1 **SCIENTIFIC OPINION**
2

3 Guidance on the safety assessment of *Enterococcus faecium* in animal nutrition[1]

4 **EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)[2,3]**

5 European Food Safety Authority (EFSA), Parma, Italy
6

7 ENDORSED FOR PUBLIC CONSULTATION on 1 February 2012
8

10

11

[1] On request from EFSA, Question No EFSA-Q-2011-01173, adopted on XXXXXXX.

[2] Panel members: Gabriele Aquilina, Georges Bories, Andrew Chesson, Pier Sandro Cocconcelli, Joop de Knecht, Noël Albert Dierick, Mikolaj Antoni Gralak, Jürgen Gropp, Ingrid Halle, Christer Hogstrand, Reinhard Kroker, Lubomir Leng, Secundino López Puente, Anne-Katrine Lundebye Haldorsen, Alberto Mantovani, Giovanna Martelli, Miklós Mézes, Derek Renshaw, Maria Saarela, Kristen Sejrsen and Johannes Westendorf. Correspondence: FEEDAP@efsa.europa.eu

[3] Acknowledgement: The Panel wishes to thank wishes to thank the members of the Working Group on Micro-organisms including Atte von Wright, Barbara E. Murray, Willem van Schaik and Johannes Huebner for the preparation of this opinion.

Suggested citation: EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Guidance on the safety assessment of *Enterococcus faecium* in animal nutrition. EFSA Journal 20YY;volume(issue):NNNN. [10 pp.] doi:10.2903/j.efsa.20YY.NNNN. Available online: www.efsa.europa.eu/efsajournal

TABLE OF CONTENTS

BACKGROUND

Regulation (EC) No 1831/2003[4] establishes the rules governing the Community authorisation of additives for use in animal nutrition. Moreover, Article 7(6) of this Regulation provides for the European Food Safety Authority (EFSA) to publish detailed guidance to assist applicants in the preparation and presentations of applications.

Among the microbial additives currently authorised, nearly one third contain strains of *Enterococcus faecium***.** Although *E. faecium* is known to be a commensal inhabitant of the human and animal gastrointestinal tract, some strains are potential human pathogens.

The FEEDAP Panel has produced a series of guidance documents for the assessment of the safety and efficacy of feed additives. However, the toxicological tests recommended in these guidance documents are not designed to identify the virulence of a microbial agent.

EFSA has received an increasing number of questions from applicants on how to assess the safety of *E. faecium* based additives. To date the Panel has relied on the demonstration of absence of putative virulence determinants identified in the scientific literature. Because of the increasing incidence of *E. faecium* infections in hospital settings and the new scientific approaches developed (e.g., genomics), there is now a far better understanding of why some strains of *E. faecium* present problems.

The Working Group on Micro-organisms of the FEEDAP Panel following discussions held with internationally recognised experts identified the potential to establish criteria for the safety assessment of *E. faecium* and to develop a Guidance document for the benefit of applicants.

TERMS OF REFERENCE

The FEEDAP Panel is requested to produce a Guidance document on the safety of the use of *Enterococcus faecium* in animal nutrition. This guidance should allow discrimination between safe strains and those more likely to cause human infections.

[4] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.

1. INTRODUCTION

Enterococci are well known commensals of the gastrointestinal tract; most people carry them normally as do many animals. Human infections caused by enterococci outside the healthcare setting are very uncommon and consist of endocarditis, urinary tract infections, or abdominal/pelvic infections resulting from contamination by the faecal microbiota (Murray, 2000).

In the modern-day healthcare setting, enterococci are commonly recovered from infections. The first "wave" (or increase) of enterococci in hospital-associated infections (mostly *Enterococcus faecalis*) followed and was generally attributed to the use of broad-spectrum cephalosporins (to which enterococci are resistant), beginning in the 1980s, as well as increased numbers of patients who are immunologically compromised. Broad-spectrum cephalosporins eliminate much of the resident microbiota in the gastrointestinal tract but the intrinsic resistance of enterococci to cephalosporins allows them to survive. Thus, enterococci are present and often more numerous in the intestinal tract of most hospitalised patients. Factors such as the presence of catheters, immunosuppression, or mucositis from chemotherapy, alter the usual host-microbe balance and facilitate infection. Antibiotic use in the patients appears to be the critical factor allowing infection by an otherwise well-controlled commensal (Murray, 2000; Ubeda et al., 2010).

