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Association of *Staphylococcus aureus* genotypes with milk or colonization of extramammary sites in Dutch dairy cattle indicates strain variation in reservoirs for intramammary infections

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ABSTRACT

Staphylococcus aureus (*S. aureus*) is a major mastitis pathogen with a detrimental impact on udder health in dairy cattle. Although *S. aureus* is generally classified as a contagious mastitis pathogen, control measures aimed at preventing contagious transmission are not always effective. Previous studies showed that various extramammary sites can be colonized with *S. aureus* and could be a reservoir for *S. aureus* intramammary infections (IMI). The aim of this study was to determine the prevalence of *S. aureus* extramammary site colonization in Dutch dairy cattle and to compare the *spa* genotypes of *S. aureus* isolates from IMI to *spa* genotypes of isolates from extramammary sites. Six herds were visited and from cows with a composite milk somatic cell count ≥200,000 cells/mL quarter milk samples and swabs from various extramammary sites (hock, groin, udder cleft, nares, and feces) were taken. Extramammary site samples were processed by a two-step high salt selective culture and presence of *S. aureus* was confirmed by *femA* PCR. *S. aureus* isolates from milk and extramammary sites were compared by *spa* typing. The cow level colonization varied from 0% to 73%, and the prevalence of IMI in the sampled cows varied from 0% to 61% between herds. The extramammary site with the highest prevalence of colonization was the hock (23%) and the lowest prevalence of colonization was found for the nares (5%) and feces (5%). *Spa* typing of *S. aureus* isolates from either extramammary sites or milk showed that in most herds there were one or two predominant *S. aureus spa* genotypes present. Different *S. aureus spa* genotypes could be categorized into three groups based on the distribution between milk or extramammary sites: i) predominantly milk associated, ii) associated with both milk and extramammary sites, and iii) associated with extramammary sites. In conclusion, we showed that the prevalence of extramammary site colonization differed significantly between herds and extramammary sites and that specific *S. aureus spa* genotypes were associated with milk (IMI) or extramammary site colonization. Extramammary *S. aureus* reservoirs could be a source for IMI that cannot be eradicated by intervention measures aimed at contagious mastitis pathogens.

1. Introduction

Mastitis, caused by intramammary infections (IMI), is an important disease in dairy cattle worldwide. *Staphylococcus aureus* (*S. aureus*) is one of the major causes of mastitis in dairy cattle and can be highly contagious, causes persistent infections, and has a low cure rate following antibiotic treatment (Abebe et al., 2016; Barkema et al., 1999; Zadoks et al., 2000). *S. aureus* is classified as a contagious mastitis pathogen, where infected quarters are the main reservoir from which new *S. aureus* IMI cases arise (Keefe, 2012). Therefore, control measures against *S. aureus* IMI are aimed at reducing and preventing transmission from infected quarters to healthy quarters (Barkema et al., 2006; Keefe, 2012) and include treating infected cows, culling chronic cases, and applying disinfecting teat dips. Although control measures aimed at preventing transmission from infected quarters are generally effective in reducing the prevalence of *S. aureus* IMI, these control measures are not always

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effective (Sommerhäuser et al., 2003). Previous research has established that various extramammary sites can be colonized with *S. aureus* (Anderson et al., 2012; Capurro et al., 2010; Haveri et al., 2008; Leuenberger et al., 2019; Matos et al., 1991; Roberson et al., 1994; Svennesen et al., 2019), including the nares, groin, vagina, perineum, udder skin, teat skin, and hock skin. Extramammary site colonization with *S. aureus* could serve as a reservoir for intramammary infections that are not controlled by measures aimed at contagious mastitis.

Although many studies have shown that various extramammary sites can be colonized, it is important to determine if the same or different genotypes can be found in milk and extramammary sites as this can give insight into whether extramammary sites may serve as a reservoir for *S. aureus* IMI. Haveri et al. (2008) used pulsed-field gel electrophoresis (PFGE) typing of *S. aureus* isolated from milk and teat skin. The results showed that in each herd one pulsotype was dominant in milk and that this type was also dominant on the teat skin. Additional studies also found an association between the genotypes of *S. aureus* isolated from milk and teat skin (Anderson et al., 2012; Capurro et al., 2010), whereas others did not find an association (da Costa et al., 2014; Mørk et al., 2012; Zadoks et al., 2002). For teat skin, it is unknown whether *S. aureus* causes persistent colonization or whether there is transient colonization resulting from contact with infected milk or contaminated milk liners. Additionally, it is unknown whether teat skin colonization with *S. aureus* is a risk factor for IMI or whether teat skin is colonized following an IMI.

