ORIGINAL ARTICLE 10.1111/j.1469-0691.2008.02663.x

Antibiotic susceptibility among *Staphylococcus epidermidis* isolated from prosthetic joint infections with special focus on rifampicin and variability of the *rpoB* gene

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Abstract

Staphylococcus epidermidis is the most important pathogen in infections related to implanted foreign materials, especially prosthetic joint infections (PJIs). The aim of this study was to investigate the antimicrobial activities of 16 antibiotics against *S. epidermidis* isolated from PJIs, with special focus on rifampicin and *rpoB* variability. Ninety-one per cent of the isolates were multiresistant (i.e. resistant to members of more than three classes of antibiotics). Thirty-nine per cent were resistant to rifampicin, associated with one or two single-nucleotide polymorphisms (SNPs) in *rpoB*. Using IsoSensitest agar with supplements, 61% were resistant to oxacillin, and using Mueller–Hinton II agar with supplement, 84% were resistant. Using the Etest, 58% were resistant to cefoxitin, and using the disk diffusion test, 91% were resistant. The *mecA* gene was detected in 85% of the isolates. Regarding recently available antibiotics, all isolates were susceptible to tigecycline and linezolid, and 97% were susceptible to daptomycin. In addition, two novel antibiotics, dalbavancin and ceftobiprole, were tested, although not yet available for routine use. The MIC_{50} and MIC_{90} values of these novel antibiotics were 0.032 and 0.047 mg/L and 0.5 and 1.5 mg/L, respectively. Among the other antibiotics, the rates of resistance varied between 0% (vancomycin) and 82% (trimethoprim–sulphamethoxazole). *S. epidermidis* strains causing PJIs often show multiresistance, including resistance to rifampicin, which is mainly caused by one or two SNPs. Some of the newer antimicrobial agents may provide alternatives for monotherapy or combination therapy with rifampicin. Detection of *mecA* is necessary before initiating treatment of infections due to *S. epidermidis* when it displays intermediate susceptibility to cefoxitin.

Keywords: Antibiotic susceptibility/resistance, prosthetic joint infections, rifampicin, *rpoB*, *Staphylococcus epidermidis*
Original Submission: 5 June 2008; **Revised Submission:** 23 July 2008; **Accepted:** 23 July 2008
Editor: D. Raoult
Clin Microbiol Infect 2009; **15:** 238–244

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Introduction

Staphylococcus epidermidis is a commensal of the human skin flora. Although the pathogenic potential and virulence factors of this bacterium are relatively unknown [1,2], it has emerged as the most important pathogen in infections related to implanted foreign materials, especially prosthetic joint infections (PJIs) [3].

Joint replacement surgery is one of the major medical advances during the 20th century and has improved the quality of life for hundreds of thousands of patients. In Sweden, approximately 14 000 hip joint replacements and 10 500 knee arthroplasties are performed annually. The main reasons for arthoplasty revisions in Sweden are aseptic loosening or infection, which may be of early, delayed or late onset (Swedish National Hip Arthroplasty Register, Annual report 2006, http://www.jru.orthop.gu.se; Swedish National Knee Arthroplasty Register, Annual report 2007, http://www.knee.nko.se). Among the patients who have had primary hip joint replacements, 1.5% will be re-operated on within 2 years, 0.6% because of infection (Swedish National Hip Arthroplasty Register).

Overall, the prevalence of primary infections following joint replacements in Sweden has been reported to be approximately 0.5% for hip PJI and 1% for knee PJI [4]. Although rare, implant infections represent a diagnostic and therapeutic challenge for the doctor and cause considerable suffering for the patient, with pain and disability and even increased mortality. In addition, the costs are significantly

increased due to prolonged hospitalization, revision surgery and long-term antimicrobial treatment [5].