Prior to the early 1990s, 90-95 % of enterococcal clinical isolates in the hospital setting were *E. faecalis* and only about 5 % were *E. faecium*. In the USA, isolation of *E. faecium* from healthcare-associated infected sites has increased markedly over the past 15-20 years and this species now accounts for ~ 35 % of enterococci from infections in the hospital setting. Coincident with this increase, it was recognised that hospital-associated isolates of *E. faecium* were more frequently resistant to ampicillin and piperacillin than those found in the community setting. In the USA, it was amongst this ampicillin-resistant group of *E. faecium* that vancomycin resistance emerged. Currently, about 70 % of *E. faecium* isolates in US hospitals are vancomycin resistant while 90 % are ampicillin resistant. In contrast, very few *E. faecalis* (1-5 %) are resistant to either of these antibiotics, which probably explains the increase of *E. faecium* relative to *E. faecalis* in the hospital setting where antibiotics active against *E. faecalis* are frequently used (Hidron et al., 2008; Bertics et al., 2009).

In the EU, vancomycin-resistant *E. faecium* (VRE) strains were first detected in the 1980s but these were mostly ampicillin-susceptible strains found in faecal samples from animals on farms using the glycopeptide avoparcin. VRE strains have also been isolated from foods of animal origin and faecal samples of healthy individuals in the community. However, infections with these *E. faecium* strains are rare outside the hospital settings. More recently, ampicillin-resistant strains of *E. faecium* have emerged in hospitalised patients in the EU. Some of these strains, as earlier in the USA, have now also acquired resistance to vancomycin and their frequency as a cause of infection is increasing in the health-care setting and about 40 – 50 % of enterococcal nosocomial infections are now attributable to *E. faecium* (Bonten et al., 2001; Leavis et al., 2003; Top et al., 2007; Werner et al., 2008).

It is now recognised that *E. faecium* consists of two distinct subpopulations, or clades, that may have diverged many hundreds of thousands of years ago. These clades have been differentiated by Multi-Locus Sequence Typing (MLST), by sequence comparisons of individual shared core genes, by the presence of insertion sequence IS*16*, other acquired elements, and in their resistance to ampicillin. One subpopulation (referred to as the community-associated clade) consists almost entirely of isolates from the faeces of animals, healthy individuals and food, and is characterised by susceptibility to ampicillin. The other subpopulation (clade) contains most of the clinical isolates and is commonly referred to as the hospital-associated or hospital-predominant clade. It is the latter clade that contains ampicillin-resistant strains and, indeed, ampicillin resistance is the major phenotypic marker of this hospital-associated subpopulation (Leavis et al., 2007; Willems & van Schaik, 2009; Galloway-Peña et al., 2011).

2. PHYLOGENETICS AND GENOMICS OF *E. FAECIUM*

Analysis of the evolutionary relatedness of *E. faecium* has mostly been performed by MLST (Homan et al., 2002) in which allelic profiles are determined based on the sequence of seven housekeeping genes. The first study using MLST of *E. faecium* population structure characterised a global collection of human (hospital- and community-acquired) and non-human (isolated from animals and the environment) strains and defined 175 sequence types (STs). STs were grouped with eBURST which divides an MLST data set of any size into groups of related isolates and clonal complexes (CCs) and predicts the founding genotype of each CC. This clustering indicated that the majority of the globally representative hospital isolates were genotypically and evolutionary closely related and belonged to a single CC, which was termed CC17 (Willems et al., 2005).