One of the few studies that genotyped *S. aureus* isolates from extramammary sites other than teat skin was by Leuenberger et al. (2019). This study found that in Swiss dairy herds (average herd size of 30 cows, with 10 out of 21 herds having tie stalls) the 16S—23S ribosomal spacer genotypes of *S. aureus* were herd specific and isolates with genotype B were associated with IMI and contagious transmission, whereas isolates with genotype C were associated with extramammary site colonization and sporadic cases of IMI. This was the first evidence showing that in Swiss dairy herds, *S. aureus* isolates with specific genotypes can be associated with IMI or extramammary site colonization and transmission characteristics. However, results from this study cannot be generalized to Dutch dairy cattle, as the husbandry conditions in Swiss dairy herds are different and the 16S—23S ribosomal *spa*cer genotyping method is not commonly used.

The objectives of this study were to determine the prevalence of *S. aureus* colonization of extramammary body sites in Dutch dairy cows with a high composite milk somatic cell count and to compare *spa* genotypes of isolates from IMI to *spa* genotypes of isolates from extramammary sites. Results from this study will give insight into the role of extramammary site colonization with *S. aureus* as a reservoir for *S. aureus* IMI.

2. Material & methods

2.1. Herd and animal selection

Herds were selected based on enrollment in a dairy herd improvement program, the history of *S. aureus* IMI, and willingness of the herd manager to cooperate with the study. Six dairy herds meeting the inclusion criteria were visited for sampling between March 2021 and December 2021 (Table 1). Herd I was a teaching dairy herd, and the five others were commercial dairy herds. Herds were located in the provinces Utrecht or Flevopolder and herd size varied from 42 to 194 cows (Table 1).

Between 5 and 30 cows with a composite milk somatic cell count (SCC) ≥200,000 cells/mL at the last milk recording were sampled, during milking. If more than 30 cows had a high composite milk SCC, a maximum of 30 cows were sampled per visit. In herd VI, cows with a composite milk SCC ≥200,000 cells/mL or a history of *S. aureus* IMI were milked in a separate group and since there was a long period between the results of DHI and sampling (25 days), all cows of this group were sampled and this included cows with a composite milk SCC < 200,000 cells/mL on the last DHI. As for all other herds, milk samples were only taken from quarters with a positive California mastitis test result, as explained under 'Sampling'. Cows receiving antibiotic treatment during the time of sampling and cows from which the wrong ID number was noted were excluded. Data from milk samples collected was excluded if the milk sample was contaminated.

To characterize *S. aureus* strain variation within extramammary sites, 13 cows from Herd I with extramammary sites that were positive for *S. aureus* in the previous sampling were resampled. In total 36 extramammary sites were sampled from these 13 animals, as detailed in Table S1. Extramammary sites were sampled with three separate swabs consecutively at the same site during a single sampling moment.

2.2. Sampling

All procedures, performed according to national and European regulations, were approved by the Ethical Committee for Animal Experiments of Utrecht University and assessed to be below the threshold of pain, suffering, distress, or lasting harm that requires an animal license (Working protocol number 10803–2019-6). Sampling was performed

Table 1
Characteristics of the sampled herds.

Herd	Breed	Herd size (lactating cows)	Housing system	Milking system	Number of milkings per day	Sample visits (n)	Number of cows sampled	Remarks
I	Holstein Friesian	42	Free stall with daytime grazing	Herringbone	2	2	11	Teaching herd of the Faculty of Veterinary Medicine Utrecht University
II	Holstein Friesian	157	Free stall with daytime grazing	Rotary	2	2	28	
III	Holstein Friesian	191	Free stall with daytime grazing	Herringbone	2	2	49 (19 cows milk only and 30 both milk and extramammary sites)	
IV	Holstein Friesian, Fleckvieh	177	Free stall with daytime grazing	Herringbone	2	2	50 (8 cows milk only and 42 both milk and extramammary sites)	Midline system, 22 cows per side
V	Blister head, Fleckvieh, MRIJ	194	Free stall with daytime grazing	Herringbone	2	1	20	Midline milking system, 11 cows per side.
VI	Holstein Friesian	192	Free stall	Rotary	2	1	22 cows (3 cows only extramammary sites and 19 cows both milk and extramammary sites)	

Table 2
Overview of the sequence of the primers used for either the *femA* PCR or the *spa* PCR.

Gene	Sequence 5′ – 3′	Product size (bp)	Reference
femA	F: tgcctttacagatagcatgcca R: agtaagtaagcaagctgcaatgacc	142	Francois et al. (2003)
spa	F: taaagacgatccttcagtgagc R: cagcagtagtgcttggctt	*	Hallin et al. (2009)

* Size of the PCR product depends on the *Spa* type of the *S. aureus spa* genotype.

during either the morning milking or evening milking, except for fecal and nose samples which were taken after milking when cows were fixated at a self-locking feed barrier. The expected *S. aureus* IMI prevalence was low and to increase the change of collecting *S. aureus* positive milk samples, milk samples were collected from more cows than extramammary site samples in some herds (Table 1). Gloves were worn during sampling and if gloves became visibly dirty they were changed. Samples were processed directly after arrival in the lab.

