Enterococcus faecium infections: where bacterial virulence meets innate immunity
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ENTEROCOCCAL SURFACE PROTEIN AGGRAVATES ENTEROCOCCUS FAECIUM INDUCED URINARY TRACT INFECTION IN MICE

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Submitted
Abstract

Background. *Enterococcus faecium* has globally emerged as a multi-resistant nosocomial pathogen causing a wide spectrum of infections including urinary tract infections (UTI) and peritonitis. Knowledge about the role of virulence factors in these infections is limited. The enterococcal surface protein Esp, linked to nosocomial *E. faecium*, has been identified as a putative virulence factor involved in biofilm formation. Methods. An Esp expressing strain of *E. faecium* (E1162) and its isogenic Esp-deficient mutant (E1162Δesp) were tested for their adherence capacity to uroepithelial cells *in vitro* and *in vivo* in mouse models for UTI and peritonitis. Results. Esp expression by *E. faecium* enhanced *in vitro* binding to bladder and kidney epithelial cells. In mice, higher numbers of E1162 were cultured from kidneys and bladders one and three days after induction of UTI compared to E1162Δesp. This was accompanied by a higher frequency of bacteremia, higher levels of proinflammatory cytokines in kidney tissue, and renal insufficiency. No differences in urine cultures were found. Esp expression had no impact on the course of or inflammatory response during a model of *E. faecium* peritonitis. Conclusions. These results indicate that Esp of *E. faecium* negatively influences the course of an UTI, thereby facilitating subsequent bloodstream infection.
Introduction

Enterococci have globally emerged as important multidrug resistant nosocomial pathogens. They can cause a wide spectrum of infections including device-related-infections, bacteremia, and endocarditis, but also urinary tract infections (UTI) and peritonitis [1-3]. Enterococci rank third among pathogens isolated from hospitalized patients with UTI [4] and have been associated with increased mortality in peritonitis [5, 6]. In the past, most enterococcal infections were caused by Enterococcus faecalis, but infections with Enterococcus faecium have increased dramatically in the last two decades. This can largely be attributed to the ability of E. faecium to adapt to the abundant use of antibiotics in hospitals by acquiring resistance to high dose aminoglycosides, β-lactam antibiotics and vancomycin [7, 8]. Molecular epidemiological studies have indicated that E. faecium clones responsible for the majority of nosocomial infections and hospital outbreaks are genetically distinct from indigenous intestinal isolates [8, 9]. Additionally to antibiotic resistance genes, these E. faecium clones acquired several genes and elements encoding potential virulence factors and adaptive mechanisms that are thought to enhance survival in hospitalized patients [10-15].

It is not known, which virulence factors play a role in the pathogenesis of E. faecium UTI and peritonitis. The enterococcal surface protein Esp, located on a putative pathogenicity island (PAI), is specifically enriched in hospital-acquired E. faecium and has been identified as a potential virulence factor involved in biofilm formation [8, 10, 14-16]. A significant relationship was found between E. faecium urinary isolates and the presence of esp, suggesting a role of Esp in UTI [17]. So far, nothing is known about the role of Esp in E. faecium peritonitis.

In this study we investigated the virulence potential of E. faecium expressing Esp in UTI and peritonitis, by comparing an Esp expressing strain of E. faecium and its isogenic Esp-deficient mutant, we recently generated [16]. We tested the adherence capacity to uroepithelial cells in vitro and colonization and persistence in vivo in a mouse model for UTI. Furthermore, we tested a possible pathogenic role of Esp in a murine peritonitis model we recently developed to investigate the normal immune response during primary E. faecium peritonitis [18].
Materials and Methods

Bacterial strains and growth conditions
E. faecium strains E1162 and E1162Δesp were used. E1162 is a clinical blood isolate and positive for Esp expression. The isogenic Esp-deficient mutant, E1162Δesp, was previously constructed by introduction of a chloramphenicol resistance cassette (cat) resulting in an insertion-deletion mutation of the esp gene [16]. E. faecium strains were grown in either Todd-Hewitt (TH) or brain heart infusion (BHI) broth or on Tryptic Soy Agar (TSA) with 5% sheep red blood cells (Difco, Detroit, MI). Slanetz-Bartley (SB) agar plates were used to selectively grow enterococci.

