Characterization of Dutch *Staphylococcus aureus* from bovine mastitis using a Multiple Locus Variable Number Tandem Repeat Analysis

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ABSTRACT

Current typing methods for *Staphylococcus aureus* have important drawbacks. We evaluated a Multiple Locus Variable Number Tandem Repeat Analysis (MLVA) scheme with 6 loci which lacks most drawbacks on 85 bovine mastitis isolates from The Netherlands. For each locus the number of repeat units (RU) was calculated. Each combination of repeat units was assigned a MLVA-type (MT). We compared the MLVA typing result with Multi Locus Sequence Typing (MLST), *spa*-typing and Pulsed-Field Gel Electrophoresis (PFGE). MLVA typing resulted in 18 MTs, although 3 loci could not always be amplified. *Spa*-typing distinguished 10 *spa*-types including 3 dominant and 2 new types. PFGE showed 5 dominant profiles with 15 related profiles and 6 unique profiles. MLST showed 4 dominant STs. Some types appeared to be bovine specific. The Simpson's Indices of diversity for PFGE, MLST, *spa*-typing and MLVA were 0.887, 0.831, 0.69 and 0.781, respectively, indicating that discriminatory power of MLVA was between MLST and *spa*-typing, whereas PFGE displayed the highest discriminatory power. However, MLVA is fast and cheap when compared to the other methods. The Adjusted Rand index and Wallace's coefficient indicated that MLVA was highly predictive for *spa*-type, but not vice versa.

Analysis of the region neighboring SIRU05 showed a difference in the genetic element bordering the repeats of SIRU05 that explained the negative SIRU05 PCRs. PFGE, MLST, and MLVA are adequate typing methods for bovine-associated *S. aureus*.

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1. Introduction

Staphylococcus aureus is a major agent of contagious mastitis in diary cattle. The sources of bovine mastitis cases mostly are from bovine origin, but *S. aureus* originating from the farmer are another important source (Zadoks et al., 2002). In 2005 it was reported that each year at least 25% of all milking cows in The Netherlands suffer from clinical mastitis which is not only due to *S. aureus* (32%) as a causative agent (Lam, 2005). Many different typing methods have been used. From a "gold standard" Pulsed-Field Gel Electrophoresis (PFGE) to Multi Locus Sequence Typing (MLST) and *S. aureus*-specific staphylococcal Protein A typing known as *spa*-typing (Enright et al., 2000; Harmsen et al., 2003; Struelens et al., 1992). However, not all methods can be used in all centers because some methods are still limited to well-equipped laboratories. Furthermore, both MLST and *spa*-typing have insufficient discriminatory power to allow accurate delineation of outbreaks and PFGE is a fingerprinting

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method which makes interlaboratory comparison difficult (van Belkum et al., 1995).

A limited number of molecular studies have been published that explored the population structure and genetic relationships of *S. aureus* causing bovine mastitis (de Sousa et al., 2007; Hata et al., 2006; Jørgensen et al., 2005; Katsuda et al., 2005; Reinoso et al., 2008). The results of our recent study demonstrated that a Multiple Locus Variable Number Tandem Repeats (VNTR) Analysis (MLVA) could be used as a fast, inexpensive, highly discriminatory, reproducible, stable and portable typing method for epidemiological tracing of human *S. aureus* (Ikawaty et al., 2008). Therefore, we aimed to expand the use of this novel MLVA scheme to *S. aureus* isolated from clinical cases of bovine mastitis and to get insight in their genetic relationship with the human *S. aureus* population.

2. Methods

2.1. Strain collection

Eighty five *S. aureus* isolates of clinical or subclinical cases of mastitis were included. Thirty five isolates were obtained by the Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands from at least 26 farms near Utrecht. Isolates from farms sampled twice were taken at least one year apart. Another 50 isolates were collected by the Animal Health Service in Deventer, The Netherlands for the Central Veterinary Institute, Lelystad, The Netherlands from farms throughout The Netherlands. The sources were individual teat milk samples from dairy cattle from all over the country from clinical or subclinical cases of mastitis. Each isolate from CVI represents one farm, the location of the farms is unknown but they are distributed all over the Netherlands. All isolates were methicillin-susceptible *S. aureus* (MSSA) and collected between 1988 and 2005.

2.2. Genomic DNA preparation

The isolates were grown on blood agar (Trypticase soy agar II containing 5% sheep blood) overnight at 37 °C prior to DNA isolation. Preparation of bacterial genomic DNA was performed using the NucleoSpin kit (Macherey-Nagel) following the protocols from the manufacturer with the exception that bacterial pellet is resuspended in buffer T1. In our method, T1 buffer was replaced by freshly made lysis buffer that contained 20 mM Tris/HCl, 2 mM EDTA, 1% Triton X-100, and supplemented with lysostaphin, achromopeptidase and RNase.

