Widespread Distribution of Disinfectant Resistance Genes among Staphylococci of Bovine and Caprine Origin in Norway

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We demonstrate here a widespread distribution of genes mediating efflux-based resistance to quaternary ammonium compounds (QACs) in staphylococci from unpasteurized milk from 127 dairy cattle herds and 70 dairy goat herds. QAC resistance genes were identified in 21% of the cattle herds (*qacA/B*, *smr*, *qacG*, and *qacJ*) and in 10% of the goat herds (*qacA/B* and *smr*). Further examination of 42 QAC-resistant bovine and caprine isolates revealed the following genes: *qacA/B* (12 isolates) was present in four different species of coagulase-negative staphylococci (CoNS), *smr* (27 isolates) was detected in eight different CoNS species and in *Staphylococcus aureus* on a previously reported plasmid (pNVH99), *qacG* (two isolates) was detected on two plasmids (pST94-like) in *Staphylococcus cohnii* and *Staphylococcus warneri*, and *qacJ* (two isolates) was found in *Staphylococcus hominis* and *Staphylococcus delphini* on a plasmid (pNVH01) previously found in equine staphylococci. Isolation of indistinguishable pulsed-field gel electrophoresis (PFGE) CoNS types from tank milk and mammary quarter milk samples in a dairy cattle herd suggested that these QAC-resistant staphylococci were of intramammary origin. Indistinguishable or closely related PFGE types of bovine QAC-resistant CoNS were observed in different herds. One particular bovine *S. warneri* PFGE type was isolated repeatedly from samples collected during a 30-month period in a herd, showing long-term persistence. In conclusion, it seems that the widespread distribution of staphylococci carrying QAC resistance genes in Norwegian dairy cattle and goat herds is the result of both the intra- and interspecies spread of QAC resistance plasmids and the clonal spread of QAC-resistant strains.

Staphylococcus aureus and coagulase-negative staphylococci (CoNS) are common causes of bovine and caprine intramammary infections. *S. aureus* infections, which can be clinical or subclinical, frequently persist for a long time, and infected mammary glands thus serve as reservoirs from which the organism may spread to other cows within a herd and occasionally to other herds (4, 8, 30). CoNS are mainly causing subclinical mastitis, particularly in prepartum heifers and primiparous cows (12, 15, 16, 31). CoNS are part of the normal skin microflora of dairy ruminants and occur in their environment. Together with organisms of extramammary origin, bacteria causing subclinical intramammary infections are present in tank milk shipped from dairy farms. As part of quality assessment of raw milk, bacteriological examination of tank milk is performed regularly. Human pathogens are occasionally found in raw milk, giving rise to some concern regarding the consumption of products from unpasteurized milk (28). In addition, attention should be paid to the possible presence in raw milk of bacteria carrying antimicrobial resistance determinants (27). Both antibiotic resistance genes and disinfectant resistance genes should be considered.

Disinfectants based on quaternary ammonium compounds (QACs) have various applications in veterinary medicine and play an important role in the control of animal disease. The

QACs benzalkonium chloride (BC) and cetyltrimethylammonium bromide (CTAB) are active components in various teat preparations commonly used for mastitis prevention in dairy herds in Norway and other countries (5, 6). Efflux-mediated resistance to QACs has been observed in staphylococci of various origins, and several staphylococcal QAC resistance genes have been identified (2, 5, 6, 7, 9, 10, 11). These genes are in general plasmid-borne (21), encoding efflux proteins capable to expel hydrophobic drugs including QACs, the intercalating dye ethidium bromide (EtBr), and some other cationic biocides. The nearly identical genes *qacA* and *qacB* (denoted *qacA/B* gene here) are normally harbored by large plasmids of >20 kb, whereas the remaining QAC resistance genes have most frequently been found within their gene cassettes on small plasmids of less than 3 kb, where they encode efflux proteins belonging to the small multidrug resistance family (1, 14, 17, 18).

Little is known about the occurrence of QAC-resistant staphylococci in dairy herds and in raw milk from such herds. The QAC resistance determinants involved and the extent to which such determinants are spread between herds remain unclear. Typing of *S. aureus* resistant to various antibiotics has shown that certain strains are widely disseminated in dairy herds, clearly demonstrating that clonal spread of resistant organisms is important (30). Moreover, apparently identical plasmids carrying antibiotic resistance determinants have been found in different *S. aureus* strains recovered from various dairy cattle herds and horse operations, suggesting that mech-

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organisms other than clonal spread are also involved in the dissemination of resistance genes (7, 30).

Studies in Norway of human clinical staphylococci and food-related staphylococci have shown that a linkage between QAC resistance and penicillin resistance occurs quite frequently (23, 24). *qacA/B* and the β -lactamase gene (*blaZ*) were found to reside on common plasmids. Thus, it is conceivable that the use of QACs for disinfection in hospitals and farm animal operations could select for penicillin-resistant staphylococci and vice versa.

