

## Duplex Real-Time PCR Assay for Rapid Detection of Ampicillin-Resistant *Enterococcus faecium*

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**Rapid and accurate identification of carriers of resistant microorganisms is an important aspect of efficient infection control in hospitals. Traditional identification methods of antibiotic-resistant bacteria usually take at least 3 to 4 days after sampling. A duplex real-time PCR assay was developed for rapid detection of ampicillin-resistant *Enterococcus faecium* (ARE). Primers and probes that are used in this assay specifically detected the D-Ala-D-Ala ligase gene of *E. faecium* and the modified penicillin-binding protein 5 gene (*pbp5*) carrying the Glu-to-Val substitution at position 629 (Val-629) in a set of 129 tested *E. faecium* strains with known *pbp5* sequence. Presence of the Val-629 in the strain set from 11 different countries was highly correlated with ampicillin resistance. In a screening of hospitalized patients, the real-time PCR assay yielded a sensitivity and a specificity for the detection of ARE colonization of 95% and 100%, respectively. The results were obtained 4 h after samples were harvested from overnight broth of rectal swab samples, identifying both species and the resistance marker mutation in *pbp5*. This novel assay reliably identifies ARE 2 to 3 days more quickly than traditional culture methods, thereby increasing laboratory throughput, making it useful for rectal screening of ARE. The assay demonstrates the advantages of real-time PCR for detection of nosocomial pathogens.**

Enterococci are a major cause of nosocomial infections (15) constituting a significant threat to debilitated patients (17). Ampicillin-resistant *Enterococcus faecium* (ARE) represents a therapeutic challenge, especially when combined with aminoglycoside and glycopeptide resistance. Endemic hospital strains of enterococci colonize patients rapidly upon admission, predisposing them to endogenous infections (3). Prudent use of antibiotics and barrier precautions must be reinforced to control outbreaks (9). There is a need for a rapid diagnostic test to identify ARE carriers in order to facilitate infection control measures.

Ampicillin resistance can be caused by the overproduction of the essential penicillin-binding protein 5 (PBP5) (2, 6, 10). A decrease in the affinity of PBP5 to  $\beta$ -lactam antibiotics has also been linked to increased ampicillin MICs (6, 10, 26). The PBP5 synthesis repressor (*psr*) seems to be associated with the decreased expression of PBP5 (11) and other cell wall components (13), although its role is not completely understood (18). In addition to PBP5-mediated ampicillin resistance,  $\beta$ -lactamase production has also been reported as a cause of ampicillin resistance but is uncommon (14).

Penicillin binding occurs in the C-terminal part of the PBP5 protein, and several polymorphisms in amino acid positions in the vicinity of conserved regions important for penicillin binding have been linked to increased ampicillin resistance. Potentially significant amino acid polymorphisms that confer ampi-

illin resistance are methionine to alanine at position 485 (23, 26), an additional serine at position 466 (23, 26), and replacement of a polar amino acid with a nonpolar one (alanine or isoleucine) at position 558, 562, or 574 (12, 24). A glutamate-to-valine substitution at position 629 (Val-629) has also been associated with increased ampicillin MICs (8, 19). Sauvage et al. (21) reported the crystal structure of the acyl-enzyme complex of PBP5 with benzylpenicillin, demonstrating that the topology of the loop from positions 451 to 465 defining the left edge of the active-site cavity differs from other PBPs. The 629 position is part of a loop connecting strands  $\beta_{C3}$  and  $\beta_{C4}$  close to the active site but was invisible in the electron density map and therefore not possible to model (21).

The introduction of a PCR method targeting the D-Ala-D-Ala ligase genes of *E. faecium* (*ddlfm*) and *E. faecalis* by Dutka-Malen et al. (4) increased the speed and reliability of species identification compared to traditional methods. Nevertheless, culture methods have until now been necessary for the identification of ampicillin resistance in enterococci. These are time-consuming and laborious, and results are usually not obtained before 3 to 4 days.

