FULL PAPER *Bacteriology*

Characterization of *Aerococcus viridans* isolated from milk samples from cows with mastitis and manure samples

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ABSTRACT. Thirty-eight *Aerococcus viridans* isolates were obtained from milk from 478 cows with clinical mastitis in a farm during the periods between November 2011 and February 2012, and between December 2012 and March 2013. Additional isolates were obtained from processed manure (a mixture of composted manure, straw and hydrated lime) and bedding materials. The processed manure was later used to cover the floor of the stalls in barns as bedding materials. The temperatures recorded in the composted and processed manure were not as high as those generally observed during satisfactory composting. To reveal the association of *A. viridans* in manure-related products with intramammary infection in cows, isolates were characterized by their DNA fragment patterns as determined by pulsed-field gel electrophoresis (PFGE) and antimicrobial susceptibility testing. Isolates obtained from milk, processed manure and bedding materials had identical DNA fragment patterns. Antimicrobial susceptibilities were determined for 29 isolates from milk, processed manure and bedding materials. Of these, 26 (89.7%) were resistant to clindamycin, whereas virtually all the isolates were susceptible to 12 other antimicrobials including cefalosporins that have been used to treat bovine mastitis in Japan. *In vitro*, three *A. viridans* isolates from milk and an isolate from processed manure survived for 3 hr in Good's buffer (pH 9) at high temperature (50°C). The results suggest that the processed manure and bedding materials in this farm were possible sources of *A. viridans* that caused infection in the cows with mastitis.
KEY WORDS: *Aerococcus viridans*, cattle, compost, manure, mastitis

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Bovine mastitis is characterized by inflammation of mammary glandular tissues and reduces quality and quantity of milk. It is frequently caused by microbial infections including bacteria, viruses and fungi [3, 17].

Aerococcus viridans is a Gram-positive, non-motile, microaerophlic coccus [7]. It has been occasionally isolated as the only species from subclinical intramammary infections in dairy cows, although the pathogenic importance of this organism in bovine mastitis has been unclear [6, 19]. Woodward *et al.* [18] reported the presence of *A. viridans* together with bacteria belonging to additional ten genera on the teat skin and inhibitory effects of these bacteria against mastitis pathogens. However, *A. viridans* was recently implicated in bovine mastitis, as 12 *A. viridans* strains were isolated from clinical and subclinical cases in Slovakia [12]. No significant genetic variability was detected in these isolates by molecular DNA-based methods, although their resistance to antibiotics varied greatly.

Compost dairy barns were first built in Minnesota [2]. This type of housing system has been used in dairy farms in Japan for improved cow comfort and recycling of litters. We isolated *A. viridans* from dairy cow milk samples with clinical mastitis in a farm in western Japan where the cows were housed in compost barns and this organism was additionally isolated from bedding materials. To clarify whether processed manure, which was later used as bedding materials, was a possible source of *A. viridans* causing intramammary infection in cows, in the present study, the isolates were further characterized by their DNA fragment patterns as determined by pulsed-field gel electrophoresis (PFGE), antimicrobial susceptibility testing and survival properties in artificial conditions similar to the processed manure in this farm.

MATERIALS AND METHODS

Farm: Over a 17-month period from November 2011 to March 2013, milk samples were obtained from 478 dairy cows with clinical mastitis that were raised on a farm (farm A) located in western Japan to isolate the causative bacteria of mastitis. All the samples were collected before the cows had been treated with antibiotics. Clinical symptoms observed included induration and swelling of udders, occurrence of milk clots, fever, anorexia and reduced milk production.

In December 2011, two additional milk samples were obtained at another farm (farm B) approximately 11 km west-northwest of farm A.

Manure-related samples: Barnyard manure at farm A was composted in a storehouse for 2 months. This manure (hereafter referred to as "composted manure") was moved to another storehouse, and mixed with straw and 3% hydrated lime (hereafter referred to as "processed manure"). The mixture was used as bedding to cover the floor of stalls in barns.

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Samples of processed manure that had been used as bedding are referred to as "bedding materials".

Temperature and pH: In January 2013, the temperature of 6 piles of composted manure and 4 piles of processed manure was measured at farm A. At one point of each pile, the temperature was measured at depths from 20 to 100 cm from the surface. To measure pH, 10 g from each of the piles of processed manure and 10 g of 4 bedding materials samples were diluted tenfold in phosphate-buffered saline (PBS) and mixed. pH was measured with a Horiba pH meter (Horiba Ltd., Kyoto, Japan).