2.3. MLVA typing

A combination of 6 loci (SIRU01, 05, 07, 13, 15 and 21 (*spa*)) from a previous study by Hardy et al. (2006) were used for MLVA typing. Amplification of SIRUs (Staphylococcal Interspersed Repeat Units) was performed as described before (Ikawaty et al., 2008).

2.4. Assignment of MLVA type (MT)

The number of repeats for each locus was determined by subtracting the size of the flanking regions from the size of the amplicon followed by division by size of the repeat (Table 1). The repeat number obtained was rounded up or down to the closest integer copy number. A number string resulted from combination of repeat units from SIRU01, 05, 07, 13, 15 and 21 was obtained after calculating the number of repeat units of all loci. This was considered an allelic profile and used for the assignment of an MLVA type (MT).

2.5. Spa-typing

Amplification and sequencing of the repeat region of the *S. aureus* Protein A gene (*spa*) was performed by using a specific primer set as described (Harmsen et al., 2003). The amplicon was sequenced using BigDye terminator version 3.1 on ABI 3100 sequencer (Applied Biosystem). BioNumerics (version 3.5; Applied Maths) was used to analyze the obtained sequences and to assign the *spa*-types. Novel *spa*-types were submitted to the Ridom SpaServer database (www.SpaServer.ridom.de).

2.6. Pulsed field gel electrophoresis

Bacterial isolates were genotyped by PFGE as described previously (Tenover et al., 1995). Digestion of chromosomal DNA was performed overnight using the restriction enzyme *Sma*I at a temperature 25 °C. Fragments were separated on 1% gel. Isolate relatedness was determined using the Tenover criteria (Tenover et al., 1995).

2.7. MLST analysis

Determination of the sequence type of 85 *S. aureus* from bovine mastitis were performed as described (Enright et al., 2000) and data were analyzed using BioNumerics software and the MLST database (www.mlst.net). The outcome of MLST of bovine mastitis *S. aureus* was

Table 1
Size of the MLVA loci, formula for calculating the number of repeat units (RU) per locus, typeability of MLVA and variation in repeat units observed.

Locus (size in bp)	Formula	Number (%) PCR negative	No. of repeats (RU)[a]
SIRU01 (55)	$(n\text{-}157\text{-}30)/55$	0 (0.0)	1–6
SIRU05 (60)	$(n\text{-}76\text{-}78)/60$	85 (100)	–
SIRU07 (56)	$(n\text{-}27\text{-}160)/56$	78 (91.8)	2–3
SIRU13 (64)	$(n\text{-}76\text{-}78)/64$	49 (47.6)	1–5
SIRU15 (131)	$(n\text{-}48\text{-}174)/131$	0 (0.0)	0–3
SIRU21 (24)	$(n\text{-}12\text{-}81)\text{-}16)/24$	0 (0.0)	2–12

n: Size of fragment in bp.
  [a] Values are in repeat unit (RU).

compared with the total population of human *S. aureus* isolates from our database (data not shown) and the MLST.net database.

2.8. Comparison of MLVA, spa-typing, MLST and PFGE

BioNumerics software was used as a tool for clustering the observed MLVA types (MTs) and MLST types (STs). The discriminatory power of the typing methods was calculated by using EpiCompare version 1.0 (Ridom GmbH, Wurzburg, Germany) as well as for the determination of Adjusted Rand index and Wallace's coefficients.

2.9. Analysis of SIRU05 locus

Further analysis of the SIRU05 locus was performed by PCR and sequencing using specific primer sets (lysR-For: 5′-GGA AGC AGA TTT AGG TTA TG-3′ and fosB-lys-Rev: 5′-CCA GTC AAT AGC AAT TTT CC-3′ for amplification fragment A; lysR-For: 5′-TTT GTT CAT CTT GGC TTA GG-3′ and ISRX-lys-Rev: 5′-GGA AGT TAC AAT CAT TTG CG-3′ for fragment B) based on reference strain of bovine *S. aureus* RF122 (Fig. 1). DNA sequencing was performed as described for *spa*-typing.

3. Results

3.1. MLVA

Among 6 SIRUs used in this typing method, no amplifications were detected for SIRU13, 07 and 05 in 47.6%, 91.8% and 100% of the cases, respectively (Table 1). Variation in the number of repeat units ranged from 0 to 12 repeat units (Table 1). Absence of PCR amplification was considered as giving a null allele and is assigned 999 for the repeat number (Table 2). The MLVA typing of 85 isolates produced 18 different allelic profiles or MLVA types. MT102 was most common with 35 isolates, followed by MT112 ($n = 17$), and MT118 ($n = 8$).

