Japanese Journal of Veterinary Research 69(4): 195-203, 2021

JJVR

REGULAR PAPER Regional Study

Sequence type and primary structure of the *vru* gene upstream region of *Streptococcus uberis* isolated from bovine clinical mastitis in Japan

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Received for publication, February 8, 2021; accepted, August 23, 2021

Abstract

In the present study, we investigated the variability of bovine clinical mastitis isolates of *Streptococcus uberis* in Japan by multilocus sequence typing. We also investigated the variations in the primary structure of the *vru* gene upstream region of the isolates to elucidate the association of this region with the occurrence of clinical mastitis. Eighty-two isolates were recovered from 62 dairy farms in Japan; these isolates were associated with 57 sequence types (STs), including 54 novel STs to which 78 isolates belonged. Thirty isolates with ST1003 (one of the novel STs) and related STs at the triple-locus variant level accounted for 37% of the isolates examined. A total 16 (20%) isolates were assigned to clonal complexes (CC143 and CC86) that are major in New Zealand and Australia. Seventy-one of the 82 isolates had 1, 3, 4 or 5 bp deletions in the *vru* gene upstream region in comparison with the corresponding region in the *S. uberis* virulent strain 0140J and the remaining 11 isolates had no deletions. These results suggest that *S. uberis* is a diverse mastitis-causing pathogen, and the integrity of the *vru* gene upstream region is not necessarily conclusive for the occurrence of bovine clinical mastitis.

Key Words: bovine mastitis, genetic diversity, multilocus sequence typing, *Streptococcus uberis*

Introduction

Streptococcus uberis is a major causal pathogen of mastitis in dairy cattle, which affects the udder health, milk quality, and production in dairy farms[7,9,15]. Intramammary infections of *S. uberis* are capable of causing clinical and subclinical mastitis during both lactating and non-

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doi: 10.14943/jjvr.69.4.195

lactating periods[16]. *S. uberis* has been historically considered an environmental pathogen, and has been isolated from environmental sources around dairy cattle, such as soil, pasture, bedding materials, and bovine feces[27]. Meanwhile, as for *S. uberis* mastitis cases in various dairy herds, molecular epidemiological studies have shown that the predominant strain or a limited number of strains may be highly likely to cause intramammary infections or transmission between cows, including possible transfer of the strains via a milking machine or the environment around the cows[2,25].

Multilocus sequence typing (MLST) is a valuable tool for examining the relationships among various strains and the recent ancestral lineage by determining the allelic profiles[17]. MLST analysis archives the exhibition of the diversity of strains and the grouping of related sequence types (STs) in clonal complexes (CCs). Examination of ST diversity is helpful for determining the mode of transmission[13,26]. For *S. uberis* mastitis isolates, a large ST diversity of isolates in a herd shows a heterogeneous environmental source of infection, while high ST homogeneity of isolates in a herd suggests an environmental point source of infection or contagious transmission[2,13]. Previous MLST analyses of the *S. uberis* isolates in the United Kingdom, New Zealand and Australia proposed three major CCs, i.e., CC5, CC86, and CC143, and their associations with pathogenicity in bovine mastitis: CC5 was proposed to be associated with clinical mastitis, CC143 with subclinical mastitis and CC86 with latent infection[1,18,22,26]. In other European and North American countries, some *S. uberis* isolates were found to belong to these CCs[12,19,20]. For Asian countries, only a relatively small number of *S. uberis* isolates have been examined in India (13 isolates) and China (28 isolates), and not enough is known about the genetic diversity and groups of STs in the mastitis isolates[21,23]. The diversity of MLST STs has not been examined in isolates of *S. uberis* mastitis in Japan.

S. uberis vru (*sub0144*) is a homolog of the *S. pyogenes* virulence regulatory gene *mga* and regulates gene expression for various virulence factors[4]. Inactivation of *vru* was reported to reduce clinical signs of mastitis compared to the wild-type strain[4]. Hossain et al. compared the deoxyribonucleic acid (DNA) sequence in the upstream region of 13 *S. uberis* strains; their results suggested that variation in this region may influence the host-pathogen interaction, since most of the strains with no deletion were clinical mastitis isolates and most subclinical mastitis isolates had 5- or 4-bp deletions in the specific *vru* upstream region, compared to the corresponding region of the clinical virulent isolate *S. uberis* 0140J[10].