However, the *E. faecium* population structure based on all STs currently available in the MLST database (http://efaecium.mlst.net/) inferred by eBURST resulted in one large CC, which includes the previously designated CC17, but also minor CCs and singletons, with 69 % of the *E. faecium* STs in the database (Willems et al., 2011). These observations and genome-based studies (van Schaik et al., 2010) indicate that the hospital-associated *E. faecium* isolates have not evolved recently from a single common ancestor and, consequently, the initial designation of CC17 as a hospital-associated CC has most likely been erroneous. Instead, hospital-associated isolates form a polyclonal *E. faecium* subpopulation harboring evolutionarily distinct clones (Willems and van Schaik, 2009; Willems et al., 2011). Comparative genomic hybridization and genome sequencing have revealed the presence of several genes that are enriched in clinical *E. faecium* isolates. One of the genes that is most clearly overrepresented in clinical isolates is the insertion sequence IS*16* (Leavis et al., 2007; van Schaik et al., 2010; Werner et al., 2011), which presumably confers a level of genomic flexibility to its host, thereby facilitating the subsequent acquisition of additional elements involved in virulence or antibiotic resistance.

MLST and genome sequences also revealed a clearly distinct cluster of strains which mostly originate from healthy humans (van Schaik and Willems, 2010; Zhang *et al*., 2011). These strains may have adapted to life as a mammalian commensal. This distinction of *E. faecium* in two major lineages was also identified by Galloway-Peña et al. (2011) and is characterised by the response to ampicillin.

3. RESISTANCE TO AMPICILLIN

The fact that most *E. faecium* isolates recovered from healthcare-associated infections belong to the same clade which differs significantly from the other clade, suggests that fundamental differences inherent to these clades may explain the difference in their occurrence in infections. One difference is resistance to ampicillin of hospital-associated isolates (often with MICs > 128 mg/L) which confers cross-resistance to piperacillin and very high-level resistance to cephalosporins. This beta-lactam resistance, together with resistance to vancomycin, provides a selective advantage to a resistant organism in the hospital environment, where vancomycin, cephalosporins and piperacillin are commonly used (Murray, 2000).

Additionally, when the gram-negative intestinal bacteria are suppressed by antibiotics, there is down regulation of the anti-enterococcal host-derived lectin RegIII gamma, which allows enterococci to proliferate (Brandl et al., 2008).

Cell-wall synthesis enzymes are often referred to as penicillin-binding proteins (PBPs), because penicillin inhibits cell wall synthesis by binding to these proteins and compromising their ability to synthesize cell wall. PBP5 is one of the cell wall synthesis enzymes of *E. faecium* and the gene encoding for this protein is part of the *E. faecium* core genome. Like many genes shared by the two clades of *E. faecium,* the gene encoding PBP5 exists in two allelic forms, *pbp5*-S and *pbp5*-R, which differ by about 5 % in their DNA sequence. The amino acid differences between PBP5-S and PBP5-R are a major factor determining ampicillin resistance in this species. Among sequenced isolates, most *E.*

faecium isolates from human infections (which belong to the hospital-associated clade) have the *pbp5*-R form of this gene, while *pbp5*-S characterises isolates of the community-associated clade. In a detailed study comparing ampicillin MICs to the *pbp5* sequence of each strain, all 32 *E. faecium* strains with an MIC of ampicillin of > 4 mg/L had the *pbp5*-R sequence while the *E. faecium* strains with an MIC of < 4 had the *pbp5*-S sequence; those *E. faecium* with an ampicillin MIC = 4 had either the *pbp5*-S or the *pbp5*-R sequence. Thus, the presence of an MIC ≤ 2 mg/L appears to reliably exclude the clade that contains most isolates from human infection and excludes strains that might have a selective advantage in the GI tract if an individual was given ampicillin, amoxicillin or similar antibiotics (Rice et al., 2004; Galloway-Peña et al., 2011).