3.2. Spa-typing

Ten *spa*-types were obtained from 85 isolates, including 2 new *spa*-types (t2112 and t2248). Seventy three isolates belonged to three dominant *spa*-types, t529 ($n = 39$), t543 ($n = 22$) and t524 ($n = 17$) (Table 2). The number of repeats obtained by *spa*-typing for all isolates tested were corresponding with the number of repeats determined by MLVA for SIRU21.

3.3. Pulsed-Field Gel Electrophoresis

DNA of 85 isolates was digested with *Sma*I and showed 26 PFGE profiles, 5 groups of closely related PFGE profiles (5 dominant profiles with 15 related to dominant profiles) and 6 unique PFGE profiles (Fig. 2).

3.4. MLST

Sequencing of the seven housekeeping genes of all isolates identified 4 dominant MLST types (STs), ST 504 ($n = 24$), ST 479 ($n = 21$), ST71 ($n = 11$), ST 151 ($n = 11$), and 11 STs that have not been described previously in the database at http://saureus.mlst.net (Table 2). A minimum spanning tree of the ST of the 85 isolates compared to the whole population of *S. aureus* showed 3 distinct clusters with bovine mastitis *S. aureus* isolates although some isolates clustered with known human sequence types (Fig. 3).

3.5. Comparison of MLVA, spa-typing, PFGE and MLST

Discriminatory power of the four typing methods, PFGE, MLST, *spa*-typing and MLVA was determined by calculating Simpson's index of diversity with 95% confidence interval (CI) of the isolates typed by these methods. PFGE showed higher discriminatory power compared to MLST, *spa*-typing, and MLVA (0.887, 0.831, 0.69 and 0.781, respectively) (Table 3), but the 95% CI of MLVA overlapped
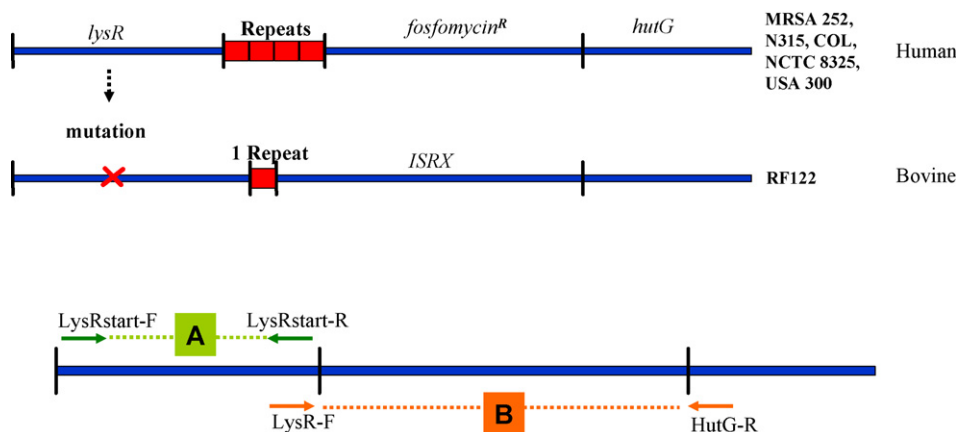
Fig. 1. Strategy for analyzing the region bordering the SIRU05 locus. The bovine *S. aureus* (BSA) RF122 reference strain showed a different genetic structure compared to human *S. aureus* reference strains. The genetic element at the right of the repeat region consists of a fosfomycin resistance gene in human *S. aureus* and was replaced by insertion site region X (IS*RX*) in RF122. A single nucleotide mutation in *lysR* gene region was present in RF122. The primers were designed to detect the presence/absence of a single nucleotide mutation (fragment A) by DNA sequencing and the presence of fosfomycin resistance gene or IS*RX* structure (fragment B).

Table 2
Comparison of PFGE, MLST, *spa*-typing and MLVA results.