In this study, we attempted to identify STs of *S. uberis* clinical mastitis isolates using MLST and to determine their diversity and distribution in Japanese dairy herds. We also aimed to determine the variations in the primary structure of the *vru* gene upstream region of the isolates, and then to verify the importance of the integrity of the *vru* gene upstream region for the occurrence of clinical mastitis.

Materials and Methods

Sample collection and DNA extraction

From 2015 to 2020, milk samples were collected from dairy cows with clinical mastitis raised in 62 local farms (62 herds) in Japan, including 44, 4, 1, 3, 6, 3 and 1 dairy herds in Hokkaido, Miyagi, Tochigi, Saitama, Okayama, Hiroshima, and Kumamoto Prefecture, respectively. The milk was cultured on sheep blood agar plates, and the isolates were preliminarily screened by colony morphology followed by *S. uberis*-specific 16S ribosomal DNA (rDNA) polymerase chain reaction (PCR)[8]. Following the shaking culture of the isolates in Todd-Hewitt broth at 37°C overnight, genomic DNA was isolated using a DNA extraction kit, ISOPLANT II (Nippon Gene, Toyama, Japan), according to the manufacturer's instructions.

Amplification of 16S rDNA and DNA sequencing

All isolates identified by the *S. uberis*-specific 16S rDNA PCR were confirmed to be *S. uberis* by 16S rDNA gene sequencing. 16S rDNA was amplified by PCR using a pair of universal primers: 16SUNI-L (5'-AGA GTT TGA TCA TGG CTC AG-3') and 16SUNI-R (5'-GTG TGA CGG GCG GTG TGT AC-3')[14]. The PCR product was purified using illustra GFX PCR DNA and a Gel Band Purification Kit (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). The DNA sequence of the PCR product was determined by the Sanger method using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

The sequence data of the 16S rDNA were compared to other 16S rDNA sequences in the DDBJ/GenBank/EMBL database using BLAST (https://blast.ncbi.nlm.nih.gov/).

MLST analysis

Amplification of the target regions of *arcC, ddl, gki, recP, tdk, tpi, and yqiL* used for MLST analysis was performed according to Coffey et al.[1], and each of the amplified products was subjected to DNA sequence analysis as described above. The allelic DNA sequence data were queried against the PubMLST sequence and profile definitions database and unidentified alleles were submitted to the database[11]. The allelic profile of each isolate was then queried against the database. The allelic profiles that were not assigned to known sequence types (STs) were submitted to the database to generate new STs. Different STs within 3 or fewer locus differences were regarded as related STs. The STs were automatically assigned to existing clonal complexes (CC5, CC86, and CC143) by a script that runs in the *Streptococcus uberis* typing database of PubMLST. The MLST profiles of the isolates were analyzed using the goeBURST option of PHYLOViZ[5, 6]. In the goeBURST analysis, the founders of the three major CCs, i.e., ST5, ST86 and ST143, were also included for comparison[18].

DNA sequence analysis of the vru gene upstream region

Genomic DNA corresponding to nucleotides -110 to +104 from the translation initiation site of *vru* of *S. uberis* strain 0140J (DDBJ/EMBL/GenBank accession number AM946015)[24] was amplified by PCR using the KOD-Plus-Neo DNA polymerase system (Toyobo, Osaka, Japan) and a pair of primers: Vru-UR-fw (5'-CCA AAA GGA GAC AGG TTT TCT CG-3') and Vru-CR-rv (5'-CAA ATG TGG GTG TAG TCA C-3'). The DNA sequence of the PCR product was determined as described above. The DNA sequence data of the *vru* upstream region of each isolate were aligned and compared using the program MUSCLE[3].

Results

Collection of S. uberis bovine clinical mastitis isolates

Eighty-two Japanese isolates of clinical mastitis of different dairy cows were confirmed to be *S. uberis*, with 16S rDNA sequences that were 100% identical to that of the reference strain, *S. uberis* HN1 (DDBJ/EMBL/GenBank accession number AB023576). These isolates were obtained from 62 dairy herds in 7 prefectures: 54 isolates were from 44 herds in Hokkaido, 5 isolates were from 4 herds in Miyagi, 6 isolates were from 1 herd in Tochigi, 7 isolates were from 3 herds in Saitama, 6 isolates were from 6 herds in Okayama, 3 isolates were from 3 herds in Hiroshima, and 1 isolate was from a herd in Kumamoto Prefecture. From each dairy farm, 1 to 6 isolates were collected.