4. VIRULENCE FACTORS AND MARKERS ASSOCIATED WITH HOSPITAL STRAINS

Enterococci have been largely considered as opportunistic pathogens. This is particularly true for *E. faecium*, which is found almost exclusively as a cause of infections in the healthcare setting (Willems & van Schaik, 2009). Many factors potentially associated with *E. faecium* virulence have been identified but, among them, the following virulence factors and markers are now considered the most relevant for the assessment of safety:

- IS*16* (hospital associated strain marker)

IS elements are the simplest transposable elements encoding only the enzyme(s) necessary for their own transposition. Enterococci harbour numerous mobile genetic elements and IS*16* can be found e.g. as flanking the transposon Tn*1547***,** which confers resistance to vancomycin in *E. faecalis*. IS*16* is a specific marker for hospital-associated subpopulations of *E. faecium*, but has also been described in clinical *E. faecalis* strains (Hegstad et al., 2010). In the study of Werner et al. (2011), 97 % of blood culture *E. faecium* strains were IS*16* positive, whereas only 4 % of human commensal strains carried the element.

- Esp (pathogenicity island (PAI) marker)

Esp is a large (approximately 200 kDa) surface protein of *E. faecium* that is covalently linked to the cell wall through an LPxTG-type motif (Leavis et al., 2004; Heikens et al., 2007). The *esp* gene is part of a large pathogenicity island (ranging from ~60 – 100 kbp in size), which also carries genes for its mobilisation (van Schaik et al., 2010; Top et al., 2011). The *esp* gene has an important role in biofilm formation of *E. faecium* (Heikens et al., 2007) and has been experimentally proven to contribute to endocarditis (Heikens et al., 2011) and urinary tract infections (Leendertse et al., 2009) in animal models. The *esp* gene is common among ampicillin and vancomycin resistant *E. faecium* isolates (Rice et al. 2003; Vankerchoven et al. 2004).

- *hyl*-like gene

Hyl_{Efm} was initially described as a hyaluronidase but recently annotated as a putative glycosyl hydrolase. Glycosyl hydrolases facilitate intestinal colonisation in many bacterial organisms (Freitas et al. 2010). Strains from the community-associated clade almost never have very large plasmids containing a *hyl*-like gene, while hospital-associated strains often (~ 30 % in one study) harbour this gene (Rice et al., 2003). These *hyl* plasmids have been shown to increase colonisation of mice GI tracts and to increase lethality in a murine peritonitis model and, thus, might contribute to the success of at least some members of the hospital-associated clade (Panesso et al., 2011; Rice et al., 2009).

5. ASSESSMENT

The purpose of this assessment is to exclude *E. faecium* strains belonging to the hospital-associated clade from the use in animal nutrition because of the hazard they present to a vulnerable subpopulation of consumers.

Prior to the safety assessment, the strain must be identified as *E. faecium* using appropriate molecular methods. Then the MIC for ampicillin should be determined:

- If the MIC > 2 mg/L, the strain is considered unsafe and should not be used as a feed additive
- If the MIC ≤ 2 mg/L, the absence of the genetic elements IS16, hyl_{Efm}, and *esp* should be established (see annex for methods)
    - If none of the three genetic elements are detected, then the strain is considered safe for use as a feed additive
    - If one or more of the three genetic elements are detected, then the strain is considered unsafe and should not be used as a feed additive

REFERENCES

Bertics PJ, and Wiepz GJ, 2009. New developments with vancomycin-resistant enterococci: *E. faecium*-Friend or Foe? Journal of Infectious Diseases 200: 679–681. doi:10.1086/605477.

Bonten MJ, Willems R, Weinstein RA. 2001. Vancomycin-resistant enterococci: why are they here, and where do they come from? Lancet Infectious Diseases 1(5):314-25. Review.

Brandl K, Plitas G, Mihu CN, Ubeda C, Jia T, Fleisher M, Schnabl B, DeMatteo RP, Pamer EG. 2008. Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. Nature 455:804-807.

Freitas AR, Tedim AP, Novais C, Ruiz-Garbajosa P, Werner G, Laverde-Gomez JA, Cantón R, Peixe L, Baquero F, and Coque TM. 2010. Global Spread of the hylEfm Colonization-Virulence Gene in Megaplasmids of the *Enterococcus faecium* CC17 Polyclonal Subcluster. Antimicrobial Agents and Chemotherapy. 2010 54(6): 2660–2665.