Here we report on the prevalence and molecular epidemiology of QAC-resistant staphylococci in raw milk from dairy cattle and goat herds. We identified the QAC resistance genes and characterized plasmids carrying them. In staphylococci resistant to both QACs and penicillin, we examined whether *blaZ* and a QAC resistance gene were residing on common plasmids.

MATERIALS AND METHODS

Herds, animals, and bacterial isolates. During a 7-month period (June to December 2001), bulk milk samples from 127 dairy cattle herds and 70 dairy goat herds were collected. The herds were randomly selected from the dairy herd population in eight of the 19 counties in Norway. The samples were screened with regard to QAC-resistant staphylococci.

One dairy cattle herd (B-2), in which QAC-resistant staphylococci were isolated from bulk milk, bulk milk samples were collected three times during a 30-month period in order to study whether such organisms persisted in the herd. To examine whether QAC-resistant staphylococci were of intramammary origin, quarter milk samples were collected aseptically from all lactating cows in herd B-2 and four herds that used a common pasture and a common milking parlor during the summer (B-5¹, B-5², B-5³, and B-5⁴).

Screening procedure. Milk (100 μ l) was spread on blood agar plates containing BC (11 μ g/ml) and incubated for 24 h at 37°C. From plates on which growth was detected, one to five colonies of different appearance were selected for further examination with regard to efflux-mediated resistance. Isolates were grown for 24 h at 37°C on Mueller-Hinton agar containing EtBr (0.5 μ g/ml), followed by inspection for fluorescence under UV light as described previously (25). Cells accumulating EtBr had a red fluorescence and were considered QAC sensitive; cells that did not accumulate EtBr were white and were defined as QAC resistant.

Antimicrobial susceptibility. MICs of BC and CTAB were determined in a microtiter assay at 0.5- μ g/ml concentration intervals from 0 to 18 μ g/ml in Mueller-Hinton broth as described previously (25) and were carried out twice. The MICs of the following antibiotics were determined by Etest (AB Biodisk, Solna, Sweden), using *S. aureus* ATCC 29213 as a reference strain: benzylpenicillin (PEN), streptomycin (STR), tetracycline (TET), trimethoprim (TMP), sulfadiazine (SUL), fusidic acid (FUS), and oxacillin. The β -lactamase activity was detected by using the microbiological cloverleaf test (3, 20).

PCR amplification. The primers used for PCR amplification (Invitrogen, Paisley, United Kingdom) of *qacA/B* and *smr* were the same as used previously (2, 6). Primers specific for the genes *qacG*, *qacH*, *qacJ*, and *blaZ* were designed from previously known sequences (EMBL accession numbers Y16944, Y16945, AJ512814, and X52734, respectively): *qacG*-For (5'-CAACAGAAATAATCGG AACT-3')/Rev (5'-TACATTTAAGAGCACTACA-3'), *qacH*-For (5'-ATAGT CAGTGAAGTAATAG-3')/Rev (5'-AGTGTGATGATCCGAATGT-3'), *qacJ*-For (5'-CTTATATTTAGTAATAGCG-3')/Rev (5'-GATCCAAAAACGTTAA GA-3'), and *blaZ*-For (5'-TACAACTGTAATATCGGAGGG-3')/Rev (5'-AG GAGAATAAGCAACTATATCATC-3'). The following strains were used as positive controls: *Staphylococcus haemolyticus* NVH97A (*qacA/B blaZ*) (2), *S. aureus* NVH99 (*smr*) (6), *Staphylococcus warneri* ST94 (*qacG*) (10), *Staphylococcus saprophyticus* ST2H6 (*qacH*) (9), and *S. aureus* NVH01 (*qacJ*) (7). The composition of each 50- μ l PCR mixture was as recommended by the manufacturer, using DyNAzyme II DNA polymerase with supplementary reagents (Finnzymes Oy, Espoo, Finland). PCR conditions included DNA denaturation at 95°C for 60 s, followed by 30 cycles at 95°C for 60 s, annealing for 45 s at 40°C (*qacA/B*), 48°C (*smr*, *qacG*, *qacH*, and *qacJ*), or 50°C (*blaZ*), and then 72°C for 60 s. To obtain probes for DNA hybridization, PCR was carried out with the

control strains from the above-described PCR experiments as templates. The resulting PCR products were analyzed by agarose gel electrophoresis.

DNA isolation and hybridization. Plasmid DNA was isolated by using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany) modified by adding lysostaphin (Sigma-Aldrich, St. Louis, Mo.) as described previously (6). Plasmid DNA from 55 staphylococcal isolates was separated by gel electrophoresis in 0.8% SeaKem LE agarose (FMC BioProducts, Rockland, ME) with Supercoiled DNA Ladder (Life Technologies, Paisley, United Kingdom) as a molecular weight marker. The size of plasmids was estimated by using the GeneGenius gel documentation and analysis system (SynGene Laboratories, Cambridge, United Kingdom). By extrapolating a standard curve, this procedure allows initial size estimation of plasmids above the range of the molecular weight marker, which only goes up to 16 kb. The localization of QAC resistance genes and *blaZ* was determined by using the following procedure: plasmid DNA was blotted from the agarose gels to nylon membranes (Hybond N+; Amersham Biosciences, Little Chalfont, United Kingdom) by using Southern blotting according to described procedures (22). PCR products amplified with primers specific for the genes *qacA/B* (common probe), *smr*, *qacG*, *qacH*, *qacJ*, and *blaZ* were used as probes after purification with the QIAgen Gel Extraction kit (QIAGEN). The probes were labeled with [α -³²P]dCTP (Hartmann Analytic GmbH, Braunschweig, Germany) by using Ready-To-Go DNA labeling beads (Amersham Biosciences, Piscataway, NJ). Prehybridizations, hybridizations, and washing were carried out according to standard procedures (22). The membranes were finally exposed to autoradiography film (Kodak, Rochester, N.Y.) at -70°C.

