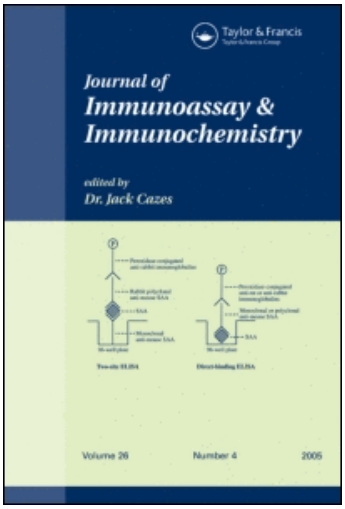

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Yun Sang Cho[a]; Hee Soo Lee[a]; Jong Man Kim[a]; Mun Han Lee[b]; Han Sang Yoo[c]; Yong Ho Park[d]; Pan Dong Ryu[d]

[a] Bacteriology and Parasitology Division, Animal Disease Research Department, National Veterinary Research and Quarantine Service, Mannan-gu, Anyang, Gyeonggi-do, Republic of Korea [b] Laboratory of Veterinary Biochemistry, College of Veterinary Medicine, Seoul National University, Seoul, Republic of Korea [c] Laboratory of Veterinary Infectious Diseases, College of Veterinary Medicine, Seoul National University, Seoul, Republic of Korea [d] Laboratory of Veterinary Pharmacology, College of Veterinary Medicine, Seoul National University, Seoul, Republic of Korea

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Immunogenic Proteins in the Cell Envelope and Cytoplasm of Vancomycin-Resistant Enterococci

Yun Sang Cho,[1] Hee Soo Lee,[1] Jong Man Kim,[1] Mun Han Lee,[2] Han Sang Yoo,[3] Yong Ho Park,[5] and Pan Dong Ryu[5]

[1]Bacteriology and Parasitology Division, Animal Disease Research Department, National Veterinary Research and Quarantine Service, Mannan-gu, Anyang, Gyeonggi-do, Republic of Korea

[2]Laboratory of Veterinary Biochemistry, College of Veterinary Medicine, Seoul National University, Seoul, Republic of Korea

[3]Laboratory of Veterinary Infectious Diseases, College of Veterinary Medicine, Seoul National University, Seoul, Republic of Korea

[4]Laboratory of Veterinary Microbiology, College of Veterinary Medicine, Seoul National University, Seoul, Republic of Korea

[5]Laboratory of Veterinary Pharmacology, College of Veterinary Medicine, Seoul National University, Seoul, Republic of Korea

Abstract: Because of the continuous advent of new modes of antimicrobial resistance, it has become difficult to control vancomycin-resistant enterococci (VRE) with standard antibiotics. Therefore, in the interest of public health, early diagnostic methods and a greater knowledge of the pathogenic process are urgently needed to prevent the spread of VRE in humans and animals. To this end, we sought immunogenic proteins suitable for the serological diagnosis of VanA-, VanB-, VanC1-, and VanC2-type VRE. Proteins were extracted from cell envelope (CE) and cytoplasm (CP), and anti-VRE guinea pig serum was used to identify immunogenic proteins. Two immunogenic proteins of 129 and 29 kDa were identified in the CE and CP of VanA, respectively, while a 28-kDa protein was identified in the CP of VanB. Additionally, the CE of VanC1 contained two immunogenic proteins of 30 and 46 kDa, while the CP of VanC1 contained two

Address correspondence to Yun Sang Cho, Bacteriology and Parasitology Division, Animal Disease Research Department, National Veterinary Research and Quarantine Service, Mannan-gu, Anyang 480, Gyeonggi-do, Republic of Korea. E-mail: choys@nvrqs.go.kr

proteins of 19 and 30 kDa. The CE of VanC2 possessed one immunogenic protein of 40 kDa. These proteins, which were specific to individual subtypes of VRE, will likely prove useful in the serological diagnosis of enterococcal infections and in the study of enterococcal pathogenesis.

