MAJOR ARTICLE

Association between the Presence of Enterococcal Virulence Factors Gelatinase, Hemolysin, and Enterococcal Surface Protein and Mortality among Patients with Bacteremia Due to *Enterococcus faecalis*

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The potential virulence factors of enterococci include production of enterococcal surface protein (Esp), gelatinase, and hemolysin. Gelatinase- and hemolysin-producing strains of *Enterococcus faecalis* have been shown to be virulent in animal models of enterococcal infections. Esp production has been shown to enhance the persistence of *E. faecalis* in the urinary bladder. We determined the presence of the *esp* gene and production of gelatinase and hemolysin in 219 *E. faecalis* isolates from a larger prospective study of 398 patients with enterococcal bacteremia. Thirty-two percent of isolates carried the *esp* gene, 64% produced gelatinase, and 11% produced hemolysin. There was no significant association between 14-day mortality and any of the markers studied, singly or in combination.

Enterococci are increasingly important causes of nosocomial infection. They are intrinsically resistant to or tolerant of many antibiotics and are readily able to acquire resistance to antibiotics, either by mutation or by acquisition of plasmids or transposons containing genetic sequences that confer resistance in other bacteria [1]. Other virulence factors include the phenotypic markers gelatinase, hemolysin, and aggregation substance protein production [2–4]. Although these factors have been associated with the virulence of *Enterococcus faecalis* in animal models [2, 5–7], it is not clear that the presence of these factors in *E. faecalis* isolates from persons with bacteremia is associated with a poorer outcome.

Hemolysin is a cytolytic protein capable of lysing human, horse, and rabbit erythrocytes. Hemolysin-producing strains of *E. faecalis* have been shown to be virulent in animal models and human infections [6–8] and to be associated with increased severity of infection [3]. Gelatinase is a protease produced by *E. faecalis* that is capable of hydrolyzing gelatin, collagen, casein, hemoglobin, and other peptides [9]. Gelatinase-producing strains of *E. faecalis* have been shown to contribute to the virulence of endocarditis in an animal model [10]. Enterococcal surface protein (Esp)

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is a cell wall–associated protein in *E. faecalis* isolates. Interestingly, the frequency of the gene coding for Esp has been found to be significantly higher among clinical isolates recovered from infected patients than among other isolates [11]. More recently, Esp has been shown to enhance the persistence of *E. faecalis* in the urinary bladder during experimental urinary tract infection [12]. Antibiotic resistance, specifically high-level gentamicin resistance, has been shown to have a significant association with hemolysin-producing strains of *E. faecalis* and with a subsequent increased risk of mortality [13]. To test the hypothesis that these virulence factors are associated with a poor outcome, we evaluated enterococcal isolates from patients with enterococcal bacteremia for the presence of hemolysin, gelatinase, and Esp.

PATIENTS, MATERIALS, AND METHODS

Blood isolates of *E. faecalis* recovered from 219 of 231 consecutive patients were available. These patients were participants in a larger multicenter and prospective study of enterococcal bacteremia [14]. These isolates, which had been stored at −70°C, were grown and maintained on trypticase-soy blood agar for subsequent testing. The study was approved by the institutional review boards at 4 of 5 facilities at which patient data were collected and was considered exempt from review at the other facility.

Patients were classified as having clinically significant bacteremia if enterococci were isolated by culture from ⩾2 separately obtained blood samples or isolated by culture from a single blood sample and from a sample from a concomitant site of infection in the presence of a clinical scenario compatible with bacteremic infection [15]. Hemolysin production was detected by inoculating enterococci onto freshly prepared beef heart infusion agar supplemented with 5% horse blood. Plates were incubated overnight at 37°C in a CO_2 chamber and evaluated at 24 and 48 h. A clear zone of β-hemolysis around the stab or streak on horse blood agar was considered to be a positive indication of hemolysin production.

Gelatinase production was detected by inoculating the enterococci onto freshly prepared peptone-yeast extract agar containing gelatin (30 g/L; Difco). Plates were incubated overnight at 37°C and then cooled to ambient temperature for 2 h. The appearance of a turbid halo or zone around the colonies was considered to be a positive indication of gelatinase production.

