-73	UW6756	<i>E. faecalis</i>	Other human clinical samples	2006	Germany	T. Mikalsen
257	TUH-50-57	916/07	<i>E. faecalis</i>	NA	2007	Poland	T. Mikalsen
258	TUH 50-58	1638/07	<i>E. faecalis</i>	Other human clinical samples	2007	Denmark The	T. Mikalsen
259	TUH 50-59	3992/09	<i>E. faecalis</i>	Other human clinical samples	2009	Netherlands	T. Mikalsen
<b>Canadaian isolates</b>							
260	TUH50-22	244	<i>E. faecium</i>	Other human clinical samples	2007	Canada	
	TUH50-23	268	<i>E. raffinosus</i>				
261				Other human clinical samples	2007	Canada	
	TUH50-25	273	<i>E. casseliflavus</i>				
262				Other human clinical samples	2007	Canada	
263	TUH50-26	274	<i>E. durans</i>	Other human clinical samples	2007	Canada	
	TUH50-27	288	<i>E. gallinarum</i>				
264				Other human clinical samples	2007	Canada	
<b>New Isolates from K-Res</b>							
265	11-C-4	50708930	<i>E. faecium</i>	Human clinical bloodcultures	2012	Norway	K-Res
266	9-C-2	50660268	<i>E. faecium</i>	Other human clinical samples	2012	Norway	K-Res
267	9-F-6	50673722	<i>E. faecium</i>	Other human clinical samples	2012	Norway/India	K-Res
268	11-E-4	50710071	<i>E. faecalis</i>	Other human clinical samples	2013	Norway	K-Res
269	11-B-4	50708494	<i>E. faecium</i>	Other human clinical samples	2013	USA	K-Res
270	11-A-3	50705414	<i>E. faecium</i>	Other human clinical samples	2013	USA	K-Res
271	11-G-1	50701700	<i>E. faecium</i>	Other human clinical samples	2013	Norway	K-Res
272	10-C-9	50698098	<i>E. faecium</i>	Other human clinical samples	2013	Norway	K-Res
273	10-B-6	50691662	<i>E. faecium</i>	Other human clinical samples	2013	Norway	K-Res
274	10-E-7	50694469	<i>E. faecium</i>	Other human clinical samples	2013	Norway	K-Res

## 9.2 List of antimicrobial susceptibility for 24 selected isolates

Numb. ID / Stock nu	Alternative Species	Isolate source	Year	Origin	Reference or source	Antibiotic Susceptibility						
						Ampicillin AMP	Gentamicin CN	Linezolid LZD	Sulfasalazine RL	Trimethoprim W	Trim-Sulfa SXT	Vanco Screen Vanco
1	TUH 44-39	A0249	1999	Norway	(Johnsen <i>et al.</i> , 2005)	S	I	S	R	I	I	R
2	TUH 32-82	E0510	1998	Australia	Willems, R. J. L.	R	I	S	R	R	R	R
3	TUH 32-79	E0729	2000	The Netherlands	Willems, R. J. L.	R	I	S	R	I	I	S
4	TUH 45-22	E. faecium	1999	Norway	(Johnsen <i>et al.</i> , 2005)	S	I	S	R	I	I	R
5	K8-42	E. faecium	2003	Norway	This study	R	I	S	R	R	R	R
6	K8-50	E. faecium	2001	Norway	This study	R	R	S	R	I	I	R
7	TUH 41-76	E. faecium	2002	Italy	(Blavasco <i>et al.</i> , 2007)	S	I	S	R	I	I	R
8	K17a	E. faecium			P. Butaye	R	I	S	R	R	R	S
9	K120a	E. durans			P. Butaye	S	I	S	R	I	R	R
10	K73a	E. hirae			P. Butaye	R	I	S	R	I	I	S
11	V70b	E. hirae			P. Butaye	R	I	S	R	I	I	S
12	K58-46	E. faecalis	2008	Norway	Rosvoll <i>et al.</i> 2013	S	R	S	R	R	R	S
		U0262/X22										
		09/04-										
13	TUH 50-48	575201-01	2002	The Netherlands		R	R	S	R	R	R	S
14	TUH 50-73	UW6756	2006	Germany		R	R	S	R	R	R	S
15	K59-05	3347	2008	Norway	Rosvoll <i>et al.</i> 2013	S	R	S	R	R	R	S
16	K59-20	5977	2008	Norway	Rosvoll <i>et al.</i> 2012	R	R	R	R	R	R	S
17	K59-32	54271	2008	Norway	Rosvoll <i>et al.</i> 2012	R	R	S	R	R	R	S
18	9-C-2	50660268	2012	Norway	K-res	R	R	R	R	R	R	R
19	9-F-6	50873722	2012	Norway/India	K-res	R	R	R	R	I	I	R
20	TUH50-22	244	2007?	Canada		R	R	S	R	I	I	R
		E. casseliflavus										
21	TUH 50-54	340/07	2007?	Canada		S	R	S	R	I	I	R
22	TUH 51-67	LR(vre)	1998	Spain	T. Mikalsen	R	R	S	R	R	R	S
		E1463/A5F	2006	Poland	T. Mikalsen	S	R	S	R	R	R	S
23	TUH 50-62	VRE-106	2007	Denmark	T. Mikalsen	R	R	S	R	I	I	R
24					T. Mikalsen	R	R	S	R	R	R	R