Keywords: Cell envelope, Cytoplasm, Immunogenic proteins, Vancomycin-resistant enterococci, VanA, VanB

INTRODUCTION

Resistance of nosocomial enterococci to antibiotics, such as streptomycin, gentamicin, penicillin, and vancomycin, is on the rise,[1,23] and effective treatments of enterococcal disease have been limited by the emergence of multidrug resistance.[33] Early diagnosis followed by immunotherapy may be an effective means of controlling enterococcal infection[16] and identifying immunogenic proteins in enterococci will be of crucial importance in the development of serological diagnostic tests and treatments.[1,36] While the microbiological and genetic features of antimicrobial resistance in enterococci have been studied intensively, investigations of enterococcal pathogenesis and serological diagnosis have been limited.[2,36] Two possible targets for serological diagnosis are pathogenic factors and protein antigens found in the cell membrane. For example, some pathogenic factors, such as proteases, hyaluronidase,[27,34] pheromones,[29] AS-48,[13] lipoteichoic acid,[35] cytolysin,[17] and aggregation substance,[8,20,31] may be common to all enterococci; however, not all of them are necessarily immunogenic.

Bacterial cell membranes play a critical role in adhesion, colonization, adaptation to changes in environment, and resistance against host immune systems. Significantly, Huebner et al.[15] found that enterococcal surface antigens are targeted by opsonin and immunogens in rabbits, and that anti-enterococcal rabbit serum had a protective effect in mice infected with enterococci.[16]

To determine how best to treat a vancomycin-resistant enterococci (VRE) infection, it is important to be able to identify the subtype with some specificity. Most VRE are classified VanA-, VanB-, or VanC-type, according to their resistance to vancomycin (Va) and teicoplanin (Tp).[10] VanA-type enterococci are frequently *Enterococcus faecalis* or *E. faecium* and are resistant to high concentrations of Va (64–1,000 μg/mL minimum inhibitory concentration [MIC]) and Tp (16–512 μg/mL MIC). VanB-type enterococci are resistant to varying levels of Va (4–1,000 μg/mL MIC), and to low levels of Tp (< 2 μg/mL MIC), while VanC-type enterococci are resistance to low levels only of both Va (2–32 μg/mL MIC) and Tp (< 2 μg/mL MIC). There are three

subtypes of VanC-type enterococci: VanC1, VanC2, and VanC3, found in *E. gallinarum*, *E. casseliflavus*, and *E. flavescens*, respectively.

Specific, sensitive diagnosis of VRE is essential when estimating VRE prevalence and determining if vancomycin resistance is transferable from animals to humans, a major public health concern. Although VRE may be diagnosed by isolating and biochemically identifying enterococci, estimating MIC, and/or detecting specific genes using multiplex PCR,[6,10,18] these methods can be time-consuming, labor intensive, and exacting,[19] When screening for infectious disease, enzyme-linked immunosorbent assays (ELISA) have several advantages, such as speed, high throughput, and ease of use.[19] Additionally, detection by immunochromatography[21] can be used to diagnose the disease even more rapidly and conveniently[3,19,30] than conventional serological methods. To identify targets for the detection of VRE, we examined the immunogenic proteins in VRE by Western blot analysis using guinea pig antiserum.

EXPERIMENTAL

Bacterial Strains and Growth Conditions

Enterococcus faecium B7641, *E. faecalis* V583, *E. gallinarum* GS, and *E. casseliflavus* ATCC 25788 were used as representatives of VanA-type *E. faecium* (VanA-VREFM), VanB-type *E. faecalis* (VanB-VREFS), and VanC1- and VanC2-type enterococci,[14,26,28,32] respectively. To compare protein profiles and immunogenic proteins, vancomycin- susceptible *E. faecium* dog isolates (VSEFM) and *E. faecalis* cattle isolates (VSEFS) were used, along with *E. casseliflavus* cattle isolates. All of the strains were isolated in the Republic of Korea and grown in Brain Heart Infusion (BHI) broth (Difco, Detroit, Mich.) for 18 h at 37°C.