Allele profiles

Sixty-seven different alleles for the 7 gene fragments were identified and 4 *arcC*, 3 *ddl*, 3 *gki*, 3 *recP*, 4 *tdk*, 3 *tpi* and 1 *yqiL* allele were found for the first time in this study. Based on the allelic profiles, the 82 *S. uberis* isolates were assigned to

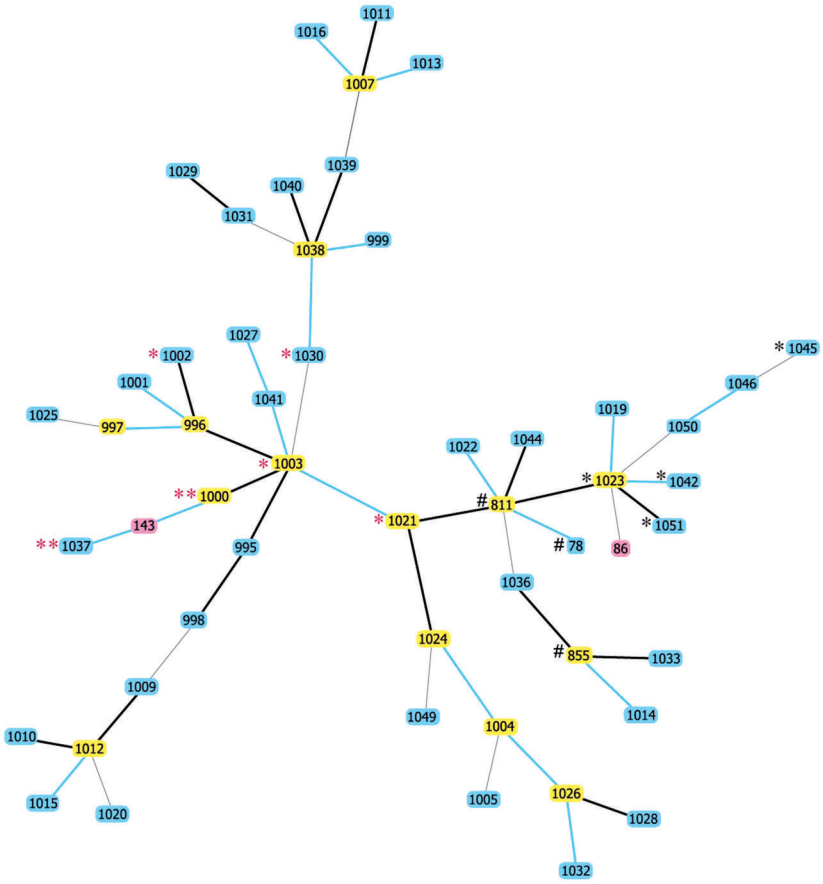

Fig. 1. goeBURST analysis of sequence types of *S. uberis* isolates of bovine clinical mastitis in Japan.
The diagram was generated by the goeBURST option PHYLOViZ[5,6] on sequence types (STs) of *S. uberis* isolates of bovine clinical mastitis in Japanese dairy herds and STs of founders of 3 major clonal complexes (ST5, ST86 and ST143). The number of each ST is shown in a yellow (regarded as the subgroup founder in the analysis), light blue, or pink node (founder of the major clonal complex included in the analysis for comparison). Thick black, light blue, and thin gray lines link single-locus variants (SLVs), double-locus variants (DLVs), and triple-locus variants (TLVs), respectively. The ST with a black asterisk indicates the TLV of ST86. The STs with double and single pink asterisks indicate the DLV and TLV of ST143, respectively. Pound signs (#) indicate the STs reported previously. ST5, ST1008, ST1034, ST1035, ST1043, ST1047, and ST1048, which were not linked to any other STs at the TLV level, are not shown in the diagram.

57 different STs (Table 1). There were 3 previously identified STs (ST78, ST811, and ST855) that included 4 isolates, and the remaining 54 STs were newly identified and included 78 isolates.

Assignment to major CCs and goeBURST analysis

A total of 9 STs were identified as CC143 at the double-locus variant (DLV) or triple-locus variant (TLV) levels; 3 isolates belonging to ST1000, and ST1037 are DLVs and 6 isolates belonging to ST1002, ST1003, ST1021, and ST1030 are TLVs of ST143. In regard to CC86, 7 isolates belonging to ST1023, ST1042, ST1045, and ST1051, which are TLVs of ST86, were found. No STs assigned to CC5 were found.

