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Does CMV infection impact the virulence of *Enterococcus faecalis*?

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Renal transplant (RTx) recipients are at a high risk of infection caused by commensal bacteria. Apart from immunodeficiency resulting from the use of immunosuppression, RTx patients often suffer from various urological malformations, increasing susceptibility to infections.[1,2] In the early phase after renal transplantation, when patients are exposed to the most intense immunosuppression and enterococcal infections are most common, even life-threatening infections can present with mild or virtually no clinical symptoms.[1,2] Difficulties in managing enterococcal infections result from the fact that this bacteria as a commensal may not be eradicated due to prophylaxis. On the other hand, there is growing evidence that *Enterococcus* spp., which is usually considered harmless commensal, can cause serious infections.[3] It seems crucial to identify patients most susceptible to UTIs, especially recurrent, symptomatic infections.[2]

The majority of the papers focus on epidemiological analysis of risk factors.[4,5] Here we evaluate correlation between kidney dysfunction predictors and expression of ASA gene, one of major enterococcal virulence trait.

Colonization of urinary tract by enterococci is epidemiologically associated with presence of ASA encoded protein and in consequence increases enterococcal adherence.[6] This protein also protects *Enterococcus faecalis* from killing by polymorphonuclear leukocytes, an important urinary tract defense element.[6] ASA is a plasmid-encoded enterococcal surface protein that interacts with enterococcal binding substance (EBS). Enterococci containing an ASA-encoding plasmid express ASA when stimulated by a peptide pheromone secreted from other enterococci.[6] However, ASA has not been directly assessed as an urovirulence factor. In our study, high expression ASA strains were isolated from material (feces and/or urine) from patients with asymptomatic bacteriuria in medical history, not with UTI (**Fig. 1**).

Asymptomatic bacteriuria is common phenomenon, with varying prevalence by age, sex, sexual activity, and the presence of genitourinary abnormalities. However outcomes of asymptomatic bacteriuria may include the short-term complications of symptomatic lower tract infection or pyelonephritis,[7] and longer-term complications, such as urolithiasis or renal failure hypertension.[7] On the other hand, colonization of the genitourinary tract by an avirulent organism could prevent infection with more virulent organisms, through competition or by eliciting a cross-protective host immune or inflammatory response.[8] The apparent lack of a role for ASA in this UTI model was also demonstrated,[6] despite the fact that ASA is epidemiologically associated with UTI and contributes to enterococcal adherence to cultured renal epithelial cells,[6] and protects enterococci from killing by polymorphonuclear leukocytes.[8]

In our study we also found that the high ASA expression is related (among other factors presented on **Fig. 2**) with CMV infection. Detailed comparison shows that the *Enterococcus faecalis* isolates from feces can be characterized by higher (but not statistically significant) expression of ASA gene than from urine, regardless the presence or lack of CMV infection (see **Fig. 3**). In **Figure 3**, we can also see the coincidence of CMV infection and presence of enterococcal isolates with high ASA gene expression. In case of feces isolates forming biofilm, the level of ASA gene expression is significantly lower in the presence of additional CMV infection. The opposite observation can be made in case of planktonic isolates (A). Such differences (as well as any others) are not observed in case of isolates from urine (B). The previous study demonstrated that the mechanisms regulating enterococcal gene expression and mRNA turnover are sensitive to the environment, identified and quantified environment-dependent changes in mRNA abundance from genes related to toxin production, surface adherence, and cell signaling.[9] It can also be clearly seen in case of ASA gene. According to the results obtained in our laboratory (shown in **Fig. 4**), etiology of end-stage renal failure (among other factors shown below) seems to be strongly connected with CMV infection. Human cytomegalovirus (HCMV), a member of the herpes virus family, is an opportunistic pathogen that causes serious health problems in transplant recipients.[10] The cytomegalovirus (CMV) infections may not only predispose to graft rejection,[11] but also can increase the susceptibility of transplant patients for other opportunistic infections.[12] Most of these infections are caused by gram-positive cocci. We