Isolation of bacteria: To isolate bacteria from milk samples, 100-μl milk was spread onto plates of heart-infusion agar (HIA) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) with 5% defibrinated sheep blood, desoxycholate hydrogen sulfate lactose agar (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) and mannitol salt agar (Nissui, Japan). From plates that yielded colonies in pure culture, these colonies were picked, and their biochemical characteristics were examined using API20E, API20Staph and API20Strep (bioMérieux, Marcy l'Etoile, France). *A. viridans* was preliminarily identified using API20Strep and confirmed by PCR targeting the 16S rRNA variable regions [9].

To isolate bacteria from the manure-related samples, 100-μl aliquots of the composted manure, processed manure and bedding materials samples diluted 10-fold in PBS were spread onto blood agar plates. Colonies suspected to be *A. viridans* were picked and identified as described above.

Antimicrobial susceptibility testing: The antimicrobial susceptibility to 13 antimicrobials was determined by the disk diffusion method according to the Clinical and Laboratory Standards Institute guideline M31–A3 [4] using Mueller-Hinton plates (Becton, Dickinson and Co., Sparks, MD, U.S.A.) supplemented with 5% defibrinated sheep blood. Antimicrobials used in this study and the disk contents were as follows: penicillin (10 IU), ampicillin (10 μg), amoxicillin-clavulanic acid (20/10 μg), cefazolin (30 μg), cefuroxime (30 μg), streptomycin (10 μg), gentamicin (10 μg), erythromycin (15 μg), clindamycin (2 μg), vancomycin (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg) and tetracycline (30 μg). *Escherichia coli* ATCC25922 and *Staphylococcus aureus* ATCC 25923 were included as quality control strains. Inhibition zone diameters were interpreted using the resistance breakpoints defined in the previous study [10] and the CLSI document M31-A3 [4]. For cefazolin and cefuroxime, we used the breakpoints recommended for testing staphylococci [5], since no specific breakpoint for aerococci is available.

PFGE: *A. viridans* DNAs prepared in an agar block were digested with *Sma*I (TaKaRa Bio Inc., Otsu, Japan), and PFGE was performed with 1% agarose by using a CHEF-DR II apparatus (Bio-Rad Laboratories, Richmond, CA, U.S.A.) in 0.5x Tris-borate EDTA (TBE) buffer at 14°C at 200 V. A linearly ramped switching time from 1 to 30 sec was applied for 21 hr [13]. Isolates were considered unrelated to each other, if their PFGE patterns were distinguished by seven or more band differences [15]. CHEF DNA Size Standard lambda ladder (Bio-Rad) was used as a molecular size standard.

Survival of A. viridans at high pH and high temperature: An overnight culture on tryptic-soya agar (TSA) plate of each isolate was suspended in PBS to obtain approximately 1×10^7 CFU/ml for each strain. One ml of each suspension was mixed with 9 ml of PBS (pH 7) or 0.1 M *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES)-NaOH buffer (pH 9) [8]. After 0, 3, 6 and 24 hr of incubation at 50°C, a 0.5 ml aliquot of the sample was serially diluted 10-fold in PBS, and 0.1 ml of each dilution was spread onto TSA plates. All plates were incubated at 37°C for 24 hr. Experiments were performed in triplicate and repeated twice. Significant (P<0.05) differences between initial viable cell numbers and those post inoculation were determined by application of the Welch's t test.

RESULTS

Isolation of bacteria from milk samples: From 478 milk samples from cows with clinical mastitis in farm A, 136 bacterial isolates were obtained: 44 *E. coli* (32.8%), 38 *A. viridians* (28.3%), 33 *Streptococcus* spp. other than *S. agalactiae* (24.6%), 15 coagulase-negative *Staphylococcus* (11.2%), 2 *Pseudomonas aeruginosa* (1.5%) and 4 Gram-negative bacteria, which were not identified to the species level. *A. viridans* was isolated between November and March, but not in the other months (Fig. 1).

Isolation of A. viridans from environmental samples: Seven strains of *A. viridans* were isolated from 3 samples of processed manure and 4 samples of bedding materials on farm A in January 2013.

Antimicrobial susceptibility: The distribution of the inhibition zone diameters of *A. viridans* isolates and percentage of resistant strains are shown in Table 1. Of representative 29 isolates, 26 (89.7%) were interpreted as being resistant to clindamycin according to the breakpoint for streptococci. Of the 26 isolates, an isolate was resistant to chloramphenicol. Of the three others, one was solely resistant to cefuroxime. All the isolates tested in the present study were susceptible to the other antimicrobials.