In the goeBURST analysis, 14 founders of subgroups were predicted; 3 subgroup founders (ST1000, ST1003, and ST1021) belonged to CC143 and 1 subgroup founder (ST1023) belonged to CC86 (Fig. 1). The previously identified STs, ST811 and ST855, were predicted to be founders of subgroups of Japanese isolates. ST5, which was added to the analysis for purpose of comparison,

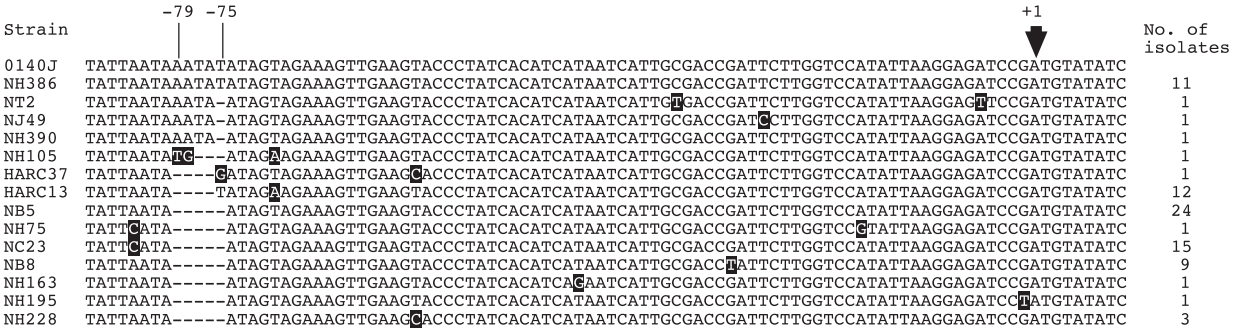

Fig. 2. Variation in the primary structure of the *vru* gene upstream region of *S. uberis* isolates of bovine clinical mastitis in Japan.

Deoxyribonucleic acid (DNA) sequences of the *vru* gene upstream region and a part of the coding region (-87 to +9 from the translation initiation site) of S. *uberis* isolated from clinical mastitis in Japanese dairy herds were aligned and compared using MUSCLE[3]. The translation initiation site (+1) is indicated by an arrowhead. The DNA sequence of the S. uberis virulent strain 0140J (DDBJ/EMBL/GenBank accession number AM946015) is also shown in the top row for comparison. The positions at -79 and -75 of the *S. uberis* 0140J strain are indicated by vertical lines above the DNA sequence. Deletions of nucleotides in comparison with the DNA sequence of *S. uberis* 0140J are shown with a dash (-). The highlighted characters are nucleotides that differ from those of *S. uberis* 0140J in the aligned DNA sequence. Examples of strain names of the isolates with their respective DNA sequences are shown on the left and the number of isolates is shown on the right.

and 6 other STs, ST1008, ST1034, ST1035, ST1043, ST1047, and ST1048, were not linked to any other STs at the TLV level. One of the subgroup founders, ST1003, and its related STs as single-locus variants (SLVs) (ST995, ST996, and ST1000), DLVs (ST998, ST1002, ST1021, and ST1041), and TLVs (ST78, ST811, ST997, ST999, ST1001, ST1004, ST1024, ST1026, ST1027, ST1030, ST1037, and ST1044), included a total of 30 isolates (Table 1, Fig. 1). The isolates with ST1003 and related STs at the TLV level accounted for 37% of the Japanese *S. uberis* clinical mastitis isolates.

In 3 farms (HK-30, ST-2, and TG-1), *S. uberis* isolates were obtained from more than 4 clinical mastitis cases. In farm ST-2, 3 isolates with ST1038 and an isolate with a SLV of ST1038 (ST1039) was obtained. In farm HK-30, 2 isolates with ST1001 and 2 isolates belonging to a TLV of ST1001 (ST1002) were obtained. Six isolates obtained in TG-1 were typed into ST1001 (2 isolates), ST1031 (2 isolates), ST1032 (1 isolate), and ST1047 (1 isolate), which were not related to each other because they were distinguished by 5 or more locus differences.