Enterococcal DNA was purified as described in detail elsewhere [11]. PCR amplification of the *esp* gene was done by use of primers Esp 11 (5′-TTGCTAATGCTAGTCCACGACC-3′) and Esp 12 (5′-GCGTCAACACTTGCATTGCCGAA-3′), which correspond to nucleotide positions 1217–1238 and 2149–2171, respectively, within the N-terminal region of Esp. The PCR reaction mixture consisted of 250 ng of DNA; 0.2 μL each of dATP (2′-deoxyadenosine 5′-triphosphate), dCTP (2′-deoxycytosine 5′-triphosphate), dGTP (2-′deoxyguanosine 5′-triphosphate), and dTTP (2′-deoxythymidine 5′-triphosphate); 2.5 m*M* $MgCl_2$; and 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer) in 1× reaction buffer. The samples underwent initial denaturation at 95°C for 2 min and then were subjected to 30 cycles of denaturation (94°C for 45 s), annealing (63°C for 45 s), and extension (72°C for 1 min). Five microliters of the amplification mixture was mixed with gel loading buffer and subjected to electrophoresis in a 1% agarose gel. The reaction products were visualized by ethidium bromide staining. DNA from *E. faecalis* isolates MMH594 (which carried the *esp* gene) and FA2-2 (which did not carry the *esp* gene) was used with each set of PCR amplifications as positive and negative controls, respectively.

Testing for susceptibility to ampicillin, vancomycin, and high-level gentamicin resistance was done with use of Etest strips (AB Biodisk North America). MIC break points recommended by the National Committee for Clinical Laboratory Standards [16] were used.

Dichotomous variables were compared by use of χ^2 or Fisher's exact test. Continuous variables were analyzed with the Mann-Whitney rank sum test. Multivariate analysis was done by logistic regression. Significance was defined as $P < .05$. Variables with a 2-tailed P value of ⩽.05 were included in stepwise logistic regression models for calculation of 14-day mortality. Presence of each virulence factor under study was entered stepwise into the logistic regression model individually, and factors were entered in combination. Statistical analysis of the data was

Table 1. Demographic and clinical characteristics of 218 patients with bacteremia due to *Enterococcus faecalis*.

Characteristic	Value
Age, median years (range)	65 (17–98)
Sex, no. male/no. female	134/85
APACHE II score, median	16
Receipt of mechanical ventilation	74 (34)
Comorbid disease or condition	
Malignancy	70 (32)
Diabetes	55 (25)
Cirrhosis	16 (7)
Renal disease	67 (31)
COPD	55 (25)
Alcoholism	34 (16)
Immunosuppression	
Due to transplantation	14 (6)
Due to HIV infection	10 (5)
Due to glucocorticoid therapy	42 (19)

NOTE. Data are no. (%) of patients, unless indicated otherwise. For 1 patient, data were not available. APACHE II, Acute Physiology and Chronic Health Evaluation II; COPD, chronic obstructive pulmonary disease.

Table 2. Univariate analysis of factors associated with 14-day mortality among patients with bacteremia due to *Enterococcus faecalis*.

Factor	Patients who survived (n = 190)	Patients who died (n = 28)	P
Patient characteristic			
Age, mean years	64.5	68.5	NS
Male sex	118 (62)	11 (39)	NS
Temperature, mean °C	38.6	37.9	.06
APACHE II score, mean	16	20	.013
ICU admission	59 (31)	14 (50)	.05
Comorbid disease or condition			
Solid malignancy	47 (25)	10 (36)	.19
Hematologic malignancy	11 (6)	2 (7)	NS
Solid organ transplant	14 (7)	1 (4)	NS
Bone marrow transplant	6 (3)	0	NS
Diabetes mellitus	50 (26)	5 (18)	NS
Cirrhosis	13 (7)	3 (11)	NS
Renal disease	64 (34)	3 (11)	.02
COPD	45 (24)	10 (36)	.14
Ethanol use	29 (15)	5 (18)	NS
Injection drug use	8 (4)	2 (7)	NS
Steroid use	39 (21)	3 (11)	NS
HIV infection	9 (5)	1 (4)	NS
Coronary heart disease	59 (31)	8 (29)	NS
Valvular heart disease	16 (8)	2 (7)	NS
Prosthetic valve	4 (2)	1 (4)	NS
Neurological disease	76 (40)	9 (32)	NS
Pressure ulcers	22 (12)	6 (21)	NS
Presence of a catheter			
Arterial	26 (14)	5 (18)	NS
Central venous	43 (23)	11 (39)	.06
Pulmonary arterial	18 (9)	6 (21)	.10
Indwelling bladder	109 (57)	21 (75)	.04
Receipt of mechanical ventilation	58 (31)	16 (57)	.005
Presence of a gastric tube	73 (38)	16 (64)	.07
Prior antibiotic therapy	117 (62)	18 (64)	NS
Isolate characteristics			
Vancomycin resistant	14 (7)	5 (18)	.07
Hemolysin production[a]	21 (11)	2 (7)	NS
Gelatinase production	124 (65)	17 (61)	NS
Presence of *esp* gene	60 (32)	11 (39)	NS