Pulsed-field gel electrophoresis analysis: DNAs of the 29 isolates used for the disk diffusion testing were analyzed by PFGE. *Sma*I-digested patterns were arbitrarily designated using lowercase letters a through l. When isolates were considered related to each other because 6 or less band differences were shown among their patterns [15], these patterns were expediently designated as a single letter. Eight different patterns (Fig. 2, lanes 1–14: patterns a to h) were detected among 14 *A. viridans* isolates obtained from cows in farm A between November 2011 and February 2012. Three patterns, designated as c, f and g, were observed in 3 (lanes 2, 8 and 12), 4 (lanes 6, 7, 13 and 14) and 2 isolates (lanes 9 and 10), respectively, from milk samples. PFGE patterns of 6 strains obtained from cows in farm A between December 2012 and March 2013 (lanes 17–22) were distinguished from those of the above 14 strains by 7 or more band differences, except for an isolate whose pattern could not be resolved (lane 21). Of the 6 strains isolated during the period between November 2012 and February 2013, 3 strains (lanes

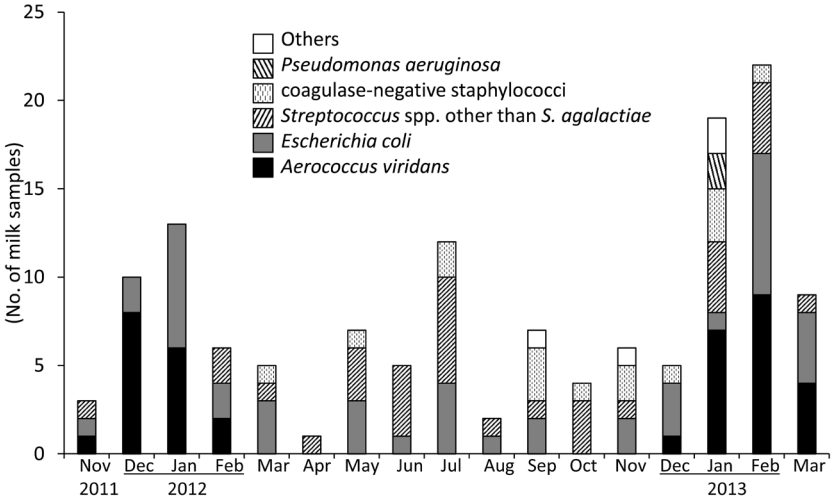

Fig. 1. Numbers of milk samples of cows from which the following bacteria were isolated: *A. viridans*, *E. coli*, *Streptococcus* spp. other than *S. agalactiae*, coagulase-negative staphylococci, *Pseudomonas aeruginosa* and others. Horizontal lines under the months represent winter season in Japan.

Table 1. Distribution of inhibition zone diameters determined with the disk diffusion testing for the 29 *A. viridans* isolates

Antimicrobials	Break-points	No. of isolates with an inhibition zone diameter of:														Resistant isolates (%)
		≤6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22	23-24	25-26	27-28	29-30	31-32	33-34	
Penicillin	14											8	5	9	6	1	0
Ampicillin	16											6	6	8	8	1	0
Amoxicillin-clavulanic acid	19											3	4	8	13	1	0
Cefazolin	14								1	10	6	10	1			1	0
Cefuroxime	14				1				2	5	8	9	4				3.4
Streptomycin	11				1	1	5	10	10	2							0
Tetracycline	12							1		1	5	11	6	4	1		0
Gentamicin	12								2	6	6	13	2				0
Chloramphenicol	17							1	1	2	7	8	7	3			3.4
Vancomycin	14								3	9	16	1					0
Erythromycin	13								1		6	9	10	2	1		0
Clindamycin	14	7	4	5	5	5		3									89.7
Ciprofloxacin	15									2	8	11	8				0

17, 19 and 22: pattern i) were possibly related to each other, because the number of difference in bands was less than 7. Of 3 strains isolated from the bedding materials covering the stall, 2 strains (lanes 23 and 25: pattern i) were closely related, because they differed by only 2 bands and also related to milk strains (lanes 17, 19 and 22). The PFGE patterns of 2 strains (lanes 26 and 27: pattern k) obtained from processed manures and those of the other 2 strains (lanes 28 and 29: pattern i) from processed manure were indistinguishable from each other, respectively. Interestingly, 4 isolates from the milk samples, processed manure and bedding materials (lanes 19, 25, 28 and 29, respectively: pattern i) were found to have identical PFGE patterns, and 3 other isolates from the milk and processed manure samples (lanes 20, 26 and 27: pattern k) were found to have PFGE patterns that were closely related to each other.