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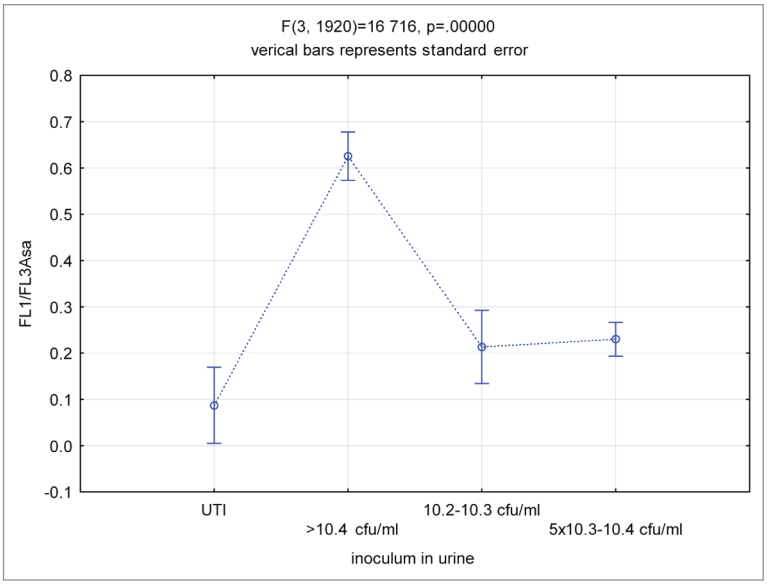

Figure 1. The expression of gene encoding ASA is higher in bacteria isolated from urine with asymptomatic bacteremia (considered as value from 10^2 to above 10^4 CFU/ml urine).

conclude that CMV not only causes substantial morbidity, but also increases the risk of bacterial infections. CMV infection in UTI was maintained on the level of 40.82% and in healthy donors (without UTI) on level of 15% (P = 0.007).[2] What is also important, it is believed that UTI reactivates latent CMV, as pro-inflammatory cytokines trigger CMV replication.[2]

CMV infection is quite common with approximately 40 to 70% of adults being infected in the latent state.[13] CMV commonly infects fibroblasts, endothelial cells, and myeloid cells.[14] It can also reside in monocytes and T lymphocytes.[15] Virus has developed methods for evading host immune system, such as latency and inhibition of apoptosis, and this ability enables reactivation of virus in the immune-suppressed hosts for example undergoing immunosuppressive therapy due to renal transplantation and leading to or increasing the risk of graft rejection.[11,16] In our experiments the high expression of ASA gene is connected with CMV infection in RTx patients. Level of ASA gene expression is higher in feces from CMV^+ patients than from CMV^- people. Carlier et al.[17] shows that the dendritic cells which develop from monocytes harboring latent CMV have an altered phenotype and functional defects which prevent stimulation of antiviral T lymphocytes. These functional defects relate to, e.g., phagocytosis. Dendritic cells which developed from CMV-infected monocytes have strongly decreased ability to perform phagocytosis (up to 50%) which allows CMV to escape host immune system. Adding to it the fact that the aggregation substance encoded by ASA gene in *Enterococcus faecalis* also prevent the phagocytosis,[8,18] we can speculate that this phagocytosis resistance shown by *E. faecalis* strains may be partly