The objective of the present study was to design a real-time multiplex PCR assay for rapid detection of ampicillin-resistant *E. faecium* to facilitate quick clinical screening. Primers and probes developed for this assay are based on the *ddlfm* gene and on the C-terminal region of a modified *pbp5* gene carrying the Val-629 substitution as a putative marker for ampicillin resistance. In the first part of our study we evaluated probe hybridization to enterococci with wild-type and modified *pbp5* C-terminal sequences and compared the presence of the Val-629 substitution with ampicillin susceptibility. In the second

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TABLE 1. Distribution of valine in position 629 in the C-terminal part of *pbp5* in *E. faecium* and the hybridization with a *pbp5* Val-629-specific probe in a real-time PCR assay

Origin	No. of isolates			No. of isolates with Val-629 <sup>b</sup>		No. of samples <sup>c</sup>		
	Total	ARE <sup>a</sup>	ASE	ARE	ASE	Rectal	Clinical	Animal
Norway	42	30	12	27	0	42	0	0
The Netherlands	35	7	28	7	1	13	8	12
United States	11	11	0	11	0	7	4	0
United Kingdom	10	4	6	4	2	5	4	0
Spain	4	2	2	2	0	0	4	0
Germany	5	5	0	5	0	0	5	0
Portugal	4	4	0	4	0	0	4	0
Italy	6	5	1	5	0	0	4	0
Finland	6	2	4	2	0	2	4	0
Australia	2	2	0	2	0	0	2	0
Tanzania	4	2	2	2	0	0	4	0
Total	129	74	55	71	3	69	43	12

<sup>a</sup> Ampicillin MICs for resistant strains were ≥16 µg/ml (NCCLS guidelines).

<sup>b</sup> As determined by sequencing. Of the 71 ARE strains, Val629 probe did not bind to 6 strains due to an AAG→AAA silent mutation at position 630 or an AAA→CAA mutation (Lys→Glx) at position 632.

<sup>c</sup> ARE and ASE strains are grouped together. The origin of five samples was unknown.

part of the study we tested the real-time PCR assay as a rapid screening tool for ARE in a clinical setting.

MATERIALS AND METHODS

**Setting.** Haukeland University Hospital in Bergen, Norway, has 1,100 beds and serves as an emergency and referral hospital for 300,000 and 1 million people, respectively. ARE has presented a nosocomial infection control challenge at the hospital since 1995, infecting approximately 100 patients annually.

**Bacterial isolates and samples.** For the evaluation of Val-629 as a marker for ampicillin resistance, 74 ARE and 55 ampicillin-susceptible *E. faecium* (ASE) strains with known *pbp5* C-terminal sequences were tested. The species identity was verified by *ddlfm* (D-Ala-D-Ala ligase gene of *E. faecium*) PCR (4). The isolates were recovered from hospitalized patients, clinical and stool isolates, nonhospitalized persons (stool), and animals (feces) and originated from Europe, the United States, Africa, and Australia (Table 1). Of these 129 isolates, 63 were vancomycin resistant. All isolates were stored frozen until used.

In the patient screening, 61 rectal swabs (Copan Italia, Brescia, Italy) were collected by gently rotating the swab in the anal orifice of inpatients on two

medical wards at Haukeland University Hospital. The Regional Committee for Ethics in Medical Research approved of the protocol.

**Susceptibility testing.** MICs (in micrograms per milliliter translated to twofold dilutions) were determined by Etest (AB Biodisk, Solna, Sweden) according to the instructions of the manufacturer. Isolates for which ampicillin MICs were of ≥16µg/ml were considered resistant as recommended by the NCCLS (16). β-Lactamase production was tested by using nitrocefin disks in a 2 McFarland turbidity cell suspension in 0.5 ml of a 0.85% NaCl solution as described by the manufacturer (AB Biodisk).

**Broth inoculation.** The rectal swabs from the patient screening (Copan Italia) were inoculated overnight at 35°C in Enterococcosel broth (Becton Dickinson Microbiology Systems, Cockeysville, Md.) with or without 8 µg of ampicillin/ml, referred to in the present study as selective or unselective broth, respectively. Broths with a black discoloration indicating hydrolysis of esculin were considered positive.

**DNA extraction.** DNA used for sequencing and evaluation of the assay was extracted as previously described by Willems et al. (25) with the addition of a final ethanol precipitation step to further purify the DNA. DNA from the clinical rectal swab screening was extracted as follows: 200 µl of vortexed Enterococcosel broth was centrifuged, and the pellet was resuspended in 100 µl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), lysed for 30 min in 1 mg of lysozyme/ml and 0.1 mg of RNase A/ml at 37°C, and then treated with 2.5 mg of proteinase K/ml and 0.1% sodium dodecyl sulfate for 20 min at room temperature. Subsequently, the lysate was heated to 100°C for 10 min and centrifuged, and then 1 µl of the supernatant was used as a template for PCR.