Cell lines, media, and culture conditions
Human bladder carcinoma T24 cells were obtained from American Type Culture Collection (ATCC, HTB-4) and cultured in Eagle’s minimal essential medium (EMEM; BioWithaker, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (Integro B.V., Zaandam, The Netherlands) and 50 μg/ml gentamicin (Gibco, Invitrogen, Paisley, UK). Madin-Darby canine kidney (MDCK) epithelial cells were obtained from ATCC as well (CCL-34) and cultured in Dulbecco’s modified Eagle’s minimal essential medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal calf serum (Integro B.V.), 1% non-essential amino acids (Gibco), 2 mM glutamine (Gibco), and 50 μg/ml gentamicin (Gibco). T24 and MDCK cells were prepared by seeding cells in 12-wells tissue culture plates (Costar, Corning, NY) at 1 x 10^5 and 0.5 x 10^4 cells/ml, respectively, in EMEM or DMEM. One ml of these suspensions was added to each well and plates were incubated at 37°C in 5% CO₂ for 3 days to a confluent monolayer.

Adhesion assay
Overnight-grown cultures of E1162 and E1162Δesp in BHI broth were diluted (1:50) and grown at 37°C to an OD₆₆₀ of 0.4, while shaking. Bacteria were harvested by centrifugation (6,500 x g; 3 min) and resuspended in EMEM or DMEM to a concentration of 1 x 10⁷ CFU/ml. For each strain, 1 ml bacterial suspension was added to the wells. Plates were centrifuged (175 x g; 1 min) and incubated for 1 h at 37°C in 5% CO₂. After incubation, non-adherent bacteria were withdrawn from the wells, cells were washed 3 times with EMEM or DMEM, and subsequently lysed with 1% Triton X-100 (Merck, Darmstadt, Germany) in PBS for approximately 5 min at room temperature. Adherent bacteria were quantified by plating serial dilutions onto TSA plates and counting resultant colonies. Also the inoculum was plated to determine initial viable counts. Adherence was calculated for both E1162
and E162Δesp as follows: (CFU/ml t=1 / CFU/ml t=0) × 100, where CFU/ml t=0 refers to the initial number of viable bacterial cells and CFU/ml t=1 to adhesion of viable bacterial cells after 1h. The proportion E1162Δesp relative to E1162 was calculated by using the equation: (percentage adherence E1162Δesp × 100) / percentage adherence E1162, where the percentage adherence of E1162 was transformed to 100. The assay was performed simultaneously in 3 separate wells in duplicate and repeated on 3 different days. The results of all individual experiments were combined.

Mice
Specific pathogen-free 10-wk-old female C57BL/6 mice were purchased from Harlan Sprague-Dawley (Horst, The Netherlands). The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

Induction of urinary tract infection
Parent (E1162) and esp-mutant (E1162Δesp) E. faecium strains were tested in a mouse UTI model. Mice were transurethrally infected, similar to a UTI model described by Kau et al. [19]; an inoculum volume of 200 μl is used to facilitate direct delivery of the bacteria to both the bladder and kidneys. Both E. faecium strains were grown overnight on TSA plates and then grown for approximately 3.5 h in Todd-Hewitt broth (Difco, Detroit, MI) to midlogarithmic phase at 37˚C, while shaking, then washed in sterile saline to clear the bacteria of medium. The UTI was induced under general anesthesia (0.07 mL/10g mouse of FFM mixture, containing 1.25 mg/mL midazolam (Roche, Mijdrecht, The Netherlands), 0.08 mg/mL fentanyl citrate and 2.5 mg/mL fluanisone (Janssen Pharmaceutica, Beerse, Belgium). Urinary bladders were emptied by gently pressing the abdomen, before transurethral administration of 10⁸ CFU of each E. faecium strain, through a 0.55 mm catheter (Abbott, Zwolle, The Netherlands). The inoculum was plated immediately after inoculation to determine viable counts. Ten to twelve mice per group were sacrificed 1, 3, and 5 days after the procedure.