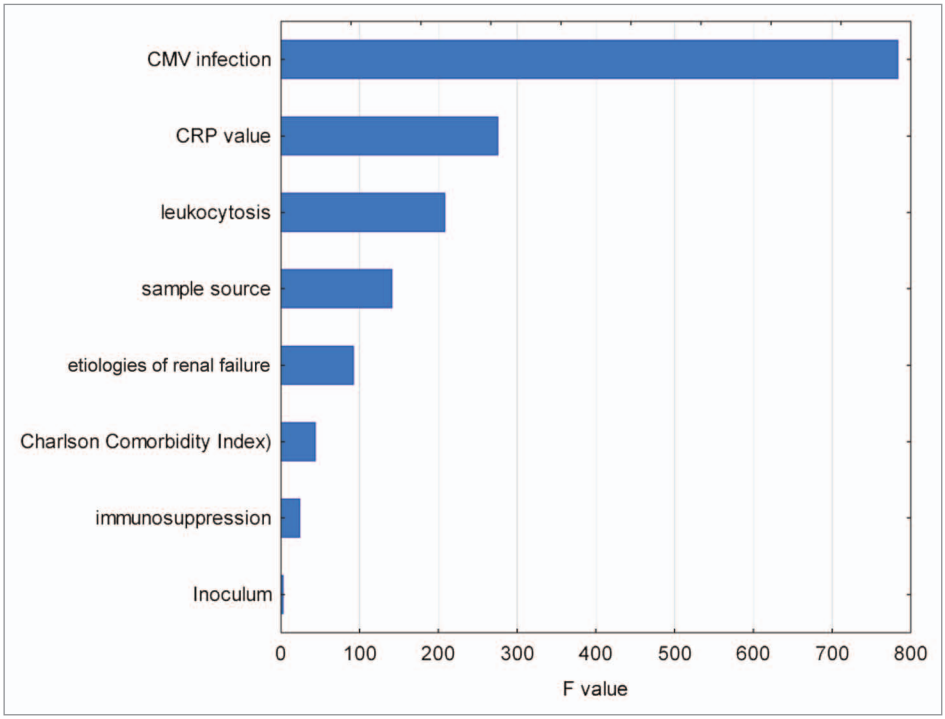

Figure 2. Influence of clinical predictors on presence of enterococcal strains with high ASA expression.

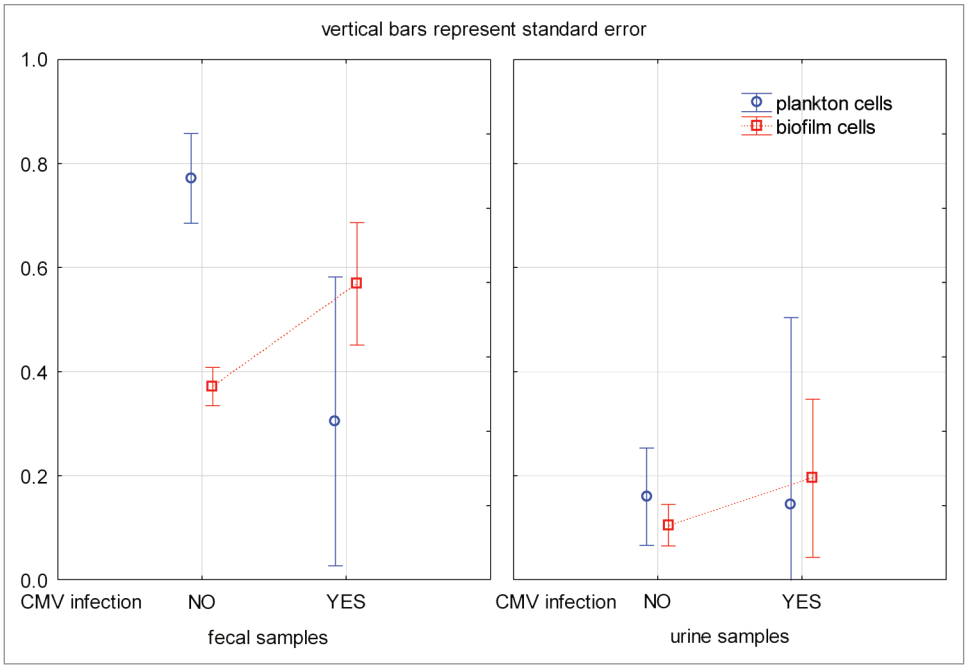

Figure 3. The relation between the presence and absence of CMV infection and the level of ASA gene expression.

dependent on the fact that dendritic cells performing phagocytosis are defective.