The antimicrobial treatment of PJIs has, during the last decades, consisted primarily of cell wall synthesis-inhibiting antibiotics such as isoxazolyl-penicillins or glycopeptides [6]. However, without prosthetic joint exchange surgery, the long-term outcome has been disappointing with finite treatment regimens [7,8]. The main reason for this is that *S. epidermidis*, which is the most common cause of PJIs [3], when accumulating on a surface such as an implant, produces biofilm and switches to a stationary growth phase that is influenced by high cell population densities [9,10]. Slow-growing bacteria in a stationary phase, as compared with planktonic bacteria, show reduced metabolic activity and, thereby, increased resistance to killing by growth-dependent antibiotics, e.g. cell-wall-active antibiotics [11,12]. In addition, the biofilm reduces the accessibility of antibiotics and host immune components such as complement and antibodies [13].

Optimal antibiotics for the treatment of foreign-body infections have been proposed [14]. Experimental studies [15,16] and clinical trials [17,18] have demonstrated impressive results with rifampicin. However, the risk of rapid development of resistance is a major problem [6], and rifampicin should not be used as monotherapy. Accordingly, various antibiotics, e.g. fluoroquinolones [8], fusidic acid [17,19], clindamycin [18] and linezolid [20], have been proposed to be combined with rifampicin in order to reduce the risk of development of rifampicin resistance. Furthermore, some recently available antibiotics, e.g. daptomycin, tigecycline and dalbavancin, may be suitable for treatment of foreign-body infections, including PJIs, caused by sessile and biofilm-producing bacteria such as *S. epidermidis* [21,22].

The aim of the present study was to investigate the antimicrobial activities of 16 antibiotics against *S. epidermidis* isolated from PJIs, with special focus on rifampicin and on *rpoB* variability.

Materials and methods

Bacterial isolates

Thirty-three *S. epidermidis* isolates obtained during revision surgery for PJIs with extraction or exchange were analysed. Multiple tissue biopsy samples, usually five or more, were taken, most of which yielded growth of *S. epidermidis*. Eleven isolates were collected from patients with infected hip prostheses treated at Linköping University Hospital, Sweden (centre A) from 1993 to 2003, and 22 isolates were obtained from patients with infected hip (n = 13), knee (n = 8) and elbow joint prostheses (n = 1) treated at Örebro University Hospital, Sweden (centre B) from 2000 to 2005.

At centre A, the tissue samples were immediately placed into thioglycollate medium broth (3.0% BBL Thioglycollate Medium (BD Diagnostic Systems, Sparks, MD, USA)) and cultured at 37°C for 5 days under aerobic conditions and for 10 days under anaerobic conditions. At centre B, the samples were cultured on Difco GC Medium Base (BD Diagnostic Systems) supplemented with 1% haemoglobin powder, 10% horse serum (SVA, Uppsala, Sweden) and 1% IsoVitalex Enrichment (BD Diagnostic Systems) at 36°C under 5% CO_2 for 2 days and on anaerobe agar medium (Fastidious anaerobe agar (Acumedia; Neogen Corporation, Lansing, MI, USA) supplemented with 5% horse blood (SVA)) at 36°C anaerobically for up to 5 days, as well as in enrichment broth (29.7% fastidious anaerobic broth (Lab M, Bury, UK) supplemented with 10% D-glucose (J. T. Baker, Deventer, The Netherlands)) for 7 days.

The isolates were stored at −70°C in glycerol storage broth [23] or in preservation medium (Trypticase soy broth (BD Diagnostic Systems) supplemented with 0.3% Yeast extract (BD Diagnostic Systems) and 29% horse serum (SVA)) until further analysis.

The *S. epidermidis* isolates were verified to species level using the ID32Staph system (bioMérieux, Marcy l'Etoile, France) and sequencing of *rpoB*, as previously described [24].