Galloway-Peña JR, Rice LB, and Murray BE. 2011. Analysis of PBP5 of early U.S. isolates of *Enterococcus faecium*: sequence variation alone does not explain increasing ampicillin resistance over time. Antimicrobial Agents and Chemotherapy. 55:3272-3277.

Hegstad K, Mikalsen T, Coque TM, Werner G, Sundsfjord A. 2010. Mobile genetic elements and their contribution to the emergence of antimicrobial resistant *Enterococcus faecalis* and *Enterococcus faecium*. Clinical Microbiology and Infection.16:541-554.

Heikens E., Bonten MJM, and Willems RJL. 2007. Enterococcal surface protein Esp is important for biofilm formation of *Enterococcus faecium* E1162. Journal of Bacteriology 189:8233-8240.

Heikens E, Singh KV, Jacques-Palaz KD, van Luit-Asbroek M, Oostdijk EAN, Bonten MJM, Murray BE, and Willems RJL. 2011. Contribution of the enterococcal surface protein Esp to pathogenesis of *Enterococcus faecium* endocarditis. Microbes and Infection 13:1185-1190.

Hendrickx AP, van Wamel WJ, Posthuma G, Bonten MJ, and Willems RJ. 2007. Five genes encoding surface-exposed LPXTG proteins are enriched in hospital-adapted *Enterococcus faecium* clonal complex 17 isolates. Journal of Bacteriology. 189:8321-8332

Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA, Fridkin SK; National Healthcare Safety Network Team; Participating National Healthcare Safety Network Facilities. 2008. NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. Infection Control and Hospital Epidemiology. 29:996-1011

Homan, WL., Tribe D, Poznanski S, Li M, Hogg G, Spalburg E, Van Embden JDA, and Willems RJL. 2002. Multilocus sequence typing scheme for *Enterococcus faecium*. Journal of Clinical Microbiology. 40:1963-1971.

247 Leavis HL, Willems RJL, Top J, Spalburg E, Mascini EM, Fluit Ac, Hoepelman A, de Neeling AJ,
248 Bonten MJM, 2003. Epidemic and nonepidemic multidrug-resistant *Enterococcus faecium*.
249 Emerging Infectious Diseases journal. 9:1108–1115.

250 Leavis H, Top J, Shankar N, Borgen K, Bonten M, van Embden J, and Willems RJL. 2004. A novel
251 putative enterococcal pathogenicity island linked to the *esp* virulence gene of *Enterococcus*
252 *faecium* and associated with epidemicity. Journal of Bacteriology. 186:672-682.

253 Leavis HL, Willems RJL, van Wamel WJB, Schuren FH, Caspers MPM, and Bonten MJM. 2007.
254 Insertion sequence-driven diversification creates a globally dispersed emerging multiresistant
255 subspecies of *E. faecium*. PLoS Pathogens. 3:e7.

256 Leendertse Heikens E, Wijnands LM, van Luit-Asbroek M, Teske GJD, Roelofs JJTH, Bonten MJM,
257 van der Poll T, and Willems RJL. 2009. Enterococcal surface protein transiently aggravates
258 *Enterococcus faecium*-induced urinary tract infection in mice. Journal of Infectious Diseases.
259 200:1162-1165.

260 Murray, BE. 2000. Vancomycin-Resistant Enterococcal Infections. The New England Journal of
261 Medicine. 342:710-721.

262 Panesso, D, Montealegre MC, Rincon S, Mojica MF, Rice LB, Singh KV, Murray BE, Arias CA.
263 2011. The hyl_{Efm} gene in $pHyl_{Efm}$ of *Enterococcus faecium* is not required in pathogenesis of
264 murine peritonitis. BMC Microbiology. 11:20.

265 Rice LB, Carias L, Rudin S, Vael C, Goossens H, Konstabel C, Klare I, Nallapareddy SR, Huang W,
266 and Murray BE. 2003. A potential virulence gene, *hyl*Efm, predominates in *Enterococcus faecium*
267 of clinical origin. Journal of Infectious Diseases. 187(3):508-12.

