Production of Exfoliative Toxin A by *Staphylococcus aureus* Isolated from Mastitic Cow's Milk and Farm Bulk Milk

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ABSTRACT. The production of exfoliative toxins A and B (ETA and ETB) by *Staphylococcus aureus* isolated from mastitic cow's milk and farm bulk milk was examined by the reverse passive latex agglutination method (RPLA). ETA was detected in 2 (1.2%) of 162 isolates from mastitic cow's milk and in 1 (0.6%) of 166 isolates from farm bulk milk. RPLA titers of these isolates were much lower than in human isolates. No ETB was detected in any of the isolates tested. These ETA-positive isolates belonged to bovine ecovar. They were non-typable using the international phage set for human strains. When these ETA-positive isolates were subcutaneously inoculated into neonatal mice, general exfoliation of the epidermis accompanied by the so-called Nikolsky sign was not recognized. By the immunoblotting and PCR methods, however, ETA and *eta* gene were recognized in the ETA-positive isolates from mastitic cow's milk and farm bulk milk. These data suggest that ETA is also produced by bovine isolates of *S. aureus*, but in smaller quantities. — KEY WORDS: bovine mastitis, exfoliative toxin A, *Staphylococcus aureus*.

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Exfoliative toxin, produced by *Staphylococcus aureus*, is the major causative agent of staphylococcal scalded-skin syndrome in children. The toxin has been divided into two serotypes, exfoliative toxins A and B (ETA and ETB) [2, 6, 11]. They caused equally general exfoliation of the epidermis accompanied by the so-called Nikolsky sign when inoculated into neonatal mice, but they differ in amino acid composition, amino acid sequence, and heat resistance [2, 6, 15]. ETA gene (*eta* gene) is located on the chromosomal DNA of *S. aureus*, whereas ETB gene (*etb* gene) is plasmid-encoded [4, 8, 12–14, 19]. In addition, a new type of exfoliative toxin was purified from a *S. aureus* strain isolated from a horse with phlegmon and was designated exfoliative toxin C (ETC) [18]. ETC caused the Nikolsky sign in both 3-day-old mice and 1-day-old chicks, and moreover the toxin was serologically different from ETA and ETB.

The production of ETA and ETB has been examined in *S. aureus* strains isolated from patients affected with staphylococcal scalded-skin syndrome [5, 7, 9–11]. But, it has not been fully understood whether the toxins are produced only by human strains or whether animal strains also produce them. Recently, Adesiyun *et al.* [1] reported that 3.9% of the animal strains of *S. aureus* produced ET and that 91.1% of the ET-producing strains produced ETA alone. In addition, they mentioned that animal species could also act as carries of ET-producing staphylococci and may act as reservoirs of infection in human beings, especially children. However, little is known about the production of ETA and ETB by *S. aureus* strains of domestic animal origins. The purpose of this study was to examine the production of ETA and ETB by *S. aureus* strains isolated from mastitic cow's milk and farm bulk milk

A total of 328 isolates of *S. aureus* were used in the present experiment. Of these isolates, 80, 82 and 166 were isolated from clinical, subclinical mastitic cow's milk and farm bulk milk, respectively, obtained at 71 dairy farms in Ishikawa Prefecture during the period from 1991 to 1994. Six human strains of *S. aureus*, isolated from patients affected with staphylococcal scalded-skin syndrome, were used as ETA and ETB positive controls.

The organisms were inoculated into 5 m*l* of brain heart infusion (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and cultured at 37°C for 18 hr. The culture fluids were centrifuged at 12,000 × g at 4°C for 5 min and then the resulting culture supernatants were tested for ETA and ETB by a reverse passive latex agglutination (RPLA) test using EXT-RPLA kit (Denka Seiken Co., Ltd., Tokyo, Japan). Briefly, 20 μl of the culture supernatants, diluted 5-fold, were placed into the wells of V-type microtiter plates. An equal volume of latex particles, sensitized with specific anti-ETA or anti-ETB immunoglobulins, was added to each well of the plates. After thorough mixing, the plates were incubated at room temperature for 24 hr. When the aggulutinating reaction was obtained, the culture supernatants of the positive isolates were serially diluted twofold in microtiter plates to determine the quantity of ETA or ETB.

ETA was detected in 2 (2.4%, strains 103, 104) of 82 isolates from subclinical mastitic cow's milk and 1 (0.6%, strain 175) of 166 isolates from farm bulk milk. No ETB was detected in any of the isolates tested. As shown in Table 1, the RPLA titers of ETA-positive isolates were much lower, from 32 to 64, in comparison to human strains (>20,000). Of the 3 ETA-positive isolates, one produced enterotoxin A when examined by a reversed passive latex aggulutination test using SET-RPLA kit (Denka Seiken), but its titer was low (10). Toxic shock syndrome toxin-1 was not detected in any of the ETA-positive isolates.

These ETA-positive isolates were yellow growth spots (type A) on crystal violet agar, β-hemolysin production-

positive and bovine plasma coagulation-positive. From the result, it seems that these ETA-positive isolates belong to bovine ecovar proposed by Devriese [3]. When the ETA-isolates were phage typed with 23 phages of the international phage set for typing human strains, they were non-typable. These ETA-positive isolates belonged to serotype IV of coagulase when examined with coagulase antisera (Denka Seiken). Rogolsky [16] described that ETA and ETB are produced predominently by bacteriophage group 2 and some phage group 1 and 3 strains of *S. aureus*. Therefore, we conjectured that these ETA-positive isolates found here may differ from ETA-positive isolates of human origin.