Primary structure of the vru gene upstream region

The *vru* gene upstream regions of 11 of 82 Japanese clinical mastitis isolates were identical to that of the clinical virulent strain *S. uberis* 0140J (Fig. 2). Fifty-four isolates had an identical 5-bp deletion at nucleotide position -79 and 13 isolates had an identical 4-bp deletion at position of -79 of this region. Three isolates had an identical 1-bp deletion at position -75. In the remaining 1 isolate, a 3-bp deletion was found at position -77. Additionally, nucleotide substitutions located at positions -83, -79, -78, -75, -70, -57, -42, -33, -28, -25, -16, -5, and -1 were found in a total of 46 isolates. Eleven isolates having an identical sequence to the *vru* gene upstream region of strain 0140J belonged to 8 STs (ST78, ST855, ST1000, ST1016, ST1020, ST1021, ST1032, and ST1049). Three isolates with ST1016 were obtained from 3 different farms in Miyagi Prefecture.

Discussion

In the MLST analysis, most of the clinical mastitis isolates in Japan were assigned to

Table 1. STs of *S. uberis* isolates of bovine clinical mastitis in Japan

ST[a]	Allele							Number of isolates	Dairy herd code[b]
	arcC	*ddl*	*gki*	*recP*	*tdk*	*tpi*	*yqiL*		
78	6	1	3	2	3	2	3	1	HK-42
811	3	1	3	2	5	2	3	1	HK-25
855	9	1	27	2	39	1	3	2	HK-4, HK-19
995	1	1	37	4	17	2	3	1	ST-1
996	1	1	37	3	17	2	3	2	HK-44, OY-4
997	1	1	3	3	3	2	3	1	HK-6
998	1	1	37	4	17	18	3	1	HK-13
999	1	1	56	2	24	39	3	1	HK-15
1000	1	1	2	2	17	2	3	2	HK-24, HK-33
1001	1	1	37	3	101	2	5	5	HK-28, HK-30, TG-1
1002	1	1	37	3	17	4	3	2	HK-30
1003	1	1	37	2	17	2	3	2	HK-31, HK-37
1004	1	15	3	2	17	18	3	1	HK-38
1005	1	15	43	2	17	1	71	1	MG-1
1007	2	1	4	2	3	1	3	1	HK-1
1008	2	5	24	2	2	40	3	1	HS-3
1009	2	1	2	4	2	18	3	1	HK-9
1010	2	2	1	4	2	18	3	2	HK-14
1011	2	1	4	2	13	1	3	3	HK-7, HK-16, HK-29
1012	2	2	2	4	2	18	3	2	HK-17, HK-36
1013	2	1	4	2	2	1	10	1	HK-20
1014	2	64	27	2	39	1	3	1	HK-23
1015	2	2	2	4	13	2	3	1	HK-35
1016	2	1	1	2	1	1	3	3	MG-2, MG-3, MG-4
1019	3	2	28	2	3	2	3	1	HK-1
1020	3	2	3	4	2	18	5	2	HK-2, HK-3
1021	3	1	3	2	17	2	3	1	HK-10
1022	3	1	3	1	9	2	3	1	HK-12
1023	3	2	3	2	5	2	3	3	HK-15, HK-21, HK-32
1024	3	15	3	2	17	2	3	1	HK-43
1025	4	2	56	3	3	2	3	1	OY-3
1026	6	15	37	2	17	18	3	2	KM-1, ST-3
1027	6	1	37	1	28	2	3	2	HK-6, HS-1
1028	6	15	62	2	17	18	3	1	OY-1
1029	6	64	56	2	104	4	3	1	OY-2
1030	6	1	56	2	17	39	3	1	HK-39
1031	6	64	56	2	3	4	3	2	TG-1
1032	6	63	37	34	17	18	3	1	TG-1
1033	9	1	27	2	39	1	10	1	HS-2
1034	9	1	2	3	103	2	10	1	OY-5
1035	9	2	3	3	3	4	13	1	HK-11
1036	9	1	27	2	5	1	3	1	HK-18
1037	20	1	5	2	17	4	3	1	HK-41
1038	56	1	56	2	3	39	3	4	HK-9, ST-2
1039	56	1	56	2	3	1	3	1	ST-2
1040	56	1	56	2	102	39	3	1	HK-34
1041	67	1	37	2	28	2	3	1	ST-1
1042	68	2	3	3	5	2	3	1	HK-5
1043	69	2	34	35	24	4	10	1	OY-6
1044	69	1	3	2	5	2	3	1	HK-8
1045	69	2	61	3	3	1	3	2	HK-22, HK-40
1046	69	2	3	1	3	18	3	1	HK-26
1047	69	62	60	33	24	4	3	1	TG-1
1048	69	62	4	34	28	38	71	1	MG-3
1049	70	15	3	2	3	2	10	1	HK-24
1050	70	2	3	2	3	18	3	1	HK-27
1051	3	2	3	4	5	2	3	1	HK-13
5	1	1	2	1	2	1	2		
86	3	2	3	3	13	1	3		
143	18	1	2	2	17	4	3		

[a] Allele profiles of ST5, ST86, and ST143 are shown in the bottom of this table for comparison.