Guinea Pig Antiserum

Antiserum against enterococci was prepared by subcutaneous injections of guinea pigs (Hartley), as described previously.[22] Each enterococcus was grown in BHI broth for 18 h at 37°C, and the cultures were inactivated with 0.5% (v/v) formalin. Inactivated whole cells were centrifuged at 10,000 × g for 10 min and resuspended in sterilized saline. Protein concentrations of inactivated whole cells (IWC) were measured using the BCA protein assay kit (Pierce, Rockford, Ill). After IWC with complete Freund's adjuvant (Sigma) was adjusted to optimal concentrations, it was injected subcutaneously into guinea pigs. An injection of IWC with incomplete Freund's adjuvant (Sigma) was given 3 wk after the first injection. If the antibody titer of immunized guinea pigs by ELISA surpassed

1:512, the immunized guinea pigs were bled terminally. Control sera were obtained from noninoculated guinea pigs, bred under the same conditions as the immunized ones. All serum was inactivated at 56°C for 30 min and stored at −20°C before being used for immunoblotting.

Cell Envelope and Cytoplasm Extraction

Cell envelope (CE) and cytoplasm (CP) proteins were extracted from enterococci by sonication and ultracentrifugation as described in Ref. [5]. Briefly, bacterial cultures in BHI broth were centrifuged at 10,000 × g for 10 min at 4°C and suspended in cold 25 mM Tris-HCl with 1 mM EDTA-Na_2 (pH 7.4). The bacterial suspensions were ultrasonicated and then centrifuged at 5,000 × g for 30 min at 4°C. The supernatants were collected and ultracentrifuged at 45,000 × g for 1 h at 4°C. The resulting clear pellets contained the CE and, following another centrifugation, the supernatants were used as CP.

SDS-PAGE and Western Blot

SDS-PAGE and Western blot were performed as described in Refs. [4,24]. Prepared antigens mixed with sample buffer were loaded onto an SDS-PAGE gel. The gel was electrophoresed at 100 V, 80 mA for 1 h 20 min and stained with Coomassie blue. The antigens were electrotransblotted from the gel to a nitrocellulose (NC) membrane (Sigma) at 100 V, 400 mA for 1 h and blocked for 30 min at 37°C in 1% skim milk (Sigma) in PBS (pH 7.4). The NC membranes were reacted for 1 h at 37°C with diluted guinea pig serum (1:2,000) in PBS (pH 7.4) containing 1% skim milk followed by anti-guinea pig IgG horseradish peroxidase conjugate (Sigma) diluted to 1:2,000 in PBS (pH 7.4) with 1% skim milk at 37°C for 1 h. They were then reacted with 3,3′-diaminobenzidine (DAB) (Sigma) susbstrate solution for 5 min. The proteins on SDS-PAGE and Western blot were analyzed using Bio1D ver 99.03 (Vilber Lourmat, France).

RESULTS AND DISCUSSION

The CE proteins of VRE, VSEFM, and VSEFS were analyzed by SDS-PAGE. The most abundant VanA-VREFM CE proteins were 8, 14, 16, 19, 21, 24, 27, 28, 34, 40, 44, 51, 54, 60, 65, 79, 92, 107, 116, 129, 137, 148 and 158 kDa (Fig. 1, lane 2). Those of the VSEFM CE were 7, 24, 27, 40, 44, 54, 60, 70, 80, 112 and 155 kDa in size (Fig. 1, lane 7). VanB-VREFS CE proteins were 14, 17, 22, 25, 28, 35, 47, 49, 54, 57,