Species determination. Identification of staphylococci was based on standard laboratory criteria (colony morphology, hemolytic zones, and production of catalase and coagulase) and the use of Staph-Zym biochemical test kit (Rosco, Tåstrup, Denmark). In addition, ca. 83% of the *sodA* gene, the internal fragment denoted *sodA*_{int}, was sequenced by using a degenerate primer pair *d1/d2* (Invitrogen) as described previously (19). Moreover, partial sequencing of the 16S rRNA gene was subsequently carried out for those not identified to species level by Staph-Zym and *sodA* sequencing, with the primers 16S rRNA For/Rev used previously (7). In order to obtain at least 85% of the 16S rRNA sequence, three additional primers were constructed and used: 16S rRNA-For357 (5'-GAAAG CCTGACGGAGCAAC-3'), 16S rRNA-Rev1068 (5'-CCAACATCTCAGCAG ACGAG-3'), and 16S rRNA-Rev1402 (5'-CAAACCTCTCGTGGTGTGACG-3'). The sequencing was carried out with the capillary sequencer 3100-Avant genetic analyzer (Applied Biosystems, Foster City, Calif.) and BigDye Terminator v3.1 Cycle Sequencing (Applied Biosystems). Sequences were edited and aligned by using the BioEdit program (<http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html>), and homology searches were performed with the NCBI Gene-Blast.

Sequencing of QAC resistance plasmids and genes. Five plasmids were selected for sequencing in order to detect the possible presence of previously characterized plasmids (6, 7, 10). Single-strand sequencing was chosen as the most appropriate method compared to plasmid restriction analysis of isolates containing more than one plasmid. The plasmids were isolated from *smr*-containing *S. aureus*, *qacG*-containing *Staphylococcus cohnii* and *S. warneri*, and *qacJ*-containing *Staphylococcus hominis* and *Staphylococcus delphini* (Table 1). Using plasmid DNA from these five strains as a template, single-strand sequencing was carried out by primer walking starting with the primer pairs *smr*-For/Rev (6), *qacG*-For/Rev, and *qacJ*-For/Rev. All sequencing, editing, alignment, and homology searches were performed as described for the species determination. In order to confirm PCR results (14 strains) indicating a previously described partial *smr* sequence duplication designated *qacC'* (13), three strains from different species were selected for plasmid-borne gene sequencing: *S. saprophyticus*, *S. warneri*, and *S. haemolyticus* belonging to PFGE types SS-2, SW-1, and SH-3b, respectively (Table 1). Primers specific for *qacC'* were designed for a final screening PCR of all *smr*-containing isolates: *qacC'*-For (5'-AATAAAATACG AAAATTTAAAGGAG-3') and -Rev (5'-ACGCCGACTATGATTTAAAC-3'). *S. aureus* NVH99 (*smr*) and *Staphylococcus pasteurii* (PFGE type SP-1) were used as negative controls. PCR was carried out under the same conditions as described for detection of the gene *smr*.

PFGE. Molecular fingerprinting of staphylococci was performed by pulsed-field gel electrophoresis (PFGE) with a CHEF-DRIII apparatus (Bio-Rad Laboratories, Hercules, Calif.) as described previously (7). PFGE patterns were compared by visual examination and were interpreted according to recommended guidelines (26). Closely related types (one- to three-band difference) were given the same PFGE type number but different lowercase letters. Different types (>3 band differences) were given different numbers.

TABLE 1. Characterization of 35 bovine and 7 caprine staphylococcal isolates resistant to QACs