The culture supernatants of the ETA-positive isolates were inoculated subcutaneously into neonatal mice. The Nikolsky sign was observed on the mice inoculated with human isolates used as positive control, but the isolates of bovine origin killed the mice within 1 hr before development of the sign. The lethality to neonatal mice seems to due to α-hemolysin, which was contained into the culture supernatants of bovine ETA-positive isolates. From the result, we were pondered whether the toxicity of ETA-positive isolates was due to a non-specific reaction between sensitized latex particles and culture supernatants.

Therefore, we attempted the detection of ETA from the bovine isolate by SDS-polyacrylamide gel electrophoresis with immunoblotting. In the experiment, ETA of bovine isolate (103) and human isolate (1897), detected by EXT-RPLA kit, were partially purified from the culture supernatants by column chromatography on Sp-Sepharose FF, gel filtration on Sephacryl S-200, column chromatography on Mono S, and gel filtration on Superose 6. These ETA preparations were applied to the gel and electrophoresed. Following electrophoresis, the separated components of the preparations were electrophoretically transferred from the gel to a nitrocellulose membrane. After transfer, the immunoblotting assay of the membrane was performed by Immun-blot assay kit (Bio-Rad, Hercules, California), according to the protocol of the manufacturer. Anti-ETA serum, which was kindly provided by Dr. S. Sakurai, was used in the immunoblotting assay.

Figure 1 shows the immunoblotting assay of the ETA preparations from bovine and human isolates. The ETA preparation (lane 1) from bovine isolate formed one band in the same position as that of the ETA preparation (lane 2) from human isolate. But the band of ETA preparation from bovine isolate was very weak because the concentration of ETA is low (0.02 optical density at 280 nm).

In addition, the *eta* gene of ETA-positive isolates was examined by PCR amplification. Bacterial cells producing ETA-positive isolates were suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) with lysostaphin (Sigma, 20 units/m*l*) and incubated at 37°C for 30 min. After centrifugation, nucleic acids in the supernatants were extracted twice with phenol and once with phenol-chloroform-isoamyl-alcohol (50–48–2) and then precipitated with ethanol. The DNA samples were dissolved in TE buffer and used in PCR amplification.

Fig. 1. Immunoblot analysis after SDS-PAGE of ETA preparations from bovine and human isolates. Lane 1: bovine isolate (103); lane 2: human isolate (1897).

PCR amplification was perfomed in a total volume of 100 μ*l* containing about 15 ng of the bacterial DNA samples, 20 pmol of each primer, 2.5 mM of each of the four deoxynucleotides, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, and 2.5 units of *Taq* polymerase (Takara Shuzou Co., Ltd., Japan). The sequence of synthesized primer 1 was 5' -CTATTTACTGTAGGAGCTA G-3' and primer 2 was 5'-ATTTATTTGATGCTCTCTAT-3' [17]. The reaction mixtures were overlaid with mineral oil and subjected to 30 cycles of amplification in a PCR thermal cycler (Takara Shuzou, Co., Ltd.). Each amplification cycle perfomed was for 1 min at 94°C for denaturing, 1 min at 46°C for annealing, and 1 min at 72°C for extension. After amplification, the PCR products were analyzed by agarose gel electrophoresis.

Figure 2 shows the electrophoretic pattern of the PCR products obtained with DNA samples from bovine isolates (lanes 2 to 4) and human isolates (lanes 5 to 7). The 741 bp band of *eta* gene was observed in both human and bovine isolates, but was not detected in any of the ETA-negative isolates tested (date not shown). From the result, it seems that the bovine isolates of ETA-positive have the same *eta* gene as the human isolates used as control.

In conclusion, our results indicate that ETA is produced by bovine isolates of *S. aureus*, but in smaller quantities. Moreover, Nikolsky sign was not recognized in mice inoculated subcutaneously with the ETA-positive isolates because the amount of ETA, produced by them, is so small. We are studying the putative promoter and expressive regulator regions of *eta* gene of bovine isolates in order to clarify the reason for the difference of amounts produced

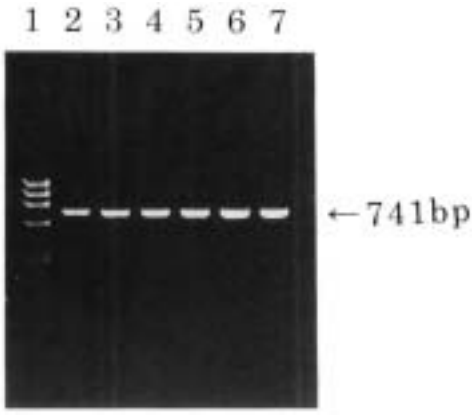

Fig. 2. Agarose gel electrophoresis of the PCR products. Lane 1: DNA molecular size marker (1,353, 1078, 872 and 603 bp) ; lanes 2–4: bovine isolates (103, 104 and 175); lanes 5–7: human isolates (1897, 1898 and 2086).

by bovine and human isolates. Futher studies are needed to examine the ETA-production of bovine mammary isolates which were collected from many dairy farms in various areas in order to clarify the roles of ETA in the occurrence of mastitis or the infection of *S. aureus* in cows.

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