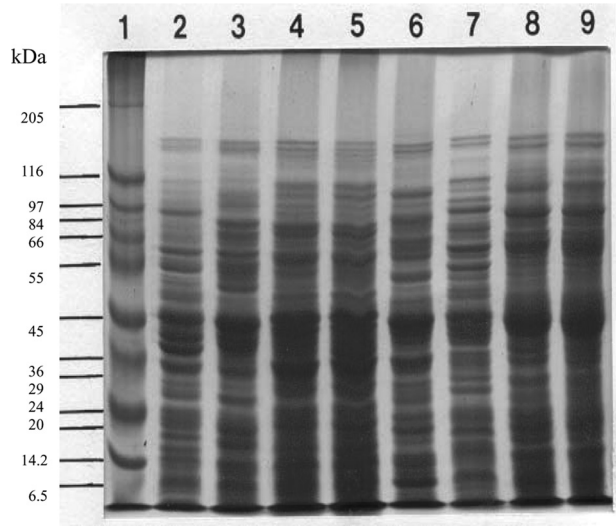

Figure 1. Electrophoretic analysis of the CE of VRE, VSEFM, and VSEFS by 10% SDS-polyacrylamide gel electrophoresis. Lanes: 1, Molecular weight marker (Sigma wide marker); 2, *E. faecium* B7641 (VanA-VREFM); 3, *E. casseliflavus* cattle isolate 2 (VanC2); 4, *E. faecalis* V583 (VanB-VREFS); 5, *E. faecalis* cattle isolate (VSEFS); 6, *E. gallinarum* GS (VanC1); 7, *E. faecium* dog isolate (VSEFM); 8, *E. casseliflavus* ATCC 25788 (VanC2); 9, *E. casseliflavus* cattle isolate 1 (VanC2).

70, 79, 107, 116, 152 and 163 kDa in size (Fig. 1, lane 4). Finally, VSEFS CE proteins were 12, 16, 22, 35, 49, 54, 107, 116 and 163 kDa in size (Fig. 1, lane 5). Each species had a unique size distribution pattern on the gel, and strains of the same species had similar patterns regardless of vancomycin resistance, with two exceptions: bands at 34 and 40 kDa in VanA-VREFM differed from those in VSEFM (Fig. 1, compare lanes 2 and 7), and one of the three *E. casseliflavus* strains had a size distribution pattern that was different from the other two strains (Fig. 1, lanes 3, 8, and 9). CE proteins were also analyzed by Western blot analysis using serum from guinea pigs inoculated with VRE and VSE. When comparing VanA-VREFM with VSEFM, we found that they had two immunogenic proteins of 24 and 44 kDa in common. There was one 129-kDa protein specific to VanA-VREFM and one 7-kDa protein specific to VSEFM (Fig. 2 and Table 1). VanB-VREFM and VSEFS had one immunogenic protein of 54 kDa in common. We did not find an immunogenic protein specific to VanB-VREFM, but found two specific to VSEFS, which were 12 and 16 kDa in size (Fig. 3 and Table 1). Two immunogenic proteins specific to VanC1 were 30 and 46 kDa in size, while a single VanC2-specific protein was 40 kDa in size (Table 1).

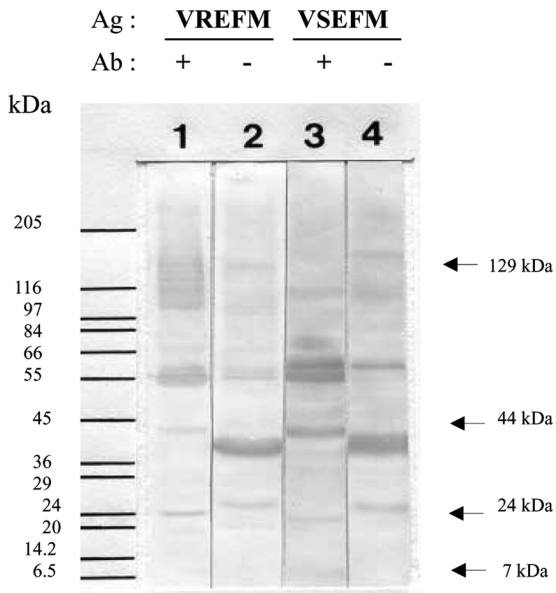

Figure 2. Western blot analysis of CE of vancomycin resistant *E. faecium* (VREFM) and VSEFM with guinea pig sera immunized with VREFM and VSEFM. Lanes: 1 and 2, CE of *E. faecium* B7641 (VREFM) with sera from guinea pigs immunized (+) and not immunized (−) with VREFM, respectively; 3 and 4, CE of *E. faecium* dog isolate (VSEFM) with guinea pig sera immunized (+) and not immunized (−) with VSEFM, respectively.