QAC resistance gene	Staphylococcal species	PFGE type ^a	Plasmid size ^b (kb)	Herd ^c	Antibiotic resistance ^d	MIC ^e (µg/ml)	
						BC	CTAB
<i>qacA/B</i>	<i>S. warneri</i>	SW-2a	23	B-5 ²	PEN ^f	3.0	6.5
	<i>S. warneri</i>	SW-2b	24	B-5 ¹	PEN ^f	2.5	6.0
	<i>S. warneri</i>	SW-4a	24	B-13	PEN ^f , STR, TET	3.0	7.5
	<i>S. warneri</i>	SW-4a	23	B-9	PEN ^f , STR	2.5	5.5
	<i>S. warneri</i>	SW-4b	25	C-4	PEN ^f , STR	3.0	8.0
	<i>S. warneri</i>	SW-5	26	B-14	PEN ^f , FUS	2.0	4.0
	<i>S. warneri</i>	SW-6	25	B-6	PEN ^f	2.5	4.0
	<i>S. warneri</i>	SW-7	18	B-2	PEN, STR	3.0	6.5
	<i>S. epidermidis</i>	SE-1	23	B-21		3.5	6.0
	<i>Staphylococcus</i> sp.	SC-1	23	B-18	PEN ^f , FUS	2.0	3.0
	<i>Staphylococcus</i> sp.	SC-1	23	B-24	PEN ^f , FUS	2.0	3.0
<i>qacA/B</i> + <i>smr</i>	<i>S. haemolyticus</i>	SH-5	23, 2.3 ^g	B-5 ²	PEN, STR, TET, TMP, FUS	3.5	8.0
<i>smr</i>	<i>S. haemolyticus</i>	SH-1b	23	B-4	PEN, SUL	1.5	2.0
	<i>S. haemolyticus</i>	SH-1b	23	B-5 ³	PEN	1.5	3.0
	<i>S. haemolyticus</i> ^h	SH-1b	23	B-5 ⁴	PEN	1.5	3.0
	<i>S. haemolyticus</i>	SH-2a	2.1	B-7		3.0	8.0
	<i>S. warneri</i>	SW-3a	2.3	C-1		2.5	8.5
	<i>S. warneri</i>	SW-3b	3.0	C-2		2.5	9.0
	<i>S. warneri</i>	SW-3c	2.4	C-3		2.5	8.0
	<i>S. caprae</i>	SP-1	3.0	C-7		3.5	10.0
	<i>S. aureus</i>	SA-1	2.2	B-20	PEN	2.5	6.5
	<i>S. epidermidis</i> ^h	SE-2	3.6	B-5 ⁴		2.5	6.5
	<i>S. hominis</i>	SO-1	2.3	B-22	PEN, STR	3.0	7.5
	<i>S. pasteurii</i>	SP-1	5.6	B-19	PEN ⁱ , STR	3.0	8.0
	<i>S. warneri</i> ^j	SW-1	5.8	B-2		2.5	8.5
	<i>S. haemolyticus</i>	SH-1a	2.4	B-1	PEN	2.0	7.0
<i>smr</i> + <i>qacC'</i>	<i>S. haemolyticus</i> ^k	SH-1a	2.3	B-2	PEN	1.5	2.5
	<i>S. haemolyticus</i>	SH-1b	2.8	B-3	PEN	1.0	3.5
	<i>S. haemolyticus</i>	SH-1b	2.8	B-23	PEN	1.0	4.0
	<i>S. haemolyticus</i> ^k	SH-1c	2.4	B-6	PEN	1.5	4.0
	<i>S. haemolyticus</i>	SH-2b	2.7	B-8		2.5	7.0
	<i>S. haemolyticus</i>	SH-3a	2.4	B-9	PEN, STR	3.5	10.0
	<i>S. haemolyticus</i>	SH-3b	2.2	B-10	PEN	3.0	10.0
	<i>S. haemolyticus</i>	SH-4	2.4	B-11	PEN	2.5	11.0
	<i>S. saprophyticus</i>	SS-1	2.4	C-5		5.0	15.5
	<i>S. saprophyticus</i>	SS-2	2.4	C-6	FUS	4.5	15.5
	<i>Staphylococcus</i> sp.	SC-2	2.4	B-16	PEN, STR	2.5	5.0
	<i>Staphylococcus</i> sp.	SC-2	2.4	B-17	PEN	1.5	3.0
	<i>S. cohnii</i>	SN-1	2.2	B-12	PEN	5.0	15.0
	<i>S. warneri</i>	SW-8	2.2	B-15	PEN, STR	3.5	11.5
	<i>S. hominis</i>	SO-2	2.7	B-5 ²		2.0	5.5
	<i>S. delphini</i> ^l	SD-1	2.7	B-2		3.5	7.5

^a SmaI restriction banding patterns: indistinguishable patterns within staphylococcal species share the same number and lowercase letter; closely related types (one to three band differences) are given the same number but different lowercase letters; different types (>3 band differences) have different numbers.

^b Plasmids that harbored QAC resistance gene. pNVH99 (5) harbored *smr* in the *S. aureus* isolate, whereas pNVH01 (6) harbored *qacI*.

^c Of bovine (B) or caprine (C) origin, with different identity numbers for different herds. The herds B-5¹ to B-5⁴ used a common pasture and a common milking parlor during the summer. The isolates from B-5¹ to B-5⁴ were recovered from quarter milk samples.

^d Resistance to PEN, STR, TET, TMP, and FUS was determined.

^e MICs were reproducible within a maximum range of 0.5 µg/ml for BC and 1.0 µg/ml for CTAB, with the highest obtained values presented in the table.

^f *blaZ* and *qacA/B* located on the same plasmid.

^g *qacA/B* on the 20.0-kb plasmid; *smr* on the 2.3-kb plasmid.

^h The strain was recovered from bulk milk and mammary quarter milk.