Different etiology of end-stage renal failure is related to difference in pre-transplantation treatment and risk of infection. Preliminary results shows the relation between the ASA gene expression and the type of end-stage renal failure which led to the need for renal transplantation. As presented, the level of ASA gene expression is almost the same in bacteria isolated from patients with all diseases leading to end-stage renal failure except ADPKD (autosomal dominant polycystic kidney disease) where that level was about 5-fold higher. ADPKD is a disease with complex etiology. In its course are observed e.g.: high levels of apoptosis and proliferation[19,20] and frequent infections.[21,22] It is also the only disease (among our patients) with genetic undercurrent.[19] ADPKD is the only disease leading to the need for renal transplantation which, in our experiments, is connected with very high ASA gene expression (approximately 5-fold higher than in other groups of patients and healthy volunteers).

Enterococci are an important cause of nosocomial infections. According to Creti et al.,[23] the aggregation substance encoded by ASA gene is mostly connected with non-invasive infections. It is also present almost always in strains derived from healthy individuals. The higher level of ASA gene expression in strains isolated from samples of healthy individuals than from samples of patients with UTI, may be connected with the dependence observed e.g., by Creti et al.[23] that the commensal strains, in contrast to the strains isolated from invasive and non-invasive infections, have always genes encoding aggregation substances. It may also be related with that the ASA protein contributes to the adhesion of the bacteria to the cells[24] and the protection of enterococci from killing by polymorphonuclear leukocytes.[8,18] It may also be associated with the formation of biofilm.[25] As found in our study, the expression of ASA gene is higher in biofilm formed by strains from healthy individuals, rather than planktonic cultures. The expression of the gene is lower, when strains are isolated from urine of people with end stage renal failure regardless the form of culture. Various authors are reporting that the enterococci participating in clinical infections express more of the virulence factors than enterococci in chronic, persistent cases[26] and healthy individuals,[23] it may suggest that the metabolic cost of expressing more genes (e.g., virulence genes and responsible for antibiotic resistance) in the same moment may cause the lower level of expression of each virulence factor overall.

To conclude, in our study we confirm role of ASA gene in colonization of urinary tract rather than in UTI. We also found out that ADPKD is related with presence of high ASA gene expressing enterococcal strain and in result, risk of enterococcal infection is higher than in other etiology of end-stage renal failure. There is also coincidence between CMV infection and presence of high ASA gene expressing enterococcal strains but additional studies are needed to develop procedure of estimation of infection risk.

Fifty-two RTx patients hospitalized at the Medical University of Gdansk was screened for the presence of enterococci in the urine and feces. Forty-four enterococcal strains were isolated from urine and feces of 19 RTx recipients with no current antibiotic therapy. We compared demographic features and clinical data of patients considering the etiology of end-stage renal disease, age, comorbidity (estimated with the use of Charlson Comorbidity index, CCI), type of immunosuppression (cyclosporine [CsA], tacrolimus [tac], everolimus, mycophenolate mofetil [MMF]/sodium [MPS]), and CMV infections. Detailed characterization of patients is presented in **Table 1.**