Induction of peritonitis
Bacteria were treated as described above, and mice were injected intraperitoneally (i.p.) with 10⁸ CFU of either E1162 or E1162Δesp in 200 μl saline. This bacterial dose of another clinical isolate of E. faecium, E155, is gradually cleared by normal C57BL/6 mice and is not associated with lethality [18]. Seven to eight mice per group were sacrificed 2 and 24 hours after the infection.
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Collection of samples
Mice were anesthetized by inhalation of isoflurane (Abbot, Laboratories Ltd., Kent, UK)/O₂ (2%/2 liter). In the UTI experiments mice were bled by cardiac puncture and blood was transferred to heparin-gel vacutainer tubes. The abdomen was opened and urine was collected by puncturing the bladder, then bladder and kidneys were harvested. In the peritonitis experiments a peritoneal lavage was performed first, with 5 ml PBS using an 18-gauge needle, then blood was drawn and liver and lungs were harvested.

Determination of bacterial outgrowth
In the UTI experiments the number of *E. faecium* CFU was determined in urine, blood, bladder, and kidney homogenates. In the peritonitis experiments CFU numbers were determined in peritoneal lavage fluid (PLF), blood, liver, and lung homogenates. The organs were homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). Serial dilutions were made and 50 μl of each dilution was plated onto SB plates and incubated at 37°C with 5% CO₂ for 44 h. Colonies were counted and the number of CFU determined after correction for the dilution factor. For cytokine measurements in kidneys and bladders, the homogenates were diluted with an equal volume of lysis buffer (300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 1% Triton X-100, and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, EDTA, Pepstatin A, and Leupeptin, pH 7.4) and incubated for 30 min. Homogenates were centrifuged (1500 x g) at 4°C for 10 min. Supernatants of organ homogenates, PLF and plasma were stored at -20°C until assays were performed.

Cell counts and differentials of PLF
Cells in the PLF samples were counted using a Coulter Counter (Beckman coulter, Fullerton, CA). Differential cell counts for the determination of neutrophils, macrophages and lymphocytes were performed on cytospin preparations, stained with Giemsa (Diff-Quick; Dade Behring, Leusden, The Netherlands).

Histology
Bladders and kidneys from three or four mice per group were fixed in 4% formalin, and embedded in paraffin. Sections of 4 μm were stained with haematoxylin and eosin. Slides were coded and scored by a pathologist without knowledge of the type of treatment. Renal inflammation and damage were scored by the amount of inflammatory cell influx and examining tubular injury, characterized by necrosis, dilatation, edema and purulent cast deposition. Inflammation of bladder was scored by the amount of neutrophil influx and thickness of the epithelium.
Role Esp in *E. faecium* infection

**Assays**
Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, IL-12p70, interferon (IFN)-γ and monocyte chemoattractant protein (MCP)-1 were measured in kidney and bladder homogenates, PLF and plasma by using a commercially available cytometric bead array multiplex assay (BD Biosciences, San Jose, CA) in accordance with the manufacturer's recommendations. Plasma levels of creatinin and urea were determined with commercially available kits (Sigma-Aldrich, St. Louis, MO), using a Hittachi analyzer (Boehringer Mannheim, Mannheim, Germany).

**Statistical analysis**
Adhesion data are expressed as the proportion adherent E1162Δesp relative to adherent E1162 ± the standard deviation (SD). Differences in proportions were tested for significance by Fisher's Exact Test. In vivo data are expressed as mean ± SEM. Differences between groups were analyzed by Mann-Whitney U test using GraphPad Prism version 4.0, GraphPad Software (San Diego, CA). A p-value < 0.05 was considered statistically significant.

**Results**

*In vitro adherence to T24 and MDCK cells*
To determine whether Esp plays a role in the ability of *E. faecium* to adhere to urinary tract epithelial cells, adherence of the Esp expressing *E. faecium* strain E1162 and its isogenic Esp-deficient mutant (E1162Δesp) to T24 (human bladder epithelial cells) and MDCK (canine kidney epithelial cells) cells was investigated. The proportion of E1162Δesp that adhered to T24 cells and to the MDCK cells was significantly lower (25% and 35%, respectively) compared to E1162 (p<0.001) (Fig. 1).