NOTE. Data are no. (%) of patients, unless indicated otherwise. For 1 patient, data were not available. APACHE II, Acute Physiology and Chronic Health Evaluation II; COPD, chronic obstructive pulmonary disease; *esp*, gene encoding enterococcal surface protein; ICU, intensive care unit; NS, not significant.

[a] For testing this factor, n = 211.

done with Prophet System (AbTech) and Epistat software (Epistat Services).

RESULTS

Patient characteristics are shown in table 1.

Production of hemolysin, gelatinase, and Esp. One hundred forty-one (64%) of 219 isolates were gelatinase producing, and 71 (32%) of 219 carried the *esp* gene. Eight isolates tested showed equivocal results for production of hemolysin despite incubation of the plates for >24 h. These 8 isolates were excluded from further analysis of hemolysin production. Of the *E. faecalis* isolates for which a determination of hemolysin production could be made, 23 (11%) of 211 were hemolysin producing.

Production of combinations of hemolysin, gelatinase, and Esp. Thirty-three (23%) of 141 gelatinase-producing isolates carried the *esp* gene; in comparison, 38 (49%) of 78 isolates that did not produce gelatinase carried the gene ($P = .0002$). The 8 isolates for which testing for hemolysin production was equivocal were excluded from further analysis, leaving a total of 211. Eight (6%) of 133 gelatinase-producing isolates also produced hemolysin, compared with 15 (19%) of 78 isolates that did not produce gelatinase ($P = .01$). Five (7%) of 69 isolates that carried the *esp* gene were hemolysin producing, compared with 18 (13%) of 142 isolates that did not carry the *esp* gene ($P = .5$).

Relationship between virulence factors and 14-day mortality. Fourteen-day mortality was not associated with production of hemolysin or gelatinase or presence of the *esp* gene (table 2). The overall 14-day mortality rate was 12% (17 of 140 patients) among patients with isolates that had any 1 of the factors present, 15% (6 of 41) among patients with isolates that had any 2 factors present, and 0% for the 1 patient whose isolate had all 3 factors present ($P > .05$); the rate was 17% (5 of 29 patients) among patients whose isolates had none of the factors. Isolates with equivocal test results for hemolysin production were excluded from this analysis. These differences were not significantly different. Factors independently associated with 14-day mortality were identified by multivariate analysis for each virulence factor individually and in combination (table 3). Severity of illness, as indicated by the Acute Physiology and Chronic Health Evaluation II (APACHE II) score, remained the independent risk factor significantly associated with 14-day mortality (for the model that included only hemolysin production: OR, 0.9; 95% CI, 0.88–0.98; $P = .005$; for the model that included only gelatinase production: OR, 1.1; 95% CI, 1.02–1.14; $P = .005$; for the model that included only presence of the *esp* gene, OR, 0.92; 95% CI, 0.86–0.98; $P = .004$; and for the model that included all 3 virulence factors: OR, 0.92; 95% CI, 0.87–0.97; $P = .002$).

Relationship between virulence factors and antibiotic susceptibility. Among the *E. faecalis* isolates tested, 6 (3%) of 219 were resistant to ampicillin, 82 (37%) of 219 had high-level resistance to gentamicin, and 19 (9%) of 219 were resistant to vancomycin. Ampicillin resistance was noted in 2 (1%) of 141 gelatinase-producing isolates, in 1 (4%) of 23 hemolysin-producing isolates, and in 2 (3%) of 71 isolates that carried the *esp* gene. High-level gentamicin resistance was noted in 62 (44%) of 141 gelatinase-producing isolates, in 7 (30%) of 23 hemolysin-producing isolates, and in 22 (31%) of 71 isolates that carried the *esp* gene. Vancomycin resistance was noted in 14 (10%) of 141 gelatinase-producing isolates, in 2 (9%) of 23 hemolysin-producing isolates, and in 4 (6%) of 71 isolates that carried the *esp* gene (table 4).