No.	Isolate	Date of isolation	Source	PFGE profile	MLST type	Spa-type	MLVA type	SIRU[a]					
								01	05	07	13	15	21
1	S0416	04/2004	FVM	E	504	t529	102	1	999	999	999	0	2
2	S0409	05/2005	FVM	E	151	t529	102	1	999	999	999	0	2
3	S0333	30/09/2005	CVI	D	504	t529	102	1	999	999	999	0	2
4	S0334	17/10/2005	CVI	D	504	t529	102	1	999	999	999	0	2
5	S0335	17/10/2005	CVI	E	151	t529	102	1	999	999	999	0	2
6	S0338	03/10/2005	CVI	E	151	t529	102	1	999	999	999	0	2
7	S0341	28/09/2005	CVI	D1	504	t529	102	1	999	999	999	0	2
8	S0343	28/09/2005	CVI	E	151	t529	102	1	999	999	999	0	2
9	S0347	10/10/2005	CVI	D	504	t529	102	1	999	999	999	0	2
10	S0350	03/10/2005	CVI	D2	151	t529	102	1	999	999	999	0	2
11	S0351	17/10/2005	CVI	D1	151	t529	102	1	999	999	999	0	2
12	S0352	14/10/2005	CVI	D2	151	t529	102	1	999	999	999	0	2
13	S0353	13/10/2005	CVI	D1	151	t529	102	1	999	999	999	0	2
14	S0357	03/10/2005	CVI	E	504	t529	102	1	999	999	999	0	2
15	S0358	28/09/2005	CVI	E	151	t529	102	1	999	999	999	0	2
16	S0364	30/09/2005	CVI	E	504	t529	102	1	999	999	999	0	2
17	S0365	30/09/2005	CVI	D1	504	t529	102	1	999	999	999	0	2
18	S0367	03/10/2005	CVI	D2	1122[b]	t529	102	1	999	999	999	0	2
19	S0368	03/10/2005	CVI	E	504	t529	102	1	999	999	999	0	2
20	S0370	28/09/2005	CVI	D1	1123[b]	t529	102	1	999	999	999	0	2
21	S0371	17/10/2005	CVI	D	504	t529	102	1	999	999	999	0	2
22	S0374	17/10/2005	CVI	D2	151	t529	102	1	999	999	999	0	2
23	S0375	17/10/2005	CVI	D3	504	t529	102	1	999	999	999	0	2
24	S0376	25/10/2005	CVI	E	504	t529	102	1	999	999	999	0	2
25	S0377	25/10/2005	CVI	D1	504	t529	102	1	999	999	999	0	2
26	S0378	25/10/2005	CVI	D	504	t529	102	1	999	999	999	0	2
27	S0379	25/10/2005	CVI	D	504	t529	102	1	999	999	999	0	2
28	S0381	25/10/2005	CVI	D	1124[b]	t529	102	1	999	999	999	0	2
29	S0389	03/1995	FVM	D	504	t529	102	1	999	999	999	0	2
30	S0390	03/1997	FVM	D	1120[b]	t529	102	1	999	999	999	0	2
31	S0396	03/2002	FVM	D1	504	t529	102	1	999	999	999	0	2
32	S0417	04/2004	FVM	D2	504	t529	102	1	999	999	999	0	2
33	S0424	01/2006	FVM	D	504	t529	102	1	999	999	999	0	2
34	S0425	01/2006	FVM	E	504	t529	102	1	999	999	999	0	2
35	S0435	1988	FVM	D	504	t529	102	1	999	999	999	0	2
36	S0340	03/10/2005	CVI	A	479	t543	112	2	999	999	2	0	3
37	S0342	28/09/2005	CVI	A	479	t543	112	2	999	999	2	0	3
38	S0344	28/09/2005	CVI	A	479	t543	112	2	999	999	2	0	3
39	S0345	10/10/2005	CVI	A	479	t543	112	2	999	999	2	0	3
40	S0346	10/10/2005	CVI	A	479	t543	112	2	999	999	2	0	3
41	S0348	10/10/2005	CVI	A	479	t543	112	2	999	999	2	0	3
42	S0349	10/10/2005	CVI	A	479	t543	112	2	999	999	2	0	3
43	S0354	03/10/2005	CVI	A	479	t543	112	2	999	999	2	0	3
44	S0355	03/10/2005	CVI	A	479	t543	112	2	999	999	2	0	3
45	S0356	03/10/2005	CVI	A	479	t543	112	2	999	999	2	0	3
46	S0360	28/09/2005	CVI	A	479	t543	112	2	999	999	2	0	3
47	S0361	28/09/2005	CVI	A	479	t543	112	2	999	999	2	0	3
48	S0369	03/10/2005	CVI	A	1118[b]	t543	112	2	999	999	2	0	3
49	S0372	17/10/2005	CVI	A	479	t543	112	2	999	999	2	0	3
50	S0373	17/10/2005	CVI	A	1118[b]	t543	112	2	999	999	2	0	3
51	S0414	09/2003	FVM	A	479	t543	112	2	999	999	2	0	3
52	S0422	01/2005	FVM	A	479	t543	112	2	999	999	2	0	3
53	S0398	10/1998	FVM	A	479	t543	114	2	999	999	1	0	3
54	S0332	12/10/2005	CVI	A1	479	t543	114	2	999	999	1	0	3
55	S0339	03/10/2005	CVI	A	479	t543	114	2	999	999	1	0	3
56	S0391	04/2002	FVM	A	479	t543	114	2	999	999	1	0	3
57	S0388	1994	FVM	F3	1119[b]	t524	118	4	999	999	3	1	2
58	S0401	01/1999	FVM	B3	1125[b]	t524	118	4	999	999	3	1	2
59	S0427	1990	FVM	B	1129[b]	t524	118	4	999	999	3	1	2
60	S0428	1989	FVM	F	71	t524	118	4	999	999	3	1	2
61	S0429	1989	FVM	G	71	t524	118	4	999	999	3	1	2
62	S0430	1989	FVM	F1	71	t524	118	4	999	999	3	1	2
63	S0432	1989	FVM	B	71	t524	118	4	999	999	3	1	2
64	S0412	06/2003	FVM	B	71	t524	118	4	999	999	3	1	2
65	S0362	30/09/2005	CVI	B6	71	t524	125	5	999	999	3	1	2
66	S0433	1989	FVM	B1	71	t524	125	5	999	999	3	1	2
67	S0434	1989	FVM	B	71	t524	125	5	999	999	3	1	2
68	S0411	06/2003	FVM	B2	1127[b]	t524	125	5	999	999	3	1	2