*In vivo model of UTI*
The role of Esp during UTI was examined by infecting mice with either E1162 or E1162Δesp via transurethral inoculation. One and three days after the infection, significantly more E1162 CFU were cultured from kidneys and bladders than E1162Δesp (p<0.05) (Fig. 2A and B). A similar, though not significant, trend (p=0.06 and 0.07 on day 1 and 3, respectively) was seen for urine cultures (Fig. 2C). Furthermore, significantly more mice infected with E1162 had positive blood cultures on day one (7/12) (p<0.05), than mice infected with E1162Δesp (1/12). On day 3 this was 3 out of 10 for mice challenged with E1162 and 1 out
of 10 for E1162Δesp challenged mice. Over time, the number of CFU of both strains in kidneys, bladders and urine declined. Five days after start of the infection, kidneys and bladders were still positive for both E. faecium strains, but with no differences between wild-type and Esp-mutant in the amount of bacteria cultured, while all urine cultures became sterile.

Figure 1. In vitro binding to bladder and kidney epithelial cells. Adhesion of E1162 (black bars) and E1162Δesp (white bars) to T24 (human bladder epithelial cells) and MDCK (canine kidney epithelial cells) cells. Adhesion levels are expressed as the proportion adherent E1162Δesp relative to adherent E1162 ± SD, * p < 0.001 versus E1162.

Cytokine production during UTI
In mice infected with E1162 significantly higher levels of TNF-α, IL-6 and MCP-1 levels were measured in kidneys 1 day after the start of the infection compared to mice infected with E1162Δesp (p<0.05), this difference was still significant for IL-6 at day 3 (p<0.05) (Fig. 3). IL-10 levels in kidneys were comparable in mice challenged with either strain and increased during the experiment, while TNF-α, IL-6, and MCP-1 levels decreased during the course of the infection. Kidney IFN-γ and IL-12p70 and plasma and bladder TNF-α, IL-6, MCP-1, IL-10, IFN-γ, and IL-12p70 were all were all below detection limit (not shown).
Figure 2. Outgrowth of Esp expressing E1162 and the isogenic Esp mutant in kidney and bladder. Mice were transurethrally infected with 10^8 CFU of E1162 (closed circles) or E1162Δesp (open circles). Mice were sacrificed 1, 3 and 5 days after the infection. Bacterial outgrowth is shown for kidney (A), bladder (B) and urine (C). Horizontal bars represent means.
The increased cytokine levels on day 1 were accompanied by higher levels of plasma creatinin and urea in mice infected with E1162 compared to mice infected with E1162Δesp (Fig. 4), indicative of a more disturbed kidney function. Histological examination of kidney tissue revealed no differences between kidneys infected with either E1162 or E1162Δesp (not shown); the pathological changes at all time points in the kidney were variable, ranging from quite extensive in some kidneys, with edema and inflammatory cells lining the entire pelvis, to small, localized spots of inflammation in other kidneys. The cellular infiltrate in the kidneys was primarily neutrophilic, determined by histological features. Tissue of the bladder displayed near to normal histology with minor neutrophil influx at all time points (data not shown), which fits the observation of undetectable cytokine levels in this organ.
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Figure 4. Plasma creatinin and urea levels during UTI. Mice were transurethrally infected with 10⁸ CFU of E1162 (closed bars) or E1162Δesp (open bars). Mice were sacrificed 1, 3 and 5 days after the infection. Creatinin (A) and urea (B) levels were measured in plasma. Data are mean ± SEM, *p < 0.05 versus E1162.