When the CPs of VRE, VSEFM, and VSEFS were analyzed by SDS-PAGE, each was found to have a unique protein size distribution pattern. The most abundant proteins in VanA-VREFM CP were 9, 13, 17, 21, 23, 27, 32, 39, 40, 44, 56, 61, 64 and 100 kDa in size (Fig. 4, lane 2), while those of VSEFM CP were 44, 48, 56 and 100 kDa in size (Fig. 4, lane 7). VanB-VREFS CP proteins were 5, 12, 16, 22, 28, 31, 35, 39, 43, 47, 49 and 53 kDa in size (Fig. 4, lane 4), while those of VSEFS CP were 6, 17, 22, 47, 53, 57 and 90 kDa in size (Fig. 4, lane 5). Each strain within a species had the same protein profile except that VanA-VREFM had 39 and 48 kDa proteins, unlike VSEFM (Fig. 4, compare lanes 2 and 7), and in VanC2, two of the three isolates had the same protein pattern while the third was different (Fig. 4, lanes 3, 8 and 9).

CP proteins were analyzed by Western blot analysis using serum from guinea pigs inoculated with VRE and VSE. When VanA-VREFM and VSEFM were compared, one 21-kDa immunogenic protein was found in VanA CP alone. No immunogenic CP proteins were shared between the two strains, and none were found in VSEFM alone (Fig. 5 and Table 1). When VanB-VREFS and VSEFS were compared, there

Table 1. Immunogenic proteins present in the CE and CP of enterococci

Strains	CE	CP
VREFM[a] (*E. faecium* B7641)	129*	21
VREFS[b] (*E. faecalis* V583)	–	28
VanC1[c] (*E. gallinarum*)	30	19, 30
VanC2[d] (*E. casseliflavus*)	40	–
VSEFM[e] (*E. faecium* dog isolate)	7	–
VSEFS[f] (*E. faecalis* cattle isolate)	12, 16	6, 17, 90
E. faecium	24, 44	–
E. faecalis	54	–

*protein size, kDa.
[a]VREFM, vancomycin resistant *E. faecium*.
[b]VREFS, vancomycin resistant *E. faecalis*.
[c]VanC1, VanC1-type enterococci.
[d]VanC2, VanC2-type enterococci.
[e]VSEFM, vancomycin susceptible *E. faecium*.
[f]VSEFS, vancomycin susceptible *E. faecalis*.