Antibiotic susceptibility testing

The MICs of antibiotics (n = 16) were determined using the Etest (AB Biodisk, Solna, Sweden), (Table 1). The disk diffusion test (DDT) was performed for cefoxitin (10 μg), moxalactam (30 μg) and norfloxacin (10 μg). All tests, except for oxacillin susceptibility, were performed on IsoSensitest agar (Oxoid, Basingstoke, UK) (ISOAP) with incubation for 16–18 h at 36°C. Oxacillin susceptibility was tested, firstly, on IsoSensitest agar (Oxoid) supplemented with 5% defibrinated horse blood (SVA) and 2% NAD (Sigma-Aldrich Inc., St Louis, MO, USA) (ISONP) that was incubated for 24 h at 30°C, and secondly, on Mueller–Hinton II agar (BD Diagnostic Systems), supplemented with 2% sodium chloride (MUHSP), that was incubated for 16–18 h at 36°C. The susceptibility testing was done and breakpoints were established according to the recommendations of the Swedish Reference Group for Antibiotics (SRGA; http://www.srga.org).

Isolation of genomic DNA from the *S. epidermidis* isolates

For DNA isolation, the MagNA pure compact system with the MagNA pure compact nucleic acid isolation kit I was used according to the instructions of the manufacturer

TABLE 1. Antibiotic susceptibility (Etest) of *Staphylococcus epidermidis* (*n* = 33) isolated from prosthetic joint infections

Antibiotic	Breakpoint MIC (mg/L)[a]	MIC (mg/L) range	MIC_{50}	MIC_{90}	Resistant (%)
Oxacillin (IsoSensitest)[b]	1/1[c]	<0.016 to 24	0.38	2	61
Oxacillin (Mueller–Hinton)[b,d]	1/1[c]	0.125 to >256	128	>256	84
Cefoxitin	4/4	0.19 to >256	6	24	58
Gentamicin	1/1	0.032 to >256	32	64	79
Erythromycin	0.5/0.5	0.047 to >256	>256	>256	67
Clindamycin	0.5/2	0.023 to >256	>256	>256	67
Fusidic acid	0.5/0.5	0.016–16	0.047	12	39
Tigecycline	0.5/0.5	0.047–0.5	0.125	25	0
Rifampicin	1/1	<0.002 to >32	0.003	>32	39
Vancomycin	4/8	0.5–3	2	3	0
Linezolid	4/4	0.047–0.38	0.19	0.25	0
Daptomycin	1/1	0.094–1.5	0.25	0.75	3
Ciprofloxacin	1/1	0.047 to >32	>32	>32	79
Moxifloxacin	0.5/1	0.016–6	2	3	64
Trimethoprim–sulphamethoxazole	16/32	0.64 to >640	>640	>640	82
Dalbavancin	NA	0.003–0.047	0.032	0.047	NA
Ceftobiprole	NA	0.094–1.5	0.5	1.5	NA

[a]Species-related breakpoints according to the Swedish Reference Group for Antibiotics (SRGA, http://www.srga.org). Breakpoints for S/I/R categorization, e.g. 4/8: $S \leq 4$ mg/L and $R > 8$ mg/L.
[b]The Etest for oxacillin was performed using both ISONP and MUHSP. The Etest for all other antibiotics were performed using ISOAP.
[c]Pharmacological breakpoint.
[d]One isolate did not grow on MUHSP.

(Roche Diagnostics GmbH, Mannheim, Germany). The DNA preparations were stored at +4°C prior to PCR.

Detection of *mecA*

The *mecA* gene was detected using a real-time PCR as described previously [25].

Nucleotide sequence determination of *rpoB* and sequence analysis

Real-time PCR amplification and sequencing of *rpoB*, as well as subsequent sequence analysis, were performed as previously described [24].

Results

The results of antibiotic susceptibility testing, using the Etest, of 16 antibiotics are summarized in Table 1.