**DNA sequencing.** Using the primers (Eurogentec, Herstal, Belgium) described in Table 2, a 778-bp C-terminal *pbp5* fragment was amplified with a Hotstar polymerase PCR kit (Qiagen, Hilden, Germany) in a thermal cycler (model 9600; PE Biosystems, Norwalk, Conn.). Cycling conditions consisted of 95°C initially for 15 min; followed by 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 2 min; followed by a final 7-min extension period at 72°C. The PCR products were purified with a Qiagen PCR purification kit (Qiagen) and used as a template for the dye termination reaction by using BigDye 2.0 as described by the manufacturer (Applied Biosystems, Foster City, Calif.). The products were analyzed on an ABI Prism 3700 DNA sequencer (PE Biosystems). Alignment was conducted with the Vector NTI Suite (v6.0; InforMax, Bethesda, Md.).

**Duplex real-time PCR assay.** The assay contained two sets of probes and primers (Eurogentec). The first probe, *ddlfm*, was designed to hybridize with the D-Ala-D-Ala ligase of *E. faecium*, thereby confirming species identification. The second probe, Val629, was designed to hybridize to *pbp5* with a valine at position 629 (i.e., Val-629) (Table 2). Duplex real-time PCR was conducted as follows: 40 µl of PCR Mastermix containing 20 µl of Hotstar PCR mix (Qiagen), 3.2 µl of 25 mM MgCl<sub>2</sub> (3.5 mM), 0.5 µl of a 25 µM concentration of primer Val629 (313 nM), 0.5 µl of a 10 µM concentration of probe Val629 (125 nM), 0.2 µl of a 25 µM concentration of primer of *ddlfm* (125 nM), 0.2 µl of a 10 µM concentration of *ddlfm* probe (50 nM), and 13.7 µl of H<sub>2</sub>O was added to 1 µl of template. After preincubation of the reaction mixture at 95°C for 15 min, thermocycling was conducted at 94°C for 10 s and 59°C for 40 s for 45 cycles (model SDS 7700; Applied Biosystems). The data handling was carried out by the Sequence Detector 1.7 program (Applied Biosystems). The following combinations of signals from the probes occurred: positive *ddlfm* and Val629 was interpreted as ARE,

TABLE 2. Primers and probes used for sequencing of PBP5 (*pbp5*) and in the real-time PCR assay to detect ARE

Gene	Primer or probe <sup>a</sup>	Reporter <sup>b</sup>	Sequence	Location (nucleotide position) <sup>c</sup>
<i>pbp5</i>	Primer fw*		5'-AACAAAATGACAAACGGG	1684-1701
<i>pbp5</i>	Primer rev*†		5'-TATCCTTGTTATCAGGG	2445-2462
<i>pbp5</i>	Primer fw†		5'-GACAAACGGGATCTACAAG	1692-1711
<i>ddlfm</i> <sup>d</sup>	Probe	TET	5'-ACTAGAACCCATATTCGCC	652-670
<i>ddlfm</i>	Primer fw		5'-TTCCTTGCTTTATCCGATGT	622-641
<i>ddlfm</i>	Primer rev		5'-CGGTTTTCTGCTTTTGTAAAT	678-696
<i>val629</i> <sup>e</sup>	Probe	FAM	5'-TTTCCCCTTACATCCTGT	2400-2418
<i>val629</i>	Primer fw		5'-TGTAATGGTACAGCACATTC	2319-2339
<i>val629</i>	Primer rev		5'-GTTGAAAGCAAACAAGAAACT	2425-2445

<sup>a</sup> fw, forward; rev, reverse; \*, primers used for amplification of C-terminal part of *pbp5*; †, primer used in *pbp5* sequence analysis.

<sup>b</sup> FAM, 6-carboxyfluorescein; TET, 6-carboxy-4,7,2',7'-tetrachlorofluorescein. 4-(4-Dimethylaminophenyl)benzoic acid (DABCYL) was used as a quencher.

<sup>c</sup> The nucleotide positions given are according to GenBank accession numbers X84860 and AF130998 for *ddlfm*.

<sup>d</sup> D-Ala-D-Ala-ligase of *E. faecium*.

<sup>e</sup> C-terminal end of *pbp5* coding for the glutamate→valine change at amino acid position 629 (GenBank accession number X84860).

TABLE 3. Detection of ARE in culture-positive Enterococcosel broth ( $n = 41$ )

Method	Description	ARE strains detected <sup>a</sup> (%)	No. of false positives	PPV <sup>e</sup> (%)
1	Reference method	22		
2	Selective broth, D-Ala-D-Ala ligase PCR <sup>b</sup> and ethidium bromide-stained gel	21 <sup>c</sup>	0	100
3	Real-time PCR assay, selective broth	21 <sup>c</sup>	0	100
4	Real-time PCR assay, unselective broth	21 <sup>d</sup>	0	100

<sup>a</sup> Of the 19 non-ARE strains, 17 were *E. faecalis* or ASE; the remaining 2 were not identified.