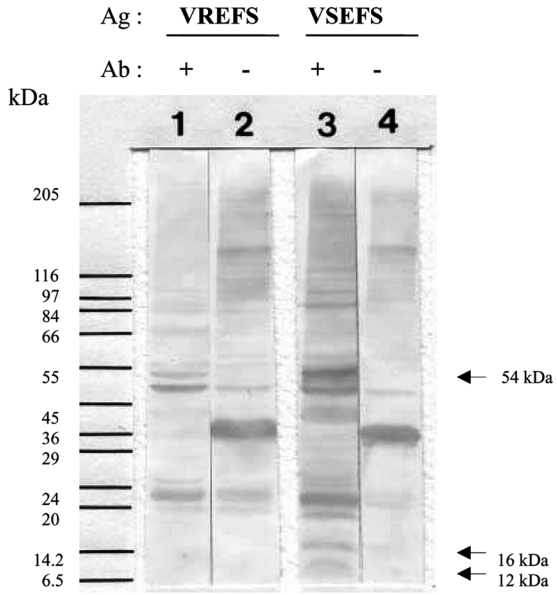

Figure 3. Western blot analysis of CE of vancomycin resistant *E. faecalis* (VREFS) and VSEFS with guinea pig sera immunized with VREFS and VSEFS. Lanes: 1 and 2, CE of *E. faecalis* V583 (VREFS) with guinea pig sera immunized (+) and not immunized (−) with VREFS, respectively; 3 and 4, CE of *E. faecalis* cattle isolate (VSEFS) with guinea pig sera immunized (+) and not immunized (−) with VSEFS, respectively.

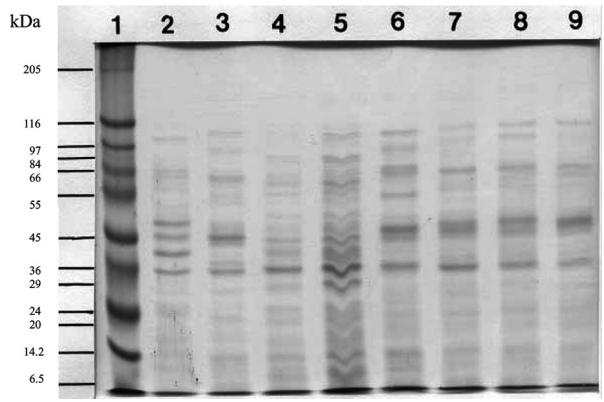

Figure 4. Eletrophoretic analysis of CP of VRE, VSEFM, and VSEFS by 10% SDS-polyacrylamide gel electrophoresis. Lanes: 1, Molecular weight marker (Sigma wide marker); 2, *E. faecium* B7641 (VanA-VREFM); 3, *E. casseliflavus* cattle isolate 2 (VanC2); 4, *E. faecalis* V583 (VanB-VREFS); 5, *E. faecalis* cattle isolate (VSEFS); 6, *E. gallinarum* GS (VanC1); 7, *E. faecium* dog isolate (VSEFM); 8, *E. casseliflavus* ATCC 25788 (VanC2); 9, *E. casseliflavus* cattle isolate 1 (VanC2).

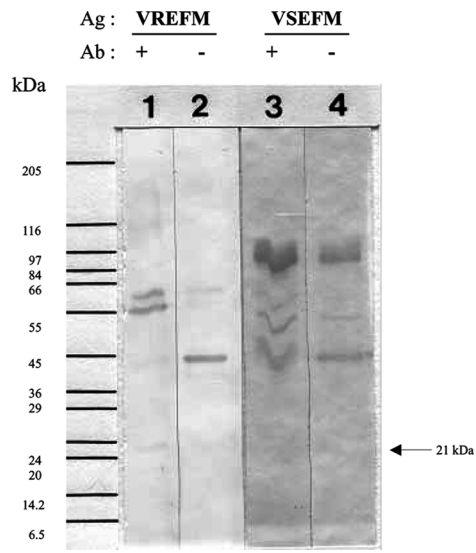

Figure 5. Western blot analysis of CP of VREFM and VSEFM with guinea pig sera immunized with VREFM and VSEFM. Lanes: 1 and 2, CP of *E. faecium* B7641 (VREFM) with guinea pig sera immunized (+) and not immunized (−) with VREFM, respectively; 3 and 4, CP of *E. faecium* dog isolate (VSEFM) with guinea pig sera immunized (+) and not immunized (−) with VSEFM, respectively.

was one 28-kDa VanB-VREFS-specific immunogenic protein, and three VSEFS-specific proteins of 6, 17, and 90 kDa in size. No immunogenic CP proteins were shared between VanB-VREFS and VSEFS (Fig. 6 and Table 1). There were two immunogenic proteins of 19 and 30 kDa in VanC1 CP. No immunogenic CP proteins were found in VanC2 (Table 1).