Thirty-nine per cent of the isolates were resistant to rifampicin (MIC >32 mg/L, *n* = 12; MIC 6 mg/L, *n* = 1). Rifampicin resistance was associated with one or two single-nucleotide polymorphisms (SNPs) in *rpoB* (Fig. 1), whereas the *rpoB* sequences were identical for all susceptible isolates (*n* = 20), except one, which had a non-synonymous SNP that resulted in the amino acid change A477V (Fig. 1). Among the resistant isolates (*n* = 13), the amino acid changes were as follows: both D471E and I527M (*n* = 9) or both S464P and A534V (*n* = 2); both combinations were associated with high-level resistance (MIC >32 mg/L). An S464P change (*n* = 1) and an H481Y change (*n* = 1), associated with low-level resistance (MIC <6 mg/L) and high-level resistance (MIC >32 mg/L), respectively, were also identified (Fig. 1).

The rate of resistance to oxacillin was 61% when the Etest was used on ISONP; it was 84% for the same drug on MUHSP and 58% for cefoxitin on ISOAP. (Table 1).

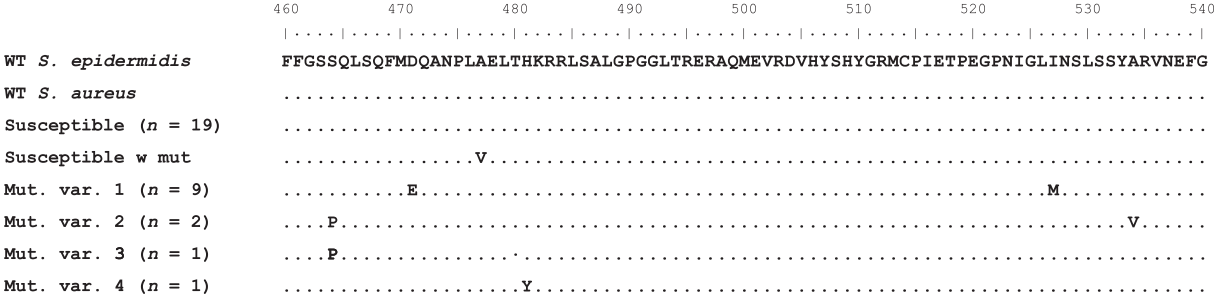

FIG. 1. Multiple alignment of amino acid sequences of an RpoB fragment from *Staphylococccus epidermidis* isolates (*n* = 33) from patients with prosthetic joint infections (numbering as for RpoB of *Staphylococcus aureus* (GenBank accession number CAA45512)).

However, the DDT with cefoxitin gave a 91% resistance rate, and for moxalactam the 50th percentile were 22 mm and the 90th percentile were 31 mm for the disc diffusion test. No S/I/R breakpoints defined.

The *mecA* gene was detected in 85% of the isolates. Three isolates, with a zone diameter of 23 mm (n = 2) or 26 mm (n = 1) for cefoxitin, were negative for *mecA* but displayed intermediate susceptibility according to the SRGA breakpoints ($R \leq 21$ mm and $S \geq 27$ mm) and one isolate was positive for *mecA*, but susceptible to cefoxitin. The two isolates with a cefoxitin zone diameter of 23 mm had moxalactam zone diameters of 28 and 31 mm.The isolate with a cefoxitin zone diameter of 26 mm had a moxalactam zone diameter of 23 mm. Overall, there was a good correlation between size of the moxalactam zone diameter and the presence of *mecA*. However, the results concerning two isolates were incongruent. One isolate with a zone diameter of 23 mm was *mecA*-negative and one isolate with a zone diameter of 36 mm was *mecA*-positive. A comparison of the results of the Etest for oxacillin, on ISONP, with the results of *mecA* detection showed that 20 isolates (61%) were resistant to oxacillin and *mecA*-positive. However, eight isolates (24%) were susceptible to oxacillin but *mecA*-positive. Accordingly, only five isolates (15%) were susceptible to oxacillin and *mecA*-negative (Table 2). The same comparison, but of the results obtained on MUHSP, showed that 27 isolates (84%) were resistant to oxacillin and *mecA*-positive, whereas only one isolate (3%) was susceptible to oxacillin but *mecA*-positive and, accordingly, four isolates (13%) were susceptible to oxacillin and *mecA*-negative. One isolate did not grow on MUHSP (Table 2). Regarding cefoxitin, 18 isolates (55%) were resistant (Etest) and *mecA*-positive, ten isolates (30%) were susceptible and *mecA*-positive, four isolates (12%) were susceptible and *mecA*-negative, and one isolate (3%) was resistant but *mecA*-negative (Table 2).