In vivo model of non-lethal peritonitis

To study additional roles for Esp in the pathogenesis of *E. faecium* infections, mice were intraperitoneally infected with either E1162 or E1162Δesp. No differences were found in *E. faecium* outgrowth in PLF (Fig. 5A), blood (Fig. 5B), liver or lung (not shown) between wild-type E1162 and the Esp mutant. Furthermore, a rapid reduction was seen in CFU counts of both strains and none of the mice died. On microscopic examination of PLF preparations comparable numbers of peritoneal neutrophils and macrophages were counted in both groups at both time points (Fig. 5C and D). The strains were found phagocytosed by peritoneal macrophages and neutrophils to a similar extent (not shown). Furthermore, no differences were observed in peritoneal and plasma cytokine levels 2 h post-infection (table I). Twenty-four hours after the infection, cytokine levels were below the level of detection.
Figure 5. E. faecium peritonitis. Mice were intraperitoneally infected with $10^8$ CFU of E1162 (closed circles) or E1162Δesp (open circles) and sacrificed 2 or 24 hours thereafter. Bacterial outgrowth in peritoneal lavage fluid (PLF) (A) and blood (B) and peritoneal neutrophil (C) and macrophage (D) influx are shown. Horizontal bars represent means.

Table I. Concentrations of cytokines in PLF and plasma in mice with E. faecium peritonitis

<table>
<thead>
<tr>
<th>Cytokines (pg/ml)</th>
<th>PLF</th>
<th>Plasma</th>
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<tbody>
<tr>
<td></td>
<td>E1162 (µg/ml)</td>
<td>E1162Δesp (µg/ml)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>132 ± 28</td>
<td>226 ± 20</td>
</tr>
<tr>
<td>IL-6</td>
<td>2733 ± 602</td>
<td>2863 ± 678</td>
</tr>
<tr>
<td>IL-10</td>
<td>223 ± 50</td>
<td>305 ± 60</td>
</tr>
<tr>
<td>MCP-1</td>
<td>1245 ± 375</td>
<td>1159 ± 209</td>
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</tbody>
</table>

Mice were infected intraperitoneally with $10^8$ CFU of E1162 or E1162Δesp and sacrificed 2 h thereafter. Data are means ± SEM.
Discussion

While infections with multi-resistant *E. faecium* are emerging, our knowledge on virulence factors expressed by *E. faecium* is very limited. Recently, some potential virulence genes have been described for *E. faecium*: esp, hyl, acm, genes encoding additional surface-exposed LPXTG proteins and genes required for the biogenesis of pili. They were all found more frequently in clinical isolates than in fecal isolates or non-human isolates, yet the exact role of the proteins expressed by these genes in the pathogenesis of *E. faecium* infections is not known [8, 12-15, 20-26]. In this study we showed for the first time an important role for Esp in the pathogenesis of *E. faecium* infection. In an experimental model of UTI in mice the *E. faecium* isogenic mutant deficient in Esp expression was clearly attenuated. Higher bacterial outgrowth of wild-type *E. faecium* relative to the Esp-deficient mutant in bladder and kidney was accompanied by higher levels of proinflammatory cytokines in kidney tissue and enhanced renal insufficiency displayed by increased plasma levels of creatinin and urea. These results indicate that Esp expressed by *E. faecium* aggravates the course of an UTI, thereby facilitating subsequent bloodstream infection. The finding that the Esp-deficient strain is still able to colonize kidneys and bladders, albeit to a lesser extent than wild-type *E. faecium*, and could still be isolated 5 days after challenge, implies important roles for additional factors as well. In addition, we demonstrated that Esp is involved in *in vitro* binding of bladder and kidney epithelial cells. Together, these data strongly suggest that *E. faecium* Esp is an important virulence factor in experimental UTI.

Esp is a cell wall-associated protein that is characterized by multiple repeat motifs, structurally similar to many bacterial surface protein adhesins involved in binding to host ligands [20, 27-31]. Esp is found in both *E. faecium* and *E. faecalis*, and located on a PAI [8-10, 14, 20]. Shankar et al. [32] demonstrated in a mouse model that Esp contributes to colonization and persistence of *E. faecalis* in the urinary tract. In this study, Esp expression in an *E. faecalis* ascending UTI resulted in more outgrowth in bladder and urine, compared to the isogenic Esp-mutant strain. In contrast to our study, they did not find significantly higher outgrowth in the kidneys, although a trend was observed. This difference in outcome may be explained by the fact that the UTI model reported by Shankar et al. differed from ours with respect to the mouse strain used and volume of bacterial inoculation. A volume of 50 μl, as used by Shankar, will only reach the bladder and from there, bacteria might cause an ascending UTI. In our own unpublished experiments, inoculation of 50 μl of *E. faecium* strain E1162 into the bladder resulted in inconsistent
Regel 1
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Regel 4
Regel 5
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Regel 30
Regel 31
Regel 32
Regel 33
Regel 34
Regel 35
Regel 36