DISCUSSION

Enterococci are an important cause of nosocomial infections. We determined the prevalence of 3 virulence factors—gelatinase production, hemolysin production, and presence of the *esp* gene—among bacteremic *E. faecalis* isolates collected during a large, prospective observational study of enterococcal bacteremia. Presence of the factors singly or in combination was not associated with a poor outcome. Our outcome of interest, mortality at 14 days, is objective and not subject to interpretation of the contribution of infection to outcome. This approach has been previously validated in several studies of the outcomes of bacteremia due to diverse bacterial genera [17–20], including *Enterococcus* [14].

We conclude that the putative virulence factors of *E. faecalis* are not associated with an increased 14-day mortality rate among bacteremic persons. Although other factors or properties of *E. faecalis* may be important to its ability to cause human disease, it is not immediately apparent from our study which factors are associated with disease severity. This is in contrast with animal studies that demonstrate a better correlation between presence of virulence factors and experimental disease [21–24]. Our study did not examine the relationship

Table 3. Multivariate analysis of factors associated with 14-day mortality among patients with bacteremia due to *Enterococcus faecalis*.

Factor	OR (95% CI)	*P*
APACHE II score[a]	1.1 (1.02–1.14)	.01
Presence of *esp* gene	1.9 (0.8–5.0)	.16
Gelatinase production	1.1 (0.4–3.0)	.79
Hemolysin production	0.8 (0.2–4.1)	.83

NOTE. APACHE II, Acute Physiology and Chronic Health Evaluation II; *esp,* gene encoding enterococcal surface protein.

[a] The odds ratio for APACHE II score is per unit change in score.

Table 4. Comparisons of antibiotic susceptibility and virulence factors among isolates of *Enterococcus faecalis* obtained from patients with bacteremia.

Antibiotic, virulence factor	Percentage of isolates (no. producing virulence factor/no. tested), by class of resistance		
	Resistant	Susceptible	*P*
Ampicillin			
Gelatinase production	1 (2/141)	99 (139/141)	.19
Hemolysin production	4 (1/23)	96 (22/23)	.51
Presence of *esp* gene	3 (2/71)	97 (69/71)	.72
Gentamicin			
Gelatinase production	44 (62/141)[a]	56 (79/141)	.008
Hemolysin production	30 (7/23)[a]	70 (16/23)	.65
Presence of *esp* gene	31 (22/71)[a]	69 (49/71)	.37
Vancomycin			
Gelatinase production	10 (14/141)	90 (127/141)	.31
Hemolysin production	9 (2/23)	91 (21/23)	.69
Presence of *esp* gene	6 (4/71)	94 (67/71)	.32

NOTE. *esp,* Gene encoding enterococcal surface protein.

[a] High-level gentamicin resistance, as defined in [16].

of virulence factors to the occurrence of disease but, rather, the relationship of the factors to outcome after the occurrence of invasive infection.

Clinical studies differ from animal studies in that, in patients with bacteremia, there are typically a number of uncontrolled variables that may affect outcome. These include presence of immunosuppression, severity of illness, and presence of comorbid conditions. We collected extensive clinical data about the patients, including the 29 clinical variables listed in table 2, and found that only severity of illness, as measured by APACHE II score, was independently associated with outcome. Thus, among patients with *E. faecalis* bacteremia, physiological severity of illness has a greater impact on outcome than any of the bacterial virulence factors studied.

All of the isolates we studied were clinically virulent, because all were associated with bacteremic illness. It is highly likely that other virulence factors are important in the occurrence of invasive disease and in clinical outcome. Recently, an enterococcal adhesin, Ace, which mediates binding to extracellular matrix proteins, has been identified in diverse isolates of *E. faecalis* [25–27]. Ace shows structural similarity to the *Staphylococcus aureus* collagen-binding protein Cna and may play a role in the pathogenesis of enterococcal endocarditis [27].

Given the growing importance of *Enterococcus* species as nosocomial pathogens and the increasing prevalence of glycopeptide resistance among enterococci, the identification of virulence factors associated with enterococcal invasiveness and disease severity will be an important subject of future investigations. Development of agents that can block enterococcal adherence or inhibit the action of other virulence factors may provide therapeutic alternatives in the face of antimicrobial resistance.

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