TABLE 2. MICs of oxacillin and cefoxitin, as related to the presence of *mecA* (detected using real-time PCR), for *Staphylococcus epidermidis* (n = 33) isolated from prosthetic joint infections

	mecA$^+$ (n = 28)	*mecA*$^-$ (n = 5)
Oxacillin-susceptible, ISONP[a]	8	5
Oxacillin-resistant, ISONP[a]	20	0
Oxacillin-susceptible, MUHSP[a]	1	4[b]
Oxacillin-resistant, MUHSP[a]	27	0
Cefoxitin-susceptible[a]	10	4
Cefoxitin-resistant[a]	18	1

[a]The Etest for oxacillin was performed using both ISONP and MUHSP. The Etest for cefoxitin was performed using ISOAP.

[b]One isolate did not grow on MUHSP.

Regarding the fluoroquinolones, 79% of the isolates were resistant to ciprofloxacin and 64% were resistant to moxifloxacin, the latter with slightly lower MICs (Table 1).

According to the DDT with norfloxacin, 79% of the isolates were resistant, and all of these were also resistant, or intermediately susceptible, to ciprofloxacin and moxifloxacin.

Twenty-two isolates (67%) were resistant to clindamycin (MIC >256 mg/L). All of these isolates were also resistant to erythromycin (MIC >256 mg/L, except for one isolate (MIC 32 mg/L)). No isolate was resistant to vancomycin, whereas 79% were resistant to gentamicin, 82% to trimethoprim–sulphamethoxazole and 39% to fusidic acid (Table 1).

Regarding recently available antimicrobial agents, all isolates (100%) were susceptible to tigecycline and linezolid, and 97% were susceptible to daptomycin. In addition, two novel antibiotics, dalbavancin and ceftobiprole, not yet commercially available for routine use, were tested. The MIC_{50} and MIC_{90} of dalbavancin and ceftobiprole were 0.032 and 0.047 mg/L and 0.5 and 1.5 mg/L, respectively (Table 1). The distributions of the MICs of dalbavancin and ceftobiprole are shown in Fig. 2.

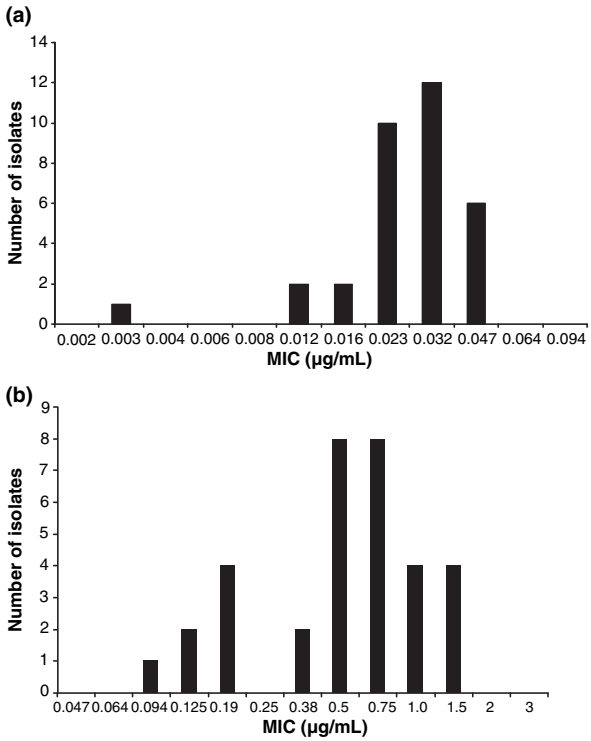

FIG. 2. Distribution of the MICs of dalbavancin (a) and ceftobiprole (b), as determined with the Etest, for 33 *Staphylococcus epidermidis* isolates from patients with prosthetic joint infections.