Abbreviations:  
R= Resistant  
I= Intermediate  
S= Susceptible

### 9.3 List of hybridization results for 24 selected isolates

Numb. ID / Stock	Alternative ID	Species	Isolate source	Year	Origin	Reference or sou	SodA Hybr.	Int1 Hybr.
1	TUH 44-39	<i>E. faecium</i>	Poultry litter	1999	Norway	(Johnsen <i>et al.</i> , 2005)	Positive	Negative
2	TUH 32-62	<i>E. faecium</i>	Other human clinical sample	1998	Australia	Willems, R. J. L.	Positive	Negative
3	TUH 32-79	<i>E. faecium</i>	Other human clinical sample	2000	The Netherlands	Willems, R. J. L.	Positive	Positive
4	TUH 45-22	<i>E. faecium</i>	Poultry litter	1999	Norway	(Johnsen <i>et al.</i> , 2005)	Positive	Negative
5	K8-42	<i>E. faecium</i>	Other human clinical sample	2003	Norway	This study	Positive	Negative
6	K8-50	<i>E. faecium</i>	Other human clinical sample	2001	Norway	This study	Positive	Negative
7	TUH 41-76	<i>E. faecium</i>	Other human clinical sample	2002	Italy	(Biavasco <i>et al.</i> , 2007)	Positive	Positive
8		<i>E. faecium</i>	Poultry litter			P. Butaye	Positive	Negative
9	K120a	<i>E. durans</i>	Poultry litter			P. Butaye	Positive	Negative
10	K73a	<i>E. hirae</i>	Poultry litter			P. Butaye	Positive	Negative
11	V70b	<i>E. hirae</i>	Pig faeces			P. Butaye	Positive	Negative
12	K58-46	<i>E. faecalis</i>	Human blood culture	2008	Norway	Rosvoll et al 2013	Positive	Negative
	U0262/X2209/04-	<i>E. faecium</i>	Human blood culture					
13	TUH 50-48			2002	The Netherlands		Positive	Negative
14	TUH 50-73	<i>E. faecalis</i>	Other human clinical sample	2006	Germany		Positive	Negative
15	K59-05	<i>E. faecalis</i>	Human blood culture	2008	Norway	Rosvoll et al 2013	Positive	Negative
16	K59-20	<i>E. faecium</i>	Human blood culture	2008	Norway	Rosvoll et al, 2012	Positive	Negative
17	K59-32	<i>E. faecium</i>	Human blood culture	2008	Norway	Rosvoll et al, 2012	Positive	Negative
18	9-C-2	<i>E. faecium</i>	Other human clinical sample	2012	Norway	K-res	Positive	Negative
19	9-F-6	<i>E. faecium</i>	Other human clinical sample	2012	Norway/India	K-res	Positive	Negative
20	TUH50-22	<i>E. faecium</i>	Other human clinical sample	2012	Norway/India		Positive	Negative
	TUH50-25	<i>E. casseliflavu</i>	Other human clinical sample	2007?	Canada		Positive	Negative
21		s	Other human clinical sample	2007?	Canada		Positive	Negative
22	TUH 50-54	<i>E. faecalis</i>	Human blood culture	2006	Poland	T. Mikalsen	Positive	Negative
	E1463/A5FLR(vr		Human blood culture					
23	TUH 51-67	<i>E. faecium</i>	Other human clinical sample	1998	Spain	T. Mikalsen	Positive	Negative
24	TUH 50-62	<i>E. faecium</i>	Other human clinical sample	2007	Denmark	T. Mikalsen	Positive	Negative
	VRE-106							