As discussed above and reported previously by Nicas et al.[25] there did not appear to be any differences in the size distribution of proteins on the SDS-PAGE gels of CE of *E. faecalis* VanB-VREFS and VSEFS. Although VanA-VREFM had a few protein differences when compared with VSEFM, e.g., 34 and 40 kDa proteins in the CE and 39 and 48 kDA proteins in the CP (Figs. 1 and 4), these were not immunospecific (Figs. 2 and 5; Table 1).

In a previous study, proteins from *E. faecalis* were detected by immunoblot analysis using serum from rabbits inoculated with *E. faecalis* isolated from patients with enterococcal infections. Serum from eight patients infected with enterococci, negative control serum, and rabbit

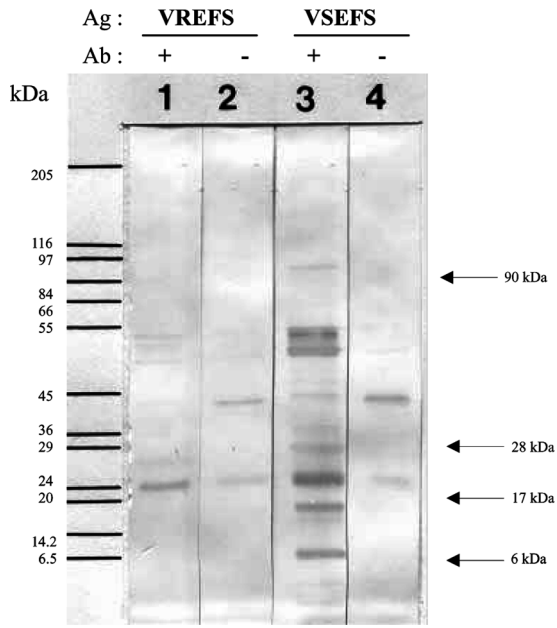

Figure 6. Western blot analysis of CP of VREFS and VSEFS with guinea pig sera immunized with VREFS and VSEFS. Lanes: 1 and 2, CP of *E. faecalis* V583 (VREFS) with guinea pig sera immunized (+) and not immunized (−) with VREFS, respectively; 3 and 4, CP of *E. faecalis* cattle isolate (VSEFS) with guinea pig sera immunized (+) and not immunized (−) with VSEFS, respectively.

anti-*E. faecalis* serum, all reacted with proteins of 36, 43, 58, and 69 kDa in size, but the negative control serum of rabbits did not react.[1] In the present study, immunogenic proteins were detected with guinea pig antiserum in CE and CP of VRE. Guinea pig antiserum also revealed that a 54-kDa immunogenic was common to the CE of both VREFS and VSEFS (Figs. 2, 3, 4, and 5; Table 1). Therefore, the immunogenic proteins identified in this study might be suitable candidates for the diagnosis and immunotherapy of VRE infection.

CONCLUSIONS

The diagnosis of VRE infections by biochemical reactions, antibiotic susceptibility tests,[18] and multiplex PCR generally takes 72–96 h. However, VRE infections can also be detected using antigen-specific antiserum, leading to a diagnosis in less time than is required for conventional diagnosis.[9,11,12] The immunogenic proteins examined in this study appear to be suitable candidates for serological diagnosis. In addition to their diagnostic utility, these proteins and the anti-enterococcus antibodies we have produced may be of use in the study of enterococcal pathogenesis. It will be of great importance to purify and further characterize the immunogenic proteins identified in this study as they may help to elucidate the pathogenesis of VRE.

ABBREVIATIONS

CE, cell envelope; CP, cytoplasm; ELISA, enzyme-linked immunosorbent assay; NC, nitrocellulose; DAB, 3,3'-diaminobenzidine; PCR, polymerase chain reaction; Tp, teichoplanin; Va, vancomycin; VRE, vancomycin-resistant enterococci; VREFM, vancomycin resistant *E. faecium;* VREFS, vancomycin resistant *E. faecalis*.

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