Survival of A. viridans at high pH and high temperature: Three milk strains (Fig. 2, lanes 6, 9 and 19) and a strain obtained from bedding materials (lane 23) were used. At pH 7 (Fig. 3A), viable cell counts of the 4 *A. viridans* strains were not significantly different from 3 at the time of inoculation, although the number of *A. viridans* ATCC700406 decreased to less than 3.0×10^2 cfu/m*l* after 3 hr. Viable cell counts of the 4 strains decreased from 3.3×10^4 to 2.1×10^5 after 6 hr to less than 3.0×10^2 cfu/m*l* 24 hr post inoculation. Viable cells of *E. coli* of both mastitis and reference strains were less than 3.0×10^2 cfu/m*l* after 6 hr, although one of the mastitis strains of *E. coli* survived until 3 hr post inoculation. At pH 9 (Fig. 3B), viable cell counts of the 4 strains decreased from 3.2×10^3 to 1.2×10^4 after 3 hr of inoculation to less than 3.0×10^2 cfu/m*l* after 6 and 24 hr. The numbers of viable cells of the reference strain of *A. viridans* and *E. coli* strains tested

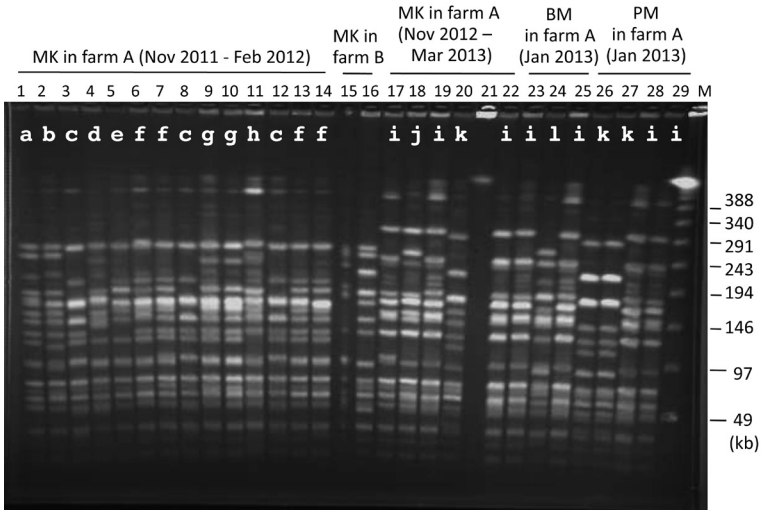

Fig. 2. *Sma*I-digested PFGE patterns of genome DNAs of representative *A. viridans* isolates. Lanes: 1 to 14, isolates from milk samples (MK) of 14 cows in farm A between November 2011 and February 2012; 15 and 16, MK of 2 cows in farm B, 17 to 22, MK of 6 cows in farm A between November 2012 and March 2013; 23 to 25, isolates from bedding materials (BM) sampled in January 2013 in farm A; 26 to 29, isolates from processed manure (PM) sampled in January 2013 in farm A. Lane M, lambda ladder. The lowercase letter below each of the lane numbers refers to the PFGE pattern (see RESULTS). The base sizes of the markers are indicated on the right of the panel.