Table 2 (*Continued*)

No.	Isolate	Date of isolation	Source	PFGE profile	MLST type	*Spa*-type	MLVA type	SIRU[a]					
								01	05	07	13	15	21
69	S0413	07/2003	FVM	B5	1128[b]	t524	125	5	999	999	3	1	2
70	S0420	10/2004	FVM	D1	504	t529	125	5	999	999	3	1	2
71	S0418	08/2005	FVM	E1	504	t529	104	1	999	999	3	1	2
72	S0410	05/2003	FVM	B4	1126[b]	t524	104	1	999	999	3	1	2
73	S0419	11/2004	FVM	C	71	t524	79	6	999	999	3	1	2
74	S0421	11/2004	FVM	D1	151	t529	79	6	999	999	3	1	2
75	S0363	30/09/2005	CVI	F4	71	t524	76	5	999	999	3	0	2
76	S0436	1990	FVM	H	97	t2174	82	6	999	2	5	2	6
77	S0387	1994	FVM	D1	504	t529	106	1	999	3	999	0	2
78	S0426	1990	FVM	B2	97	t1236	108	1	999	2	5	3	10
79	S0366	03/10/2005	CVI	I	1121[b]	t127	110	1	999	3	2	2	7
80	S0392	04/2002	FVM	A	479	t2248	116	2	999	999	1	0	6
81	S0337	13/10/2005	CVI	B2	97	t2112	120	4	999	2	5	2	11
82	S0431	1989	FVM	F2	97	t521	121	4	999	3	3	2	12
83	S0336	17/10/2005	CVI	J	71	t524	123	4	999	999	2	1	2
84	S0423	11/2005	FVM	A	479	t543	127	5	999	999	3	1	3
85	S0393	08/2001	FVM	K	124	t224	129	5	999	3	5	1	8

CVI: Central Veterinary Institute, Lelystad, The Netherlands.
FVM: Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.
999: no amplification of SIRU.
[a] Values are in repeat units (RU).
[b] New MLST type.

with those of *spa*-typing and MLST. It is remarkable that all 3 major *spa*-types contained multiple ST (Table 2).

3.6. Analysis of the neighboring region of SIRU05

SIRU05 could not be amplified for any of the isolates. Therefore, the presence of sequences bordering SIRU05 was investigated. In human-derived SIRU05-positive strains SIRU05 is flanked by a LysR regulatory protein family gene at one side and a fosfomycin resistance encoding gene (*fosB*) followed by the *hutG* gene which putatively encodes a formimidoylglutamase. In the sequenced bovine strain RF122 the fosfomycin resistance gene is replaced by insertion sequence ISRX. One primer set was used to amplify the *lysR* region (fragment A) and a second set was used to amplify the region between the *lysR* gene and *hutG* (Fig. 1). All isolates were positive for amplification of fragment A and B. Sequencing of fragment A and B of 10 isolates showed a single nucleotide mutation in the *lysR* gene resulting a premature stop-codon and the absence of ISRX. A new primer designed in the *hutG* gene which was combined with the original primer of SIRU05 in the *lysR* gene did not show differences in the number of repeat units for this locus.