Discussion

In the present study, the antimicrobial activities of 16 antibiotics against *S. epidermidis* isolated from PJIs were investigated. Ninety-one per cent of the isolates were multiresistant, whereas only two isolates were susceptible to all the antibiotics tested, and one isolate was resistant to cefoxitin only, as determined by DDT.

Among the isolates resistant to rifampicin (n = 13), one or two SNPs associated with resistance were found in *rpoB*.

Rifampicin, in combination with an additional effective antimicrobial agent, has been proposed as being ideal for the treatment of PJI [3]. However, in the present study, 39% of the isolates were resistant to rifampicin. Regarding the *rpoB* mutations associated with rifampicin resistance, some of those identified here have been described similarly for *Staphylococcus aureus* but, as far as we know, not for *S. epidermidis*. The A477V change has previously been described in *S. aureus* as resulting in low-level resistance (MIC 1 mg/L) [26]. However, the *S. epidermidis* isolate with the corresponding change in the present study exhibited an MIC value of only 0.25 mg/L, and was consequently regarded as susceptible.

Concerning the mutation variant 1 (Fig. 1), the non-synonymous substitution (C → A) in amino acid codon 471 (D471E) has previously been described in *S. aureus*, but only for *in vitro*-derived strains [27], whereas other non-synonymous nucleotide changes in the same codon have been described in clinical isolates [26,28]. However, the I527M change has resulted in high-level resistance (MIC >256 mg/L) in *S. aureus* [28]. In mutation variant 2, the non-synonymous substitution (T → C) in codon 464 (S464P) has previously been described in *S. aureus*, resulting in a low level of resistance (MIC 4 mg/L) [26]. However, the substitution (C → T) in codon 534 (A534V) has not been described previously. The non-synonymous substitutions resulting in mutation variants 3 and 4 (S464P and H481Y, respectively) have been described previously in *S. aureus*, resulting in low-level (MIC 4 mg/L) and high-level (MIC >256 mg/L) resistance, respectively [26]. Accordingly, the results of the present study clearly show that the development of rifampicin resistance in *S. epidermidis* is similar to what has been reported in *S. aureus* as well as in other bacterial species [26]. Thus, a single SNP can lead to a dramatic increase in the MIC.

The majority (85%) of isolates carried *mecA*. However, a higher proportion, depending on which agar was used for oxacillin susceptibility testing, should have been regarded as methicillin-susceptible according to the pharmacological MIC breakpoints for oxacillin and the species-related MIC breakpoints for cefoxitin, all recommended by the SRGA (http://www.srga.org). When comparing ISONP and MUHSP, the different results are a clear example of the effects of discrepant test conditions for detecting resistance. There are other studies showing that an addition of up to 5% NaCl improves the detection of methicillin resistance [29,30]. In contrast, the DDT for cefoxitin showed a higher correlation with the presence of *mecA* than the Etest. However, the species-related zone breakpoints for oxacillin using DDT have recently been invalidated by the SRGA (http://www.srga.org).

Moxalactam susceptibility was investigated in order to determine whether, with this compound, one could better discriminate between *mecA*-positive and *mecA*-negative isolates than with cefoxitin, especially in the case of isolates showing intermediate susceptibility to cefoxitin (DDT zone diameter 21–27 mm) [31]. The correlation between the moxalactam zone diameters and the presence of *mecA* was slightly better than that observed with cefoxitin, but it was still not completely reliable. Consequently, detection of *mecA* remains necessary prior to initiating treatment for infections due to *S. epidermidis* strains that are intermediately susceptible to cefoxitin.