<sup>b</sup> Described by Dutka-Malen et al. (4).

<sup>c</sup> For the ARE strains not detected, ampicillin MICs were 32  $\mu\text{g/ml}$ , but these strains did not grow in broth containing 8  $\mu\text{g}$  of ampicillin/ml. This strain was detected when grown in broth without ampicillin.

<sup>d</sup> For the ARE strains not detected, ampicillin MICs were 32  $\mu\text{g/ml}$ , and these strains carried Val-629.

<sup>e</sup> PPV, positive predictive value.

positive *ddlfm* and negative Val629 was interpreted as ASE, and a negative *ddlfm* was interpreted as a non-*E. faecium*.

**Detection of ARE in a clinical setting.** For rectal screening of patients the following four methods were evaluated for detection of ARE, including two variants of the real-time PCR assay (see Table 3). For the reference method (method 1), samples from the Enterococcosel broth without ampicillin with a positive bile-esculin reaction were plated on *E. faecium* selective cephaloxin-aztreonam-arabinose agar plates (7) with 8  $\mu\text{g}$  of ampicillin/ml. Subsequently, the *ddlfm* gene was identified by conventional PCR as described by Dutka-Malen et al. (4), and the MIC of ampicillin was determined. Method 2 was based on selective broth and species identification PCR. Detection of the *ddlfm* genes was conducted as described previously (4) on esculin-positive broths containing 8  $\mu\text{g}$  of ampicillin/ml. Method 3 utilized real-time PCR on selective broths. DNA was extracted from esculin-positive broths containing 8  $\mu\text{g}$  of ampicillin/ml as described above, and the real-time PCR was conducted. Method 4 relied upon real-time PCR on unselective broths and was identical to method 3 except that the broth did not contain ampicillin.

## RESULTS

**Evaluation of the real-time PCR method.** Determination of ampicillin susceptibility of a diverse strain collection of 129 strains from 11 countries revealed that 74 of the strains were ARE and 55 were ASE. Sequencing of the C-terminal region of the *pbp5* gene showed that 71 of 74 ARE strains and 3 of 55 ASE strains had valine in position 629 (Table 1). The ampicillin MICs for the ASE isolates with Val629 were 8  $\mu\text{g/ml}$ , i.e., higher than those for the remaining susceptible strains (data not shown). The observed association of the Val-629 substitution in *pbp5* and ampicillin resistance in *E. faecium* corresponds well with previously published studies (8, 19). A real-time PCR was developed by using primers and probes specific for the *ddlfm* gene and for the Val629-modified *pbp5* gene and tested on purified DNA of the 74 ARE strains and 55 ASE strains with known *pbp5* C-terminal sequences (Table 1). The assay correctly identified 65 of 71 strains with the Val-629 substitution in the *pbp5* gene and correctly identified all isolates as *E. faecium* (data not shown). The six ARE strains with the Val-629 substitution that did not hybridize the probe contained additional mutations in the region complementary to the probe. Of these, four were from outbreaks in The Netherlands harboring a mutation at position 632 (Lys→Gln), while two had a silent mutation at position 630.

**Clinical screening.** With the knowledge that the real-time PCR assay was able to correctly identify the Val-629 substitution of almost all ARE, we proceeded to test and evaluate this assay in a clinical setting on rectal swabs inoculated overnight

in Enterococcosel broth. Hospitalized patients were screened for rectal carriage with ARE on two occasions in February and March 2002 and on two medical wards with known endemicity for ARE. A total of 61 rectal samples were collected. No growth in Enterococcosel broths was observed in 20 samples, leaving 41 samples for further investigation.

The reference method (method 1) detected 22 samples positive for ARE among the 41 positive Enterococcosel broths (Table 3). ASE or ampicillin-susceptible *E. faecalis* was detected in 17 of the 19 samples that did not contain ARE; 2 samples contained neither *E. faecium* nor *E. faecalis*. The real-time PCR assay conducted on DNA extracted from positive Enterococcosel broths with or without ampicillin (methods 3 and 4) was able to detect ARE in 21 of the 22 samples that tested positive with the reference method. The only sample positive for ARE by the reference method but negative by the real-time PCR approach contained an *E. faecium* strain for which the ampicillin MIC was 32  $\mu\text{g/ml}$  that carried the Val-629 substitution and that did not grow in the selective Enterococcosel broth. The results were obtained within 4 h after samples were harvested from the overnight broths. The sensitivity of the real-time PCR for detection of ARE in a clinical setting as determined by using selective and unselective broths was 95%, whereas the specificity was 100%; the positive predictive value for the real-time PCR was 100%.