ⁱ *blaZ* located on a 26-kb plasmid.

^j The strain was repeatedly recovered from bulk milk and individual cows in the herd during a 30-month period.

^k The strain was repeatedly recovered in bulk milk samples from the herd during a 7-month period.

^l The strain was recovered from different mammary quarters in one animal in the herd.

RESULTS

Staphylococcal isolates. Screening of bulk milk samples on agar plates revealed staphylococci resistant to QACs in bulk milk from 27 (21.3%) of 127 cattle herds and from 7 (10.0%)

of 70 goat herds. After the screening of bulk milk and mammary quarter milk samples, 55 QAC-resistant isolates were selected for further molecular genetic studies; 37 isolates were from bulk milk, and 18 isolates were from quarter milk. Iso-

lates from the same herd having the same antibiotic resistance profile and indistinguishable PFGE patterns were considered identical; only one of such isolates was included in Table 1, where the characteristics of 42 QAC-resistant isolates are presented.

Species determination. The distribution of the 42 isolates among staphylococcal species is presented in Table 1. Staph-Zym biochemical test kit results were in agreement with *sodA* sequencing results for 32 of totally 42 isolates: *S. haemolyticus* (14 isolates), *S. warneri* (13 isolates), *S. saprophyticus* (2 isolates), *S. aureus* (1 isolate), *Staphylococcus epidermidis* PFGE type SE-1 (1 isolate), and *S. hominis* PFGE type SO-1 (1 isolate). Staph-Zym failed to assign a species name for the ten remaining isolates; six of these isolates were assigned a species name based on *sodA* sequencing results: *Staphylococcus caprae*, *S. pasteurii*, *S. epidermidis* PFGE type SE-2, *S. hominis* PFGE type SO-2, *S. cohnii*, and *S. delphini*. Unacceptable *sodA* sequence homology (ca. 90%) was obtained for the four remaining isolates: *Staphylococcus* sp. PFGE type SC-1 (two isolates) and *Staphylococcus* sp. PFGE type SC-2 (two isolates). However, pairwise alignment of the *sodA* sequences from the two *Staphylococcus* sp. PFGE types SC-1 and SC-2 showed 95% identity. Subsequent sequencing of more than 85% of the 16S rRNA gene did not result in sufficient information to identify the species of these isolates, and thus they were designated *Staphylococcus* spp. These findings indicate the possible presence of one or two novel staphylococcal species.

Genes and plasmids identified by PCR and sequencing. PCR detected four different QAC resistance genes (Table 1). The gene *smr* was found in 21 bovine and six caprine isolates, and the *qacC'* sequence duplication was detected in 12 bovine and 2 caprine isolates; the presence of *qacC'* sequence was confirmed by partial plasmid sequencing and showed 100% identity to the corresponding sequence in pSK108 (13). Single-strand primer walking sequencing of five selected plasmids resulted in complete plasmid sequences and identification of the two previously sequenced *smr*- and *qacJ*-containing plasmids pNVH99 (6) in *S. aureus* and pNVH01 (7) in *S. hominis* and *S. delphini* based on 99 to 100% sequence identity. *qacG* was harbored by two different plasmids, both showing ca. 90% sequence identity to the previously sequenced *qacG*-containing plasmid pST94 (10) and therefore considered as pST94-like plasmids (Table 1). However, the conserved *qacG* sequence in the two pST94-like plasmids was 100% identical to the previously sequenced *qacG* (10).

***blaZ* and QAC-resistance genes on common plasmids.** Hybridization revealed that the *blaZ* and *qacA/B* genes were linked together on common plasmids of similar sizes in nine isolates (*S. warneri*, *Staphylococcus* sp.), including one caprine *S. warneri* isolate. However, in one *S. pasteurii* isolate, *smr* resided on a 5.6-kb plasmid, and a 26-kb plasmid harbored *blaZ* but not *qacA/B* (Table 1).

Susceptibility. MICs for BC and CTAB are presented in Table 1. A total of 29 isolates were resistant to PEN (all β -lactamase producing); 27 of these had MICs from 0.25 to 1.0 μ g/ml, while the MICs for two isolates (*Staphylococcus* sp. PFGE type SC-2) were 0.125 μ g/ml (the cutoff value for PEN-susceptible strains). Plasmid-borne *blaZ* was present in 10 isolates. Resistance to STR was detected in 10 isolates, with MICs ranging from 96 to >256 μ g/ml. Two isolates were resistant to

TET, and four isolates were resistant to FUS. One *S. haemolyticus* isolate carrying both *qacA/B* and *smr* was resistant to PEN, STR, TET, and FUS, and moderately resistant to TMP (MIC = 24 μ g/ml). One *S. haemolyticus* isolate (herd B-4) was resistant to SUL (MIC > 1,024 μ g/ml) and moderately resistant to TMP (MIC = 16 μ g/ml). All isolates were susceptible to oxacillin.