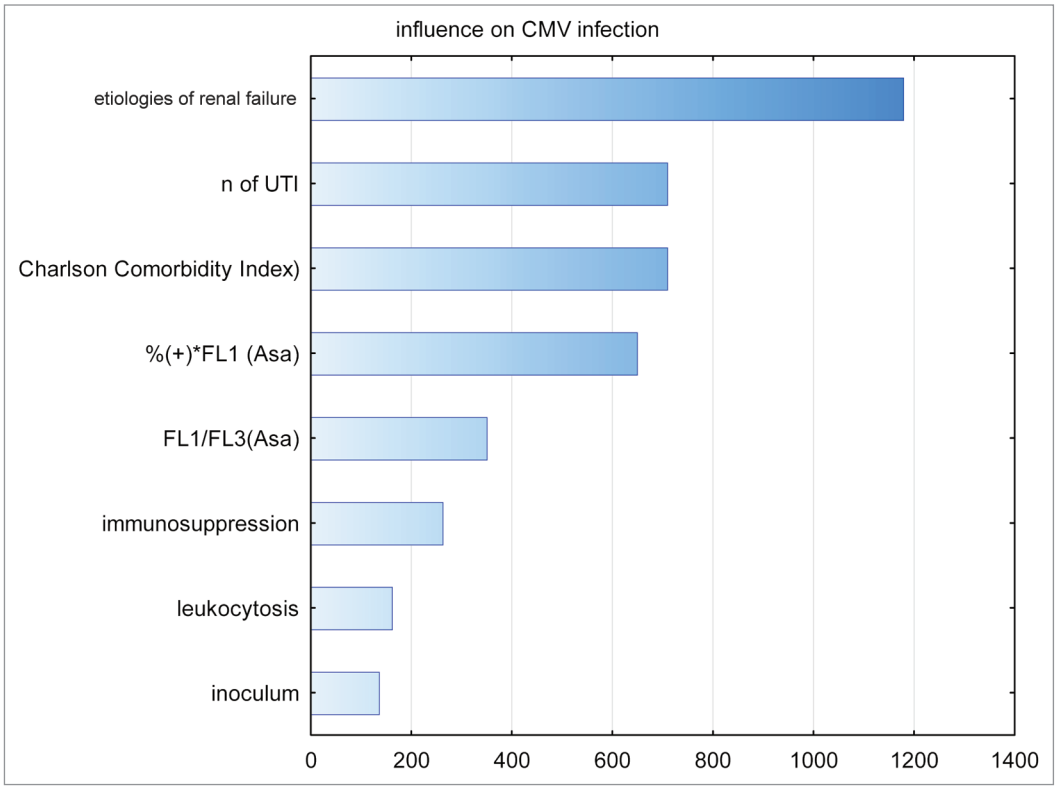

Figure 4. The effect of CMV infection on various parameters listed below.

Table 1. Characteristic of RTx patients

No. of patients	Years after transplantation	Catheter	No. of UTI	CMV infection (Y/N)	Comorbidity	Cause of renal failure
19	1.08 ± 1.03	0	0 – 7 (0: 12 p., 1: 3 p., 2: 0 p., 3: 0 p., 4: 1 p., 5: 1 p., 6: 1 p., 7: 1 p.)	4/15	4.42 ± 1.74	KZN: 6 patients ADPKD: 3 patients NN: 4 patients T: 2 patients ZN: 1 patient

p., patient; KZN, disease with unknown nephropathy; ADPKD, autosomal dominant polycystic kidney disease; NN, diabetic and hypertensive nephropathy; T, tubulointerstistial nephritis ; ZN, lupus nephritis. Mean ± SD.

The bacterial isolates were identified to species level by strep ID test (BioMerieux) and classified as different strains of *Enterococcus faecalis* by biochemical and resistance profiles. Biofilms of these strains were formed in flat-bottom wells (TRP, Switzerland). As reference group, seven commensal strains isolated from healthy volunteers and reference strains ATTC 51299 and ATTC 29212 were used in this study.

To evaluate ASA gene expression by the Flow-FISH method we used a linear locked nucleic acid (LNA) probe, AGCGATAAAC TAGACGTCAA ACATGACA-5' FITC containing nucleic acid analogs with higher affinity for DNA and RNA.[27] As a positive control, Enfl84 probe (3'-ACGTGAGTTA ACCTTTCTCC)[28] targeting 16srRNA gene was used. Oligonucleotides were synthesized commercially (Metabion), labeled with fluorescein isothiocyanate (FITC) and tested for specificity against the set of reference organisms listed above. For hybridization, the procedure described by Waar et al.[28] was adopted and modified.[7] Briefly, cell membranes were permeabilized by incubation for 30 min at 37 °C in permeabilization buffer (Tris-EDTA) consisting of 1 mg/ml lysozyme (DNA Gdansk). Then, the cells were suspended in 1 mL of 0.9% NaCl and sonicated for 2 min on ice. To ensure permeabilization of the cells, we used propidium iodide (PI, 1 μg/ml) staining of DNA. Particles without PI fluorescence (FL3) were excluded from further investigation. Fluorescence of particles was determined using a FACScan flow cytometer (Becton-Dickinson). The mean probe fluorescence (FL1) normalized by DNA fluorescence (FL3) and the median fluorescence (MFL1) weighted by percentage of probe-binding particles (FL1 positives) were analyzed. Results were tested by analysis of variance (ANOVA) by StatSoft software (Statistica 10).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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