268 Rice LB, Bellais S, Carias LL, Hutton-Thomas R, Bonomo RA, Caspers P, Page MG, Gutmann L.
269 2004. Impact of specific pbp5 mutations on expression of beta-lactam resistance in *Enterococcus*
270 *faecium*. Antimicrobial Agents and Chemotherapy. 48:3028-3032

271 Rice, LB, Lakticova V, Carias LL, Rudin S, Hutton R, Marshall SH. 2009. Transferable capacity for
272 gastrointestinal colonization in *Enterococcus faecium* in a mouse model. Journal of Infectious
273 Diseases. 199:342-9.

274 Singh KV, Coque TM, Weinstock GM, Murray BE. 1998. *In vivo* testing of an *Enterococcus faecalis*
275 *efaA* mutant and use of *efaA* homologs for species identification. FEMS Immunology and Medical
276 Microbiology. 21:323–31.

277 Süssmuth SD, Muscholl-Silberhorn A, Wirth R, Susa M, Marre R, Rozdzinski E. 2000. Aggregation
278 substance promotes adherence, phagocytosis, and intracellular survival of *Enterococcus faecalis*
279 within human macrophages and suppresses respiratory burst. Infection and Immunity. 68(9):4900–
280 4906.

281 Top J, Willems R, Blok H, de Regt M, Jalink K, Troelstra A, Goorhuis B, Bonten M. 2007. Ecological
282 replacement of *Enterococcus faecalis* by multiresistant clonal complex 17 *Enterococcus faecium*.
283 Clinical Microbiology and Infection. 13:316-319.

284 Top J, Sinnige JC, Majoor EAM, Bonten MJM, Willems RJL, and van Schaik W. 2011. The
285 recombinase *IntA* is required for excision of *esp*-containing ICEEfm1 in *Enterococcus faecium*.
286 Journal of Bacteriology. 193:1003-1006

287 Ubeda C., Taur Y, Jenq RR, Equinda MJ, Son T, Samstein M, Viale A, Socci ND, van den Brink
288 MR, Kamboj M, Pamer EG. 2010. Vancomycin-resistant *Enterococcus* domination of intestinal
289 microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in
290 humans. The Journal of Clinical Investigation. 120:4332-41.

291 van Schaik W and Willems RJL. 2010. Genome-based insights into the evolution of enterococci.
292 Clinical Microbiology and Infection. 16:527-532.

van Schaik, W, Top J, Riley D, Boekhorst J, Vrijenhoek J, Schapendonk C, Hendrickx A, Nijman I, Bonten M, Tettelin H, and Willems R. 2010. Pyrosequencing-based comparative genome analysis of the nosocomial pathogen *Enterococcus faecium* and identification of a large transferable pathogenicity island. BMC Genomics 11:239.

Vankerckhoven V, Van Autgaerden T, Vael C, Lammens C, Chapelle S, Rossi R, Jabes D, Goossens H. 2004. Development of a multiplex PCR for the detection of *asa1, gelE, cylA, esp*, and *hyl* genes in enterococci and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*. Journal of Clinical Microbiology. 42:4473-9.

Werner G, Coque TM, Hammerum AM, Hope R, Hryniewicz W, Johnson A, Klare I, Kristinsson KG, Leclercq R, Lester CH, Lillie M, Novais C, Olsson-Liljequist B, Peixe LV, Sadowy E, Simonsen GS, Top J, Vuopio-Varkila J, Willems RJ, Witte W, Woodford N. 2008. Emergence and spread of vancomycin resistance among enterococci in Europe. Euro Surveillance. 13:19046.

Werner G, Fleige C, Geringer U, van Schaik W, Klare I, and Witte W. 2011. IS element IS16 as a molecular screening tool to identify hospital-associated strains of *Enterococcus faecium*. BMC Infectious Diseases 11:80.

Willems RJL, Top J, van Santen M, Robinson DA, Coque TM, Baquero F, Grundmann H, and Bonten MJM. 2005. Global spread of vancomycin-resistant *Enterococcus faecium* from distinct nosocomial genetic complex. Emerging Infectious Diseases journal. 11:821-828.