PFGE types. PFGE types within staphylococcal species are presented in Table 1. Eight different PFGE types of *S. warneri* were identified. Isolates with indistinguishable or closely related (one- to three-band differences) PFGE patterns (SW-2 and SW-4) were recovered from different cattle herds. *S. warneri* isolates from two cattle herds (B-9 and B-13) and one goat herd (C-4) were closely related (SW-4a and SW-4b). Closely related PFGE types of *S. warneri* (PFGE type SW-3) were also identified in three different goat herds. Isolates of *Staphylococcus* sp. with PFGE type SC-2 were recovered from two different cattle herds (B-16 and B-17), and *Staphylococcus* sp. with another PFGE pattern (SC-1) was identified in two other herds (B-18 and B-24). Five PFGE types of *S. haemolyticus* were identified; indistinguishable or closely related types (SH-1, SH-2, and SH-3) were identified in different cattle herds, and one particular type (SH-1b) was found in two different cattle herds (B-5³ and B-5⁴) that shared a common pasture and milking parlor during summer.

DISCUSSION

We found staphylococci with efflux-mediated resistance to QACs in bulk tank milk from 21% of the 127 dairy cattle herds and 10% of the 70 dairy goat herds that were screened. The herds were selected at random from various parts of Norway without prior knowledge of the occurrence of QAC resistance determinants or use of QAC-containing preparations. Therefore, these figures might be an indication of the prevalence of dairy herds in Norway in which such organisms are present in bulk milk.

Infected mammary glands could be a source for QAC-resistant staphylococci isolated from tank milk, but such organisms may also originate from teat skin or other extramammary reservoirs. Besides being screened for QAC-resistant staphylococci, the quarter milk samples were examined bacteriologically using methods based on recommendations of the International Dairy Federation as previously described (6). The presence of indistinguishable PFGE types of *S. warneri* (SW-1) in mammary quarter milk and bulk milk from the same herd suggests that mammary glands were a likely source of this strain. Furthermore, indistinguishable PFGE types of *S. haemolyticus* (SH-1a) were recovered from the bulk milk in one herd and from milk from infected mammary quarters of cows in another herd. Another *S. haemolyticus* PFGE type (SH-1b), closely related to SH-1a, was recovered from bulk milk from three different herds and from infected quarters of cows in two other herds. Thus, at least some of the QAC-resistant CoNS in bulk milk were mammary pathogens.

From the agar plates used for screening, only a limited number of colonies was selected for further characterization. Nevertheless, QAC-resistant isolates of both *S. aureus*, coagulase-positive *S. delphini*, and several CoNS species were found. The most prevalent species were *S. haemolyticus* and *S. war-*

neri. *S. aureus* was found in only one cattle herd, suggesting that bovine and caprine *S. aureus* are less likely to confer QAC resistance than are certain species of CoNS.

All previously known plasmid-borne staphylococcal QAC resistance genes, except for the *qacH* gene, were identified. The *smr* gene was the most prevalent and was harbored by small plasmids (2.2 to 3.6 kb) in 23 different isolates belonging to various staphylococcal species and by large plasmids (23 kb) in three *S. haemolyticus* isolates with indistinguishable PFGE patterns. In the single *S. aureus* strain, *smr* was located on the 2.2-kb pNVH99 plasmid previously detected in bovine *S. aureus* (6). Interestingly, isolates of *S. pasteurii* and *S. warneri* harbored *smr* on medium-sized plasmids (5.6 to 5.8 kb), a phenomenon not previously reported.

Partial sequence duplication of the *smr* gene (*qacC'*) previously found in pSK108 (13) occurred in 15 of the isolates that contained *smr*. These isolates belonged to four CoNS species; two were *S. saprophyticus* isolates of caprine origin. This cassette feature appears to be quite widespread and could easily be overlooked, since common *smr* primers do not necessarily amplify the slightly larger additional PCR product in sufficient quantities. The MIC results in our study support previous findings that the partial *smr* sequence duplication is unlikely to mediate any significant effect on the resistance to QACs (13).

qacA/B was found on large plasmids (18 to 26 kb) in 12 isolates representing four different CoNS species. Eight of these were *S. warneri* isolates. With the exception of a *S. haemolyticus* isolate, which contained both *qacA/B* and *smr* located on separate plasmids, the isolates contained only one of the plasmid-borne QAC resistance genes.

The *qacG* gene, which was found in one *S. warneri* and one *S. cohnii* isolate, resided on plasmids closely related to pST94 (10). *qacJ* was found in *S. hominis* and *S. delphini* isolates, in both isolates harbored by pNVH01. Previously, *qacJ* located on this plasmid has been found in equine isolates of *S. aureus*, *S. simulans*, and *S. intermedius* (7); thus, pNVH01 is disseminated among multiple staphylococcal species of both bovine and equine origin.

PFGE typing of the CoNS showed that certain types occurred in two or more herds. Particularly widespread were three closely related *S. haemolyticus* strains (PFGE types SH-1a, SH-1b, and SH-1c), which were recovered from eight cattle herds in five different counties. Closely related PFGE types of *S. warneri* (SW-3a, SW-3b, and SW-3c) were present in three different goat herds located in two different counties. A different subset of closely related *S. warneri* PFGE types (SW-4a and SW-4b) was recovered from one goat herd and two cattle herds within the same county. The staphylococci for which the *sodA* and 16S rRNA sequences did not agree with sequences of species currently identified by these analyses belonged to two different PFGE types, and each type was found in two different cattle herds within the same county. Thus, clonal dissemination seems to be an important mode for the spread of QAC-resistant CoNS between dairy herds, including spread between cattle and goat herds.