## DISCUSSION

Traditional identification methods for resistant bacteria usually take at least 2 to 3 days after sampling. We have had in our hospital an epidemic with ampicillin-resistant enterococci, and we were challenged to rapidly identify carriers in order to start intensified infection control measures as soon as possible.

The present real-time PCR assay using DNA from rectal swabs cultured overnight in Enterococcosel with ampicillin detected 95% of the ARE strains with a specificity of 100%. The results were obtained 4 h after the bacteria were harvested from the overnight broths and thus identified ARE 2 to 3 days more quickly than traditional culture-based methods (5, 22).

Unlike in other studies (20), there was no need for purification of the PCR template, thus reducing the workload. The assay was also performed with sealed tubes, lowering the risk of laboratory contamination with PCR products.

By using a selective broth, most susceptible strains are elim-



inated, thus lowering the number of isolates from which DNA needs to be extracted. One *E. faecium* isolate for which the ampicillin MIC was 32 µg/ml from the clinical screening failed to grow in this selective Enterococcosel broth. This strain grew poorly also on cephalixin-aztreonam-arabinose plates with ampicillin in the reference method. However, this strain was detected by the real-time PCR on DNA from Enterococcosel without ampicillin. The ampicillin concentration (8 µg/ml) was set under the NCCLS breakpoint for resistance not to miss any resistant strains. This concentration was sufficient to inhibit the growth of all susceptible strains (MIC, ≤8 µg/ml) but also inhibited the growth of the one resistant strain mentioned above.

Six ARE strains from The Netherlands and Italy with Val-629 did not hybridize with the probe (Table 2). Sequencing of this region revealed that these strains contained additional polymorphisms at either position 630 or 632. The hybridization failure of the probe can be overcome by modifying the probe at the aberrant positions.

The real-time PCR assay was also used on clinical samples, bypassing the broth which functions as an enrichment step, but the method performed poorly (data not shown). Low concentrations of bacteria and the presence of inhibitors from the rectal swabs (1) are probable causes of the poor performance of the assay directly on rectal swabs. In addition to being an enrichment medium, Enterococcosel broth also dilutes inhibitors of PCR that may be present in feces.

With only a few exceptions, the real-time PCR assay was able to detect the Val-629 substitution and as a consequence yielded results that were highly consistent with sequencing results. This implies that the limitation of sensitivity and specificity of this novel assay to properly detect ARE is defined by the frequency of valine and glutamate in the polymorphic position 629 of *pbp5* in ARE and ASE strains, respectively. Valine in position 629 was chosen as a marker because of its high correlation to ampicillin resistance in *E. faecium*. A total of 129 isolates from 11 countries were sequenced, showing that this polymorphism is a valid marker beyond the endemic situation in Norway. No other polymorphisms in the C terminus of *pbp5* were as predictive as that at position 629, even though 485Met→Ala/Thr is also a potential candidate for screening purposes (data not shown). The sequencing also revealed three ASE strains with Val-629. However, the ampicillin MIC for these strains (8 µg/ml) was increased compared to those for the other ASE strains, and the significance of Val-629 cannot be ruled out on the basis of these strains. These results show a worldwide distribution of Val-629 as a marker of ampicillin resistance.

Rybkin et al. sequenced the C termini of 16 different *E. faecium* strains (19). Several variants of *pbp5* were associated with an increased ampicillin MIC, especially a change at position 485 from methionine to alanine or threonine and an additional serine at position 466. In that study, the four most ampicillin-susceptible strains (MIC, ≤16 µg/ml) had a Glu-629, whereas all strains for which MICs were ≥64 µg/ml had Val-629. Zorzi et al. have previously shown that the two resistant strains EFM-1 and H80721 with a significantly decreased penicillin affinity also had Val-629 (26). Previously described Norwegian ASE strains from poultry farmers had a glutamate at position 629 (8). The exact mechanisms conferring ampicillin

resistance are not known, and it is likely that Val-629 acts in concert with other mutations and/or various quantities of PBP5. The loop containing position 629 is close to the active site of PBP5 and may hinder the entry of the penicillin substrate into the groove (21). The significance of Val-629 on PBP5 needs to be further elucidated, preferably with site-specific mutagenesis. Nevertheless, Val-629 has proved to be an excellent marker for ampicillin resistance, which is also supported by the studies discussed above.

In conclusion, we have presented a real-time PCR assay that reliably identifies ARE 2 to 3 days faster than traditional culture methods. The assay increases laboratory throughput and can be useful for screening patients for rectal colonization of ARE.

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