Willems RJ and van Schaik W. 2009. Transition of *Enterococcus faecium* from commensal organism to nosocomial pathogen. Future Microbiology. 4:1125-1135.

Willems RJL, Hanage WP, Bessen DE, and Feil EJ. 2011. Population biology of Gram-positive pathogens: high-risk clones for dissemination of antibiotic resistance. FEMS Microbiology Rev. 35:872-900.

Zhang X, Vrijenhoek JEP, Bonten MJM, Willems RJL, and van Schaik W. 2011. A genetic element present on megaplasmids allows *Enterococcus faecium* to use raffinose as carbon source. Environmental Microbiology. 13:518-528.

APPENDIX

RECOMMENDED METHODS

Some of the control strains are not at present readily available. However, the FEEDAP Panel has been informed that the strains have been deposited in DSMZ and will be available soon.

- **Ampicillin MIC**

For the determination of ampicillin MIC, serial two-fold dilution procedures in agar or broth should be used and include relevant quality control strains. The tests should be performed according to internationally recognised standards such as European Union Committee on Antimicrobial Susceptibility Testing (EUCAST), the Clinical and Laboratory Standard Institute (CLSI), ISO standard or similar. After incubation, the MIC is defined as the lowest concentration of the antibiotic that inhibits bacterial growth. Qualitative or semi-qualitative methods to determine MIC indirectly, such as diffusion methods, are not acceptable.

- **IS*16***

It is recommended that the method of Werner et al. (2011) is used for the detection of IS*16* with the following PCR primers: IS16-F (forward) 5'-CATGTTCCACGAACCAGAG and IS16-R (reverse): 5'-TCAAAAAGTGGGCTTGGC (expected product size 547 bp from *E. faecium*). PCR analysis should contain positive and negative control strains. As a positive control strain *E. faecium* DSMZ 25390 and as a negative control strain *E. faecium* TX1330 can be used.

- ***esp***

Detection of *esp* is best performed using hybridisation techniques as they are less dependent on point mutations in primer-binding sites, which could give false negative results. The primers for the generation of the probe are esp14F: 5'-AATTGATTCTTTAGCATCTGG-3' and esp12R: 5'-AGATTTCATCTTTGATTCTTGG-3' (Leavis et al., 2003). Hybridisation conditions for Southern blotting are described in Hendrickx *et al*. (2007), whereas the hybridisation conditions for dot blotting are described in Rice et al. (2003) and in Hendrickx et al. (2007). Also colony lysates can be used in the hybridisation (Singh et al., 1998). Hybridisation analysis should contain positive and negative control strains. As a positive control strain *E. faecium* DSMZ 25390 and as a negative control strain *E. faecium* DSMZ 25389 can be used.

- ***hyl*$_{Efm}$**

The method of Rice et al. (2003) is recommended for the detection of *hyl*$_{Efm}$ with the following PCR primer: 5′-GAGTAGAGGAATATCTTAGC-3′ (nt 856 – nt 875) and the reverse primer *hyl*Efm 5′-AGGCTCCAATTCTGT-3′ (nt 1517 – nt 1503) (expected size 661 bp from *E. faecium* TX16 (=ATCC BAA-472).

As an alternative method hybridisation to colony lysates or Southern blots can be used (Rice et al. 2003, Singh et al. 1998). The primers for the generation of the intragenic probe are: forward primer *hyl*Efm (5′-GTT AGA AGA AGT CTG GAA ACC G-3′; nt 149 – nt 170) and reverse primer *hyl*Efm (5′-TGC TAA GAT ATT CCT CTA CTC G-3′; nt 876 – nt 855); expected size 727 bp from *E. faecium* TX16 (=ATCC BAA-472).

PCR and hybridisation analysis should contain positive and negative control strains. As a positive control strain *E. faecium* ATCC BAA-472 (=TX16) or *E. faecium* DSMZ 25390 and as a negative control strain *E. faecium* DSMZ 25389 can be used.