3.7. The congruence between PFGE, MLST, spa-typing and MLVA

Adjusted Rand's and Wallace's coefficients were calculated to explore the concordance between typing methods (Tables 4 and 5). The Adjusted Rand's coefficient for the comparison of the clustering by MLVA and PFGE, MLVA and MLST, and MLVA and *spa*-typing was 0.385, 0.442 and 0.758, respectively. Considering MLVA as the test typing method for comparison, the value of Wallace's coefficients showed that MLVA could only poorly predict the PFGE and MLST type. The probability of two strains having the same MLVA type and sharing the same *spa*-type was 99%

(Wallace's coefficient 0.991), whereas the reverse was reasonably predictive (Wallace's coefficient 0.699). This finding reflects that MLVA was less discriminatory than MLST and PFGE.

We observed variation of *spa*-type and MT within the same MLST type as shown by ST97 and 479 isolates, although an identical *spa*-type isolates also showed variation of STs that were closely related (single locus variants or SLVs) to possibly related (3 loci different).

Identical t524 isolates had new STs: ST1119, 1125, 1126, 1127, and 1129 that were SLVs and ST1128 that was a double locus variant (DLV) of ST71.

4. Discussion

MLVA has shown great potential for fast and reliable typing of pathogenic bacteria. Our previous study demonstrated that a newly developed MLVA scheme for human *S. aureus* had higher discriminatory power compared to PFGE, MLST and *spa*-typing (Ikawaty et al., 2008). A major advantage of the proposed MLVA scheme is that it requires only simple laboratory equipment and is fast and relatively cheap to perform. This scheme provides better and more timely access to typing of bovine mastitis. This allows more adequate surveillance of mastitis and the identification of particular virulent or epidemic strains. Early recognition of these strains may help to initiate more timely therapy and other interventions to prevent further spread. In this study, we extended the use of the MLVA typing scheme for human *S. aureus* to bovine *S. aureus* from clinical mastitis. Eighty five isolates represented regional (*n* = 35) and national (*n* = 50) *S. aureus* isolates. The isolates were not considered to belong to local outbreaks as 90% of the isolates were obtained on different farms whereas the remaining isolates were obtained at least one year apart when sampled on the same farm. The scheme showed good typeability although 3 loci (SIRU05, 07 and 13) were not always amplified. SIRU21 had the most variance in the
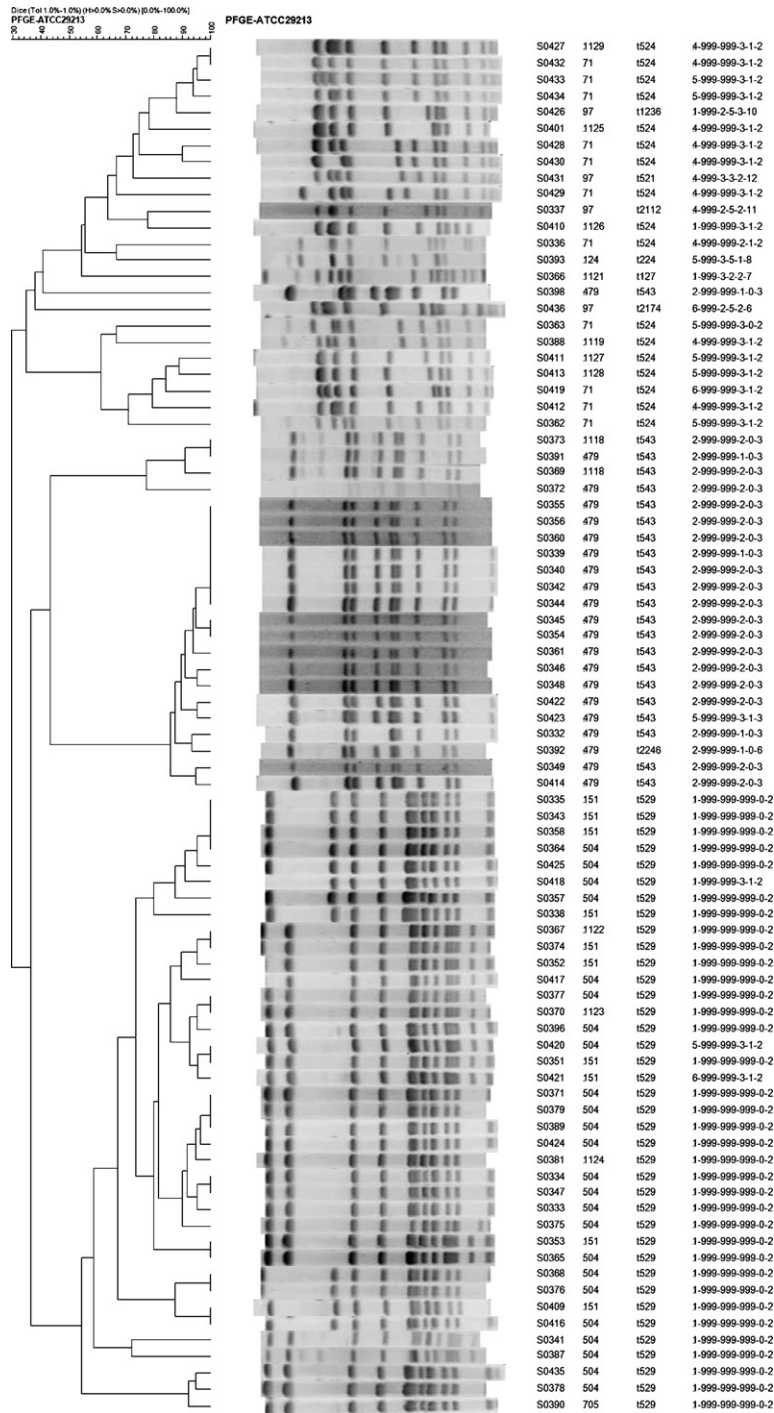
Fig. 2. Dendrogram containing PFGE patterns of 85 MSSA strains collected from bovine. At the 50% similarity level, seven branches are distinguished. ID: isolate ID; ST: MLST type.