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and only low graded kidney infection. Similar observations were described previously for
*E. faecalis* by Kau et al. [19] and Singh et al. [33]. We therefore used the model described by
Kau et al. [19] and Singh et al. [33] in which 200 μl was introduced transurethrally in order
to reach both the bladder and the kidneys. In hospitalized patients, one can imagine a
continuous bacterial challenge from a biofilm formed on a catheter placed in situ for
several days. However, such a model is difficult to simulate in mice.

Interestingly, Shankar et al. [32] did not find differences between the binding of Esp
expressing *E. faecalis* strains and the isogenic Esp-deficient mutant strains to the
porcine renal tubular cell line LLC-PK1. Our observation that, in contrast to the findings
in *E. faecalis*, the *E. faecium* Esp mutant was significantly impaired in cell line binding
might be explained by the fact that we used different cell lines or by a different role
of Esp in the two *Enterococcus* species. Although Esp of *E. faecalis* and *E. faecium*
share 89% sequence similarity [20, 34], there are some striking differences between *E. faecalis*
and *E. faecium* Esp with respect to structure and function. First, a number of sequence
differences exist within the N- and C- termini and in the arrangement of the repeat
region [20]. Second, in *E. faecium*, Esp is clearly involved in biofilm formation while the
role of Esp in *E. faecalis* biofilm formation is still a matter of debate [16, 35-39]. Third, there
are important differences in the distribution of Esp positive strains: while Esp-positive *E.
faecium* strains are confined to hospitalized patients, Esp-positive *E. faecalis* strains are
more widely distributed and are also found in the animal reservoir [14, 15, 20, 40].

As opportunistic pathogens, *Enterococcus* species are frequently isolated from urine and
may cause chronic UTI as they form biofilms on inanimate surfaces, like catheters [4,
41, 42]. Currently, they rank third among the most common pathogens isolated from
ICU patients [4]. The observation that *E. faecium* fails to elicit a strong induction of
inflammatory cytokines in the bladder and causes only minor pathology is consistent
with findings by Kau et al. [19] for *E. faecalis*-induced UTI. Furthermore, clinical data
published by Wong et al. [42] indicate that the majority of patients with *E. faecium* UTI
have an asymptomatic bacteriuria.

Knowledge on how the innate immune system deals with an *E. faecium* infection is
limited. In previous studies we described the normal immune response during primary
*E. faecium* peritonitis [18]. In a non-lethal murine model of *E. faecium* peritonitis we
found a fast and brisk peritoneal neutrophil influx and a consecutive rapid decline in
peritoneal and systemic enterococcal load [18]. This was accompanied by a modest
peritoneal and systemic cytokine response. To further investigate the pathogenesis of
*E. faecium* infections we tested the role of Esp in this peritonitis model. No differences
were found in the course of the infection between wild-type E1162 and the Esp mutant,
as shown by comparable clearance of the two isogenic species and similarly induced inflammatory responses. In *E. faecalis*, Dupont et al. [43] found that 46% (30/65) of *E. faecalis* strains isolated from peritoneal fluid of patients with peritonitis contained the esp gene. Furthermore, the presence of esp in combination with cytolysin, gelatinase, and aggregation substance was independently associated with mortality in patients with severe peritonitis [43].

Differences in levels of attenuation of the Esp mutant *E. faecium* in a model of UTI and peritonitis suggests a niche specific role of Esp in the pathogenesis of *E. faecium* infections. With the increase in multiresistance of *E. faecium*, development of antibodies directed against Esp might be a valuable addition in the treatment of UTI caused by *E. faecium*.

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References

Role Esp in *E. faecium* infection