Plasmid-borne *blaZ* was detected in 10 (34%) of the 29 penicillin-resistant isolates. A study in Denmark of bovine *S. aureus* containing *blaZ* found that this gene was located on plasmids in only 7% of the isolates (29). In the present study, *blaZ* and *qacA/B* coresided on large plasmids in nine different

isolates. Recent studies in Norway of human clinical staphylococcal isolates and food-related staphylococci suggest that there is a linkage between resistance to QACs and resistance to penicillin (23, 24). In human hospital environments, it has been shown that staphylococci resistant to BC more frequently were resistant to certain antibiotics than were BC-sensitive isolates, indicating that the presence of either resistance determinant selects for the other during antimicrobial therapy and disinfection (23). In one of the herds, where QAC preparations were used daily for teat disinfection to prevent mastitis, milk samples were collected on several occasions. Indistinguishable PFGE types of QAC-resistant *S. warneri* were isolated 30 months apart. Apparently, certain QAC-resistant CoNS can persist for long periods in herds where QACs are used routinely for disinfection.

In conclusion, QAC-resistant staphylococci occur quite frequently in bulk milk shipped from dairy cattle and goat farms in Norway. *S. warneri* and *S. haemolyticus* seem to be the most common species in unpasteurized milk but other CoNS, the coagulase-positive *S. delphini*, and *S. aureus* were also isolated. Various plasmid-borne genes were responsible for the observed QAC resistance; the genes *qacA/B*, *smr*, *qacG*, and *qacJ* were detected. The fact that certain staphylococcal PFGE types were recovered from more than one herd shows that the occurrence of QAC-resistant staphylococci in dairy herds can be the result of clonal spread. In addition, the presence of identical QAC resistance genes, and certain plasmids carrying such genes, in different staphylococcal species and PFGE types clearly suggest that transfer of QAC resistance genes among species and strains contributes to the dissemination of staphylococcal QAC resistance. The presence of *qacA/B* and *blaZ* on common plasmids in seven *S. warneri* isolates and two isolates of an unidentified *Staphylococcus* sp. seems to support the previous observation that there is a linkage between resistance to QAC and penicillin resistance in staphylococci. This may allow for coselection due to antibiotic treatment or disinfection using QACs.

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REFERENCES

1. Alam, M. M., M. Ishino, and N. Kobayashi. 2003. Analysis of genomic diversity and evolution of the low-level antiseptic resistance gene *smr* in *Staphylococcus aureus*. *Microb. Drug Resist.* 9(Suppl. 1):S1-S7.
2. Anthonisen, I. L., M. Sunde, T. M. Steinum, M. S. Sidhu, and H. Sørum. 2002. Organization of the antiseptic resistance gene *qacA* and Tn552-related β -lactamase genes in multidrug-resistant *Staphylococcus haemolyticus* strains of animal and human origins. *Antimicrob. Agents Chemother.* 46:3606-3612.
3. Bergan, T., J. N. Bruun, A. Digraanes, E. Lingaas, K. K. Melby, and J. Sander. 1997. Susceptibility testing of bacteria and fungi: report from the Norwegian Working Group on Antibiotics. *Scand. J. Infect. Dis. Suppl.* 103:1-36.
4. Bergonier, D., R. de Cremoux, R. Rupp, G. Lagriffoul, and X. Berthelot. 2003. Mastitis of dairy small ruminants. *Vet. Res.* 34:689-716.
5. Bjorland, J., and M. Sunde. 2001. Plasmid-borne *qacA/B* and *smr* genes cause resistance to quaternary ammonium compounds in bovine coagulase-negative staphylococci. In Abstracts of the summer conference on antibiotic and biocide resistance in bacteria. Society for Applied Microbiology, Bedford, United Kingdom.
6. Bjorland, J., M. Sunde, and S. Waage. 2001. Plasmid-borne *smr* gene causes resistance to quaternary ammonium compounds in bovine *Staphylococcus aureus*. *J. Clin. Microbiol.* 39:3999-4004.
7. Bjorland, J., T. Steinum, M. Sunde, S. Waage, and E. Heir. 2003. Novel plasmid-borne gene *qacJ* mediates resistance to quaternary ammonium com-