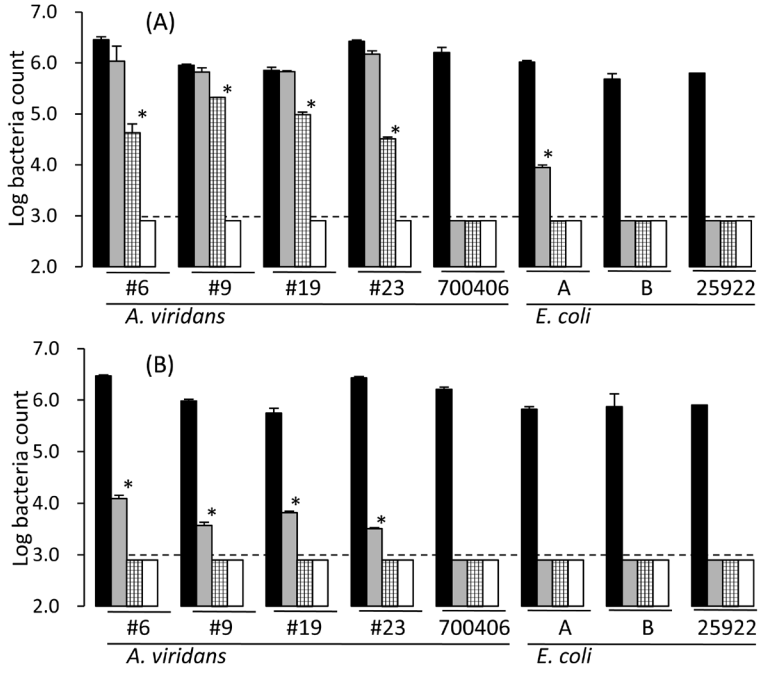

Fig. 3. Changes in viable cell counts of *A. viridans* and *E. coli* in (A) PBS (pH 7) and (B) the Good's buffer (pH 9) at 50°C after 0 (solid column), 3 (grey), 6 (grid) and 24 (open) hr. *A. viridans* isolates #6, #9, #19 and #23 correspond to those with the same numbers in Fig. 2. *E. coli* isolates A and B were from milk samples of cows with mastitis. 700406 and 25922 represent the ATCC strains of *A. viridans* and *E. coli*, respectively. Dashed lines indicate the detection limit (3.0×10^2 cfu/m*l*). Significant differences between initial viable cell numbers and those post inoculation are indicated by asterisks.

were less than 3.0×10^2 cfu/m*l* after 3, 6 and 24 hr.

pH and temperature of composted and processed manure: The temperature of composted manure varied from 10°C at a depth of 20 cm to 48°C at a depth of 100 cm, and that of processed manure varied from 50°C at a depth of 20 cm to 64°C at a depth of 100 cm. The pH of processed manure ranged from 9.5 at a depth of 20 cm to 10.1 at a depth of 100 cm, and that of bedding materials ranged from 7.5 to 9.2.

DISCUSSION

The role of *A. viridans* in the etiology of bovine mastitis has been unclear [6, 19]. On the other hand, *A. viridans* was isolated from cases of bovine mastitis [12]. In the study conducted using postcalving milk samples collected at 0 to 6 days in milk from a total of 1,091 cows from 6 commercial dairy herds in 4 different states in the United States of America between February 2011 and November 2011, *Aerococcus* spp. were the second-most common species, after coagulase-negative *Staphylococcus* spp. [1].

A. viridans was possibly associated with bovine mastitis cases in the present study. This organism was isolated in pure culture from 38 milk samples from cows with clinical mastitis. One of the sources of infection with *A. viridans* may be processed manure and bedding materials, because several strains of *A. viridans* isolated from cows with mastitis, processed manure and bedding materials were closely related as shown by their PFGE patterns and had an identical antimicrobial resistance phenotype.

Virtually, all the isolates were susceptible to the drugs tested, except for clindamycin. Only limited data are available on the antimicrobial resistance in *A. viridans* isolated from cows. In studies published in 1990 [11] and 2012 [12], substantial numbers of *A. viridans* isolates obtained from bovine intramammary infections were resistant to streptomycin, tetracycline, erythromycin and beta-lactams. Penicillin-resistant *A. viridans* strains, which were possibly induced by selective pressure by prolonged antibiotic use, were isolated from human cases [14, 16]. On the other hand, most (89.7%) of the *A. viridans* isolates in the present study were resistant to clindamycin. The reasons are unclear, because clindamycin is not used in clinical settings or in cow feed in Japan.

The *A. viridans* strains isolated in the present study may be able to survive in processed manure and bedding materials, because these strains were viable after 3 hr in alkaline conditions (pH 9) at 50°C *in vitro*. The strains isolated in the present study that survived under high temperature may have phenotypic characteristics different from those observed in most strains of *A. viridans.* Although the growth of the strains in the present study was not examined, this organism is considered to be unable to grow at 45°C according to the literature [7]. However, it is unclear whether tolerance to pH 9 was specific to the strains of *A. viridans* isolated in this study, since no information is available about the survival properties of *A. viridans* at high pH values.

In the present study, *A. viridans* strains were abundantly isolated from milk of cows with mastitis in winter. The temperatures recorded in piles of the composted manure and processed manure (10 to 64°C) were not as high as those generally observed during satisfactory composting. Thus, *A. viridans* strains that tolerate such moderately high temperature may survive in the manure under suboptimal composting conditions and thus be able to infect cows. Thus, farmers in dairy farms should consider taking steps to improve the hygiene of their bedding materials.

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