Fluoroquinolones have been recommended as the drugs of first choice for combination therapy with rifampicin for the treatment of foreign-body infections such as PJIs [8]. Three fluoroquinolones were tested in the present study, but the proportion of susceptible isolates was rather similar for all of them. However, slightly lower MICs of moxifloxacin were found, as compared with ciprofloxacin. The significance of this, however, is uncertain, as no clinical trial using moxifloxacin for the treatment of PJI has been published. Nevertheless, there are several clinical trials concerning PJIs in which ciprofloxacin or levofloxacin has been used in combination with rifampicin, displaying impressive treatment outcomes [8,18,32].

For additional commonly used antibiotics, e.g. clindamycin, gentamicin and trimethoprim–sulphamethoxazole, a high level of resistance was found. The high rate of gentamicin resistance (79%) may be a result of the mandatory use of gentamicin-containing cement in Sweden, and may also limit the utilization of local application with gentamicin-containing collagen sponges. There is only one published clinical study that has examined the combination of rifampicin and clindamycin [18], and this showed a lower cure rate than that obtained with the combination of rifampicin and levofloxacin. The cumulative risk of treatment failure with the combination of rifampicin and fusidic acid has been reported to be 12% [19]. Rifampicin is a strong inducer of the cytochrome P450 enzymes, including cytochrome 3A4 [33]. The exact metabolic pathways for clindamycin and fusidic acid are not known, but clindamycin is probably metabolized by hepatic

cytochrome P450 enzymes and fusidic acid by cytochrome 3A4 [34,35]. Theoretically, pharmacological interactions could therefore occur, resulting in subtherapeutic concentrations of clindamycin and fusidic acid, respectively, during treatment with rifampicin and, eventually, in less favourable treatment outcomes.

Almost all of the isolates were found to be highly susceptible to the recently available antibiotics daptomycin (97%), tigecycline (100%) and linezolid (100%). Several clinical trials, although open and non-randomized, have shown promising outcomes in the treatment of PJIs with linezolid, including the combination with rifampicin [20,36]. However, the clinical experience of treating PJIs with daptomycin and tigecycline is limited [37]. In the present study, one daptomycin-resistant isolate (MIC 1.0 mg/L) was found. The MIC of vancomycin for this isolate was 2.0 mg/L. A relationship between increased MICs of daptomycin and vancomycin has been reported [38], as well as an increase in MIC during long-term treatment with daptomycin [39].

The novel antimicrobial agents ceftobiprole and dalbavancin have not yet been approved by the US Food and Drug Administration or the European Medicines Agency (EMEA), but preliminary *in vitro* and *in vivo* data have indicated the potential usefulness of these antimicrobial agents for the treatment of foreign-body infections [40,41]. The MICs of these antimicrobial agents determined here indicate that they may be considered for the treatment of PJIs, at least initially until peroral antibiotics could be instituted.

In conclusion, *S. epidermidis* strains causing PJIs often show multiresistance, including resistance to rifampicin, which is mainly associated with one or two SNPs in *rpoB*. However, some of the newer antimicrobial agents may provide potential alternatives for monotherapy or combination therapy with rifampicin. Detection of *mecA* is necessary before initiating treatment of infection due to *S. epidermidis* when it displays intermediate susceptibility to cefoxitin.

Acknowledgements

The authors wish to thank Pfizer AB, Sweden, and Janssen-Cilag Medical Affairs EMEA, a division of Janssen Pharmaceutica NV, USA, for supplying the Etest strips for dalbavancin and ceftobiprole, respectively.

Transparency declaration

This study was supported by grants from the Örebro County Council Research committee, the County Council of Östergötland and the Faculty of Health Sciences of Linköping University, Sweden. B. Söderquist has served as a consultant to Pfizer AB, Sweden, but the other authors, B. Hellmark, M. Unemo and Å. Nilsdotter-Augustinsson, have no financial, personal or other relationship that could be viewed as presenting a potential conflict of interest.

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