- pounds in equine *Staphylococcus aureus*, *Staphylococcus simulans*, and *Staphylococcus intermedius*. Antimicrob. Agents Chemother. **47**:3046–3052.
8. Bramley, A. J., and F. H. Dodd. 1984. Reviews of the progress of dairy science: mastitis control: progress and prospects. J. Dairy Res. **51**:481–512.
 9. Heir, E., G. Sundheim, and A. L. Holck. 1998. The *Staphylococcus qacH* gene product: a new member of the SMR family encoding multidrug resistance. FEMS Microbiol. Lett. **163**:49–56.
 10. Heir, E., G. Sundheim, and A. L. Holck. 1999. The *qacG* gene on plasmid pST94 confers resistance to quaternary ammonium compounds in staphylococci isolated from the food industry. J. Appl. Microbiol. **86**:378–388.
 11. Heir, E., G. Sundheim, and A. L. Holck. 1999. Identification and characterization of quaternary ammonium compound resistant staphylococci from the food industry. Int. J. Food Microbiol. **48**:211–219.
 12. Jarp, J. 1991. Classification of coagulase-negative staphylococci isolated from bovine clinical and subclinical mastitis. Vet. Microbiol. **27**:151–158.
 13. Leelaporn, A., N. Firth, I. T. Paulsen, A. Hettiaratchi, and R. A. Skurray. 1995. Multidrug resistance plasmid pSK108 from coagulase-negative staphylococci; relationship to *Staphylococcus aureus qacC* plasmids. Plasmid **34**: 62–67.
 14. Littlejohn, T. G., D. DiBerardino, L. J. Messerotti, S. J. Spiers, and R. A. Skurray. 1991. Structure and evolution of a family of genes encoding anti-septic and disinfectant resistance in *Staphylococcus aureus*. Gene **101**:59–66.
 15. Myllis, V. 1995. Staphylococci in heifer mastitis before and after parturition. J. Dairy Res. **62**:51–60.
 16. Oliver, S. P., and B. M. Jayarao. 1997. Coagulase-negative staphylococcal intramammary infections in cows and heifers during the nonlactating and periparturient periods. J. Vet. Med. B **44**:355–363.
 17. Paulsen, I. T., M. H. Brown, T. G. Littlejohn, B. A. Mitchell, and R. A. Skurray. 1996. Multidrug resistance proteins QacA and QacB from *Staphylococcus aureus*: membrane topology and identification of residues involved in substrate specificity. Proc. Natl. Acad. Sci. USA **93**:3630–3635.
 18. Paulsen, I. T., M. H. Brown, S. J. Dunstan, and R. A. Skurray. 1995. Molecular characterization of the staphylococcal multidrug resistance export protein QacC. J. Bacteriol. **177**:2827–2833.
 19. Poyart, C., G. Quesne, C. Boumaila, and P. Trieu-Cuot. 2001. Rapid and accurate species-level identification of coagulase-negative staphylococci by using the *sodA* gene as a target. J. Clin. Microbiol. **39**:4296–4301.
 20. Rosdahl, V. T., Jarlöv, J. O., and A. M. Knudsen. 1986. Beta-lactamase production in coagulase-negative micrococaceae. Acta Pathol. Immunol. Microbiol. Scand. B **94**:423–427.
 21. Russell, A. D. 1997. Plasmids and bacterial resistance to biocides. J. Appl. Microbiol. **83**:155–165.
 22. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 23. Sidhu, M. S., E. Heir, T. Leegaard, K. Wiger, and A. Holck. 2002. Frequency of disinfectant resistance genes and genetic linkage with β -lactamase transposon Tn552 among clinical staphylococci. Antimicrob. Agents Chemother. **46**:2797–2803.
 24. Sidhu, M. S., E. Heir, H. Sørum, and A. Holck. 2001. Genetic linkage between resistance to quaternary ammonium compounds and β -lactam antibiotics in food-related *Staphylococcus* spp. Microb. Drug Resist. **7**:363–371.
 25. Sundheim, G., T. Hagtvædt, and R. Dainty. 1992. Resistance of meat associated staphylococci to a quaternary ammonium compound. Food Microbiol. **9**:161–167.
 26. Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33**:2233–2239.
 27. Teuber, M. 1999. Spread of antibiotic resistance with food-borne pathogens. Cell. Mol. Life Sci. **56**:755–763.
 28. van Kessel, J. S., J. S. Karns, L. Gorski, B. J. McClusk, and M. L. Perdue. 2004. Prevalence of *Salmonellae*, *Listeria monocytogenes*, and fecal coliforms in bulk tank milk on US dairies. J. Dairy Sci. **87**:2822–2830.
 29. Vesterholm-Nielsen, M., M. Olhom Larsen, J. Elmerdahl Olsen, and F. M. Aarestrup. 1999. Occurrence of the *blaZ* gene in penicillin resistant *Staphylococcus aureus* isolated from bovine mastitis in Denmark. Acta Vet. Scand. **40**:279–286.
 30. Waage, S., J. Bjorland, D. A. Caugant, H. Oppegaard, T. Tollersrud, T. Mørk, and F. M. Aarestrup. 2002. Spread of *Staphylococcus aureus* resistant to penicillin and tetracycline within and between dairy herds. Epidemiol. Infect. **129**:193–202.
 31. Waage, S., T. Mørk, A. Røros, D. Aasland, A. Hunshamar, and S. A. Ødegaard. 1999. Bacteria associated with clinical mastitis in dairy heifers. J. Dairy Sci. **82**:712–719.