[b] Codes represent the different dairy farms. The two-letter prefix indicates the prefecture where each herd was located (HK, HS, KM, MG, OY, ST, and TG represent Hokkaido, Hiroshima, Kumamoto, Miyagi, Okayama, Saitama, and Tochigi, respectively).

numbers of new STs. The STs of *S. uberis* isolates from the United Kingdom, Switzerland, Portugal, Canada, Australia, New Zealand, India, and China were reported, and most of the STs were newly found in each of these countries[1,12,18–23]. These data suggest that *S. uberis* mastitis strains indigenous to each country cause mastitis in dairy cows.

S. uberis has been suggested to have the potential to cause cow-to-cow transmission in dairy herds[2,19]. In the present study, 4 isolates obtained in farm ST-2 were assigned to ST1038 and ST1039, and ST1039 was closely related to 1038 at the SLV level. Similarly, 4 isolates from farm HK-30 belonged to ST1001 and 1002 that were related to each other at TLV level. These results may suggest that *S. uberis* occasionally transmits cow-to-cow in Japanese dairy herds. Otherwise, *S. uberis* may have been transmitted via the environment around the cows or via the milking machine. In Miyagi Prefecture, 3 isolates obtained from 3 different farms were assigned to ST1016 and had an identical sequence of the *vru* gene upstream region. However, it cannot be concluded that the same strain of *S. uberis* had been transmitted among the farms since epidemiological data—including information on the possible transport of dairy cows between these farms—was not available. It will be necessary to analyze additional cases in various dairy herds to clarify the mode of transmission of this causal pathogen.

Previous studies reported three major CCs, i.e., CC5, CC86 and CC143, and suggested that CC5 is highly associated with clinical mastitis, while CC143 is associated with clinical and subclinical mastitis[22,26]. CC86 tends to be associated with latent infection. In our study, although no isolate belonging to CC5 was found, isolates belonging to CC143 were found. However, these isolates did not seem to be derived from the primary founder ST143 in the goeBURST analysis, and they accounted for smaller proportions of total CCs (11%) than in New Zealand (41%) and Australia (approximately 26%)[18,22]. In contrast, isolates with ST1003 and related STs at the TLV level made up 37% of the Japanese *S. uberis* clinical mastitis isolates, suggesting the presence of a Japanese cluster. These results, along with the fact that most of the STs identified in this study were newly found, suggest that most of the *S. uberis* isolates of clinical mastitis in Japan were derived from a Japanese environmental source. Meanwhile, the goeBURST analysis has indicated that 2 STs, ST811 previously found in Switzerland and ST855 previously found in Canada, were founders of subgroups of the Japanese isolates. These STs of *S. uberis* may have been distributed in the Japanese environment by nature. Otherwise, the *S. uberis* strains that cause mastitis in Japan might have been slightly influenced by foreign strains.

Hossain et al. suggested that variation in the upstream regions of the *vru* gene may influence the host-pathogen interaction, because most of the strains with no deletion in this region were isolated from clinical mastitis cases and most subclinical mastitis isolates had 5- or 4-bp deletions[10]. The same deletions in the *vru* upstream region were found in 82% of the Japanese clinical mastitis isolates. We also found 1- or 3-bp deletions in the upstream region. Although the considerable importance of *vru* in mastitic pathogenesis was shown by the fact that inactivation of *vru* caused an apparent reduction in clinical signs of mastitis compared to the wild-type strain[4], the results of this study suggest that these deletions in the *vru* upstream region are not necessarily crucial for causing clinical bovine mastitis. Further studies, including analyses of isolates of subclinical mastitis and latent intramammary infection, must be conducted to determine the contribution of the integrity of the *vru* gene upstream region.

Acknowledgments

This study was supported by a Grant-in-Aid for "Regulatory Research Projects for Food Safety, Animal Health and Plant Protection" (no. JPJ008617. 17935709) from the Ministry of

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