number of repeat units, which is important in typing by MLVA.

We observed no particular difference between regionally and nationally obtained *S. aureus* bovine mastitis strains in terms of PFGE profile, MLST and *spa*-types, except for one MLVA type (MT 118) that was only present among regionally obtained isolates, but due to the limited

number of isolates further analysis using more samples is needed.

The number of repeat units obtained by amplification of SIRU21, having the smallest repeat unit, agreed completely with the number of repeats obtained by DNA sequencing for *spa*-typing indicating the reliability of the determination of the number of repeat units.
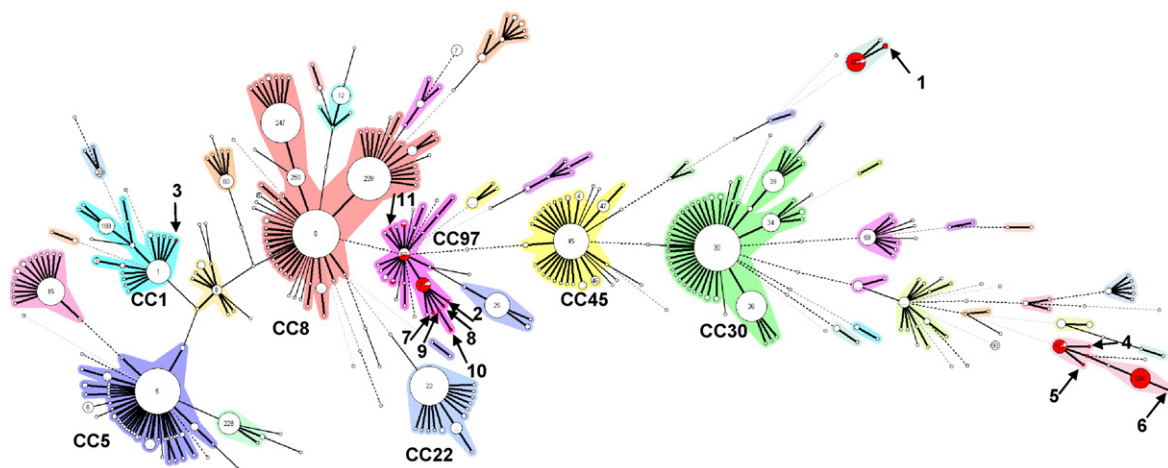
Fig. 3. Minimum spanning tree of *S. aureus* based on MLST. Five major clonal complexes are present within the *S. aureus* population: CC5, CC8, CC22, CC30 and CC45. Each circle represents a different MLST type. Single locus variant STs are connected by a thick line, double locus variant STs by a thin line, triple locus variant STs by a dark grey dashed line, and STs with more than 3 loci variant are connected by a light grey dashed line. Red circles indicate *S. aureus* isolated from bovine mastitis. Arrows indicated new MLST types observed in this study: 1: ST 1118; 2: ST 1119; 3: ST 1121; 4: ST 1122; 5: ST 1123; 6: ST 1124; 7: ST 1125; 8: ST 1126; 9: ST 1127; 10: ST 1128; 11: ST 1129.

SIRU05 was analyzed more in depth, since it could not be amplified in any of the bovine isolates including 4 isolates with ST97 that were already known as possibly human derived (Smith et al., 2005a; Sung et al., 2008). PCR of the region neighboring SIRU05 demonstrated that all isolates were fragment A and B positive. Sequencing of the fragments of 10 isolates showed that IS*RX* and the fosfomycin resistance element were lacking. The difference in the genetic element bordering the repeats of SIRU05 explains the negative result of SIRU05 PCR, since the primer chosen in the fosfomycin resistance element could not anneal to the IS*RX* element or the *hutG* gene. Amplification of SIRU05 with a primer chosen in *hutG* showed no variation in the number of repeat units. SIRU05 does not contribute to the discrimination of the isolates tested and can be omitted from the scheme for bovine mastitis isolates. It should be noted that the primer sets used in this MLVA scheme were developed based on human *S. aureus*.

LysR is a member of the largest family of bacterial activator/regulator proteins (Henikoff et al., 1988; Zaim and Kierzek, 2003). The LysR protein contains a substrate recognition site and a helix-turn-helix DNA-binding motif crucial for protein-DNA interaction (Zaim and Kierzek, 2003). In bovine *S. aureus* a stop-codon is present, which prevents transcription of the substrate domain. This should inactivate LysR leading to different expression patterns in bovine strains compared to human strains.

A minimum spanning tree based on MLST showed that clustering of bovine *S. aureus* was different than for isolates from the human population. The difference between human and bovine derived isolates has been suggested to be caused by tissue specificity by some authors (Gilbert et al., 2006; Smith et al., 2005a) while most authors assumed host specificity among *S. aureus* clones (de Sousa et al., 2007; Kapur et al., 1995). Two of the three dominant MLST types (ST151 and 504) were clustered together while ST479 was distantly related to ST151 and ST504. They were not related to human-derived *S. aureus*. Eleven isolates from both regional and national origin were ST71 which is known to be a bovine-associated strain from The Netherlands (Smith et al., 2005a). Interestingly, the 11 new STs isolates fell into a cluster which was not related to the known bovine STs. Little sharing of strains between the bovine and human population has been reported (Kapur et al., 1995). A similar finding was made by Rabello et al. for Brazilian isolates (Rabello et al., 2007).

The ST or clonal complexes (CC) of bovine mastitis isolates from The Netherlands differed from those described elsewhere with the exception of 4 isolates (S0337, S0426, S0431 and S0436) that belonged to CC97. CC97 isolates have previously been reported to be obtained from humans (Feil et al., 2003). In Brazil CC97 and CC127 were predominant (Rabello et al., 2007). CC97 was also present in isolates from the UK, the USA and Chile (Smith et al., 2005a,b). A different set of STs was obtained from

Table 3
Simpson's index of diversity and 95% confidence interval.

Typing method	Number of different types	Discriminatory index	95% confidence interval
PFGE	26	0.887	0.847–0.926
MLST	18	0.831	0.787–0.875
Spa-typing	10	0.69	0.627–0.752
MLVA	18	0.781	0.71–0.852

Total of 85 isolates tested.

Table 4
Adjusted Rand's coefficients for the methods used to characterize the 85 bovine mastitis *Staphylococcus aureus* isolates.

Typing method	PFGE	MLST	Spa-typing	MLVA
PFGE	–			
MLST	0.479	–		
Spa-typing	0.405	0.583	–	
MLVA	0.385	0.442	0.758	–

Table 5
Wallace's coefficients for the methods used to characterize 85 bovine mastitis *Staphylococcus aureus* isolates.

Typing method	PFGE	MLST	Spa-typing	MLVA
PFGE	–	0.684	0.941	0.699
MLST	0.459	–	0.957	0.63
Spa-typing	0.344	0.521	–	0.699
MLVA	0.362	0.486	0.991	–

milk in Norway where ST130 (CC3), ST133 and ST132 (CC1) were predominant (Jørgensen et al., 2005).

The highest discriminatory power was obtained by PFGE followed by MLST, MLVA and *spa*-typing. Because SIRU05 yielded no usable data a lower discriminatory power of MLVA compared to the *spa*-typing was expected. But the results demonstrated that MLVA had a higher Simpson's index of diversity than *spa*-typing.

We observed variation in related STs within the same *spa*-type as shown in Table 2. The limited number of *spa*-types might suggest selective pressure on the *spa* gene and it may explain their maintenance in the population of bovine-derived *S. aureus*. This finding suggests that *spa*-typing is not a useful method to compare bovine mastitis isolates.

Transfer of antibiotic resistance from human to animal isolates or the other way around has been a major concern (Pesavento et al., 2007; Zadoks et al., 2002). Host specificity of clones may reduce the chance that human-derived antibiotic-resistant *S. aureus* isolates are transmitted to cattle, although bovine mastitis may occasionally be caused by human-derived isolates. Typing of *S. aureus* from bovine mastitis will be required to monitor potentially changing population dynamics. Based on our data PFGE, MLST, and MLVA are adequate typing methods for international studies and local studies of bovine *S. aureus* isolates involved in mastitis. However, PFGE and MLST are more time-consuming and/or expensive than MLVA.

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