Quarter milk samples were collected aseptically from lactating cows according to the NMC guidelines. Teats were cleaned with a dry cloth and fore stripped. A California mastitis test (CMT) was performed on each quarter using BOVIVET CMT liquid (Kruuse Livestock, Langeskov, Denmark), and results were scored according to the manufacturer's recommendation. Milk was only collected from quarters positive for the CMT (scored trace or higher). Each sampled teat was cleaned with cotton pads soaked with 70% ethanol, and two to three squirts of milk were discarded before 5 to 10 mL of milk was collected.

Milk samples were vortexed for ten seconds on a vortex mixer (VV3 vortex, VWR, Radnor, Pennsylvania, USA) and ten microliters of the collected milk samples were plated on half of a Columbia Agar plate supplemented with 5% Sheep Blood (BD, Franklin Lakes, USA) using a sterile disposable loop (VWR, Radnor, Pennsylvania, USA) and incubated for 22–24 h at 37 °C. From each milk sample, 750 µL was aliquoted separately in a 1.5 mL Eppendorf and incubated for 22–24 h at 37 °C. One colony forming unit (CFU) or less was defined as no growth. If three or more different types of morphologies were observed, the sample was classified as contaminated. If there was no growth or one colony after 22–24 h of incubation, ten microliters of the incubated milk sample were plated and incubated for 22–24 h at 37 °C and growth was classified the same as for directly plated samples. Preliminary phenotype identification of *S. aureus* colonies was based on colony morphology and hemolysis. Three colonies of suspected *S. aureus* morphology were replated on Columbia Agar plate supplemented with 5% Sheep blood and incubated for 22–24 h at 37 °C.

The following extramammary sites were swabbed using a sterile viscose swab (Sarstedt, Nümbrecht, Germany): hock skin (the cleanest hock from a site without lesions), groin (the swab was inserted between the hindleg and udder), udder skin (skin of the cleft between the left and right halves of the udder), nares (the first 2 to 3 cm of the nasal duct and circumferentially rubbed against the mucosa), and feces (a small amount of feces was collected rectally). For each location, one swab was used for the sampling.

All samples were processed by washing the swab in 500 µL phosphate-buffered saline (PBS) (Life Technologies, Carlsbad, CA, USA). To selectively culture *S. aureus* from extramammary site swabs, a two-step high salt selective culture method was used. Hundred and fifty microliters of the swab eluate were added to 5 mL Mueller Hinton Broth (MHB) (Oxoid, Hampshire, UK) supplemented with 15% Sodium Chloride (NaCl)(Sigma-Aldrich, Merck, Darmstadt, Germany). Samples were incubated for 22–24 h at 37 °C. Five-hundred microliters of the incubated sample in MHB with 15% NaCl were added to 5 mL MHB with 10% NaCl, which was incubated for another 22–24 h at 37 °C. Ten microliters of the broth were plated on Columbia Agar plate supplemented with 5% Sheep blood and incubated for 22–24 h at 37 °C. Next, two to seven

Table 3
Cow level prevalence estimates with 95% confidence interval according to Wilson for IMI and colonization of extramammary sites with *S. aureus*.

	Nares		Feces		Hock		Groin		Udder cleft		Cow level colonization*		Milk	
	Pos./ Tot.	Prevalence (95% CI)	Pos./ Tot.	Prevalence (95% CI)	Pos./ Tot.	Prevalence (95% CI)	Pos./ Tot.	Prevalence (95% CI)	Pos./ Tot.	Prevalence (95% CI)	Pos./ Tot.	Prevalence (95% CI)	Pos./ Tot.	Prevalence (95% CI)
Herd I	1/11	9% (2–38)	1/11	9% (2–38)	8/11	73% (43–90)	3/11	27% (10–57)	1/11	9% (2–38)	8/11	73% (43–90)	1/11	9% (2–38)
Herd II	1/9	11% (2–44)	0/0	NA	8/28	29% (15–47)	1/28	4% (1–18)	0/28	0% (0–12)	8/28	29% (15–47)	17/28	61% (42–76)
Herd III	1/20	5% (1–24)	2/20	10% (3–30)	5/30	17% (7–34)	4/30	13% (5–30)	9/30	30% (17–48)	15/30	50% (33–67)	5/49	10% (4–22)
Herd IV	3/34	9% (3–23)	0/34	0% (0–10)	6/42	14% (7–28)	2/42	5% (2–16)	3/42	7% (3–19)	12/42	29% (17–44)	3/50	6% (2–16)
Herd V	0/20	0% (0–16)	0/20	0% (0–16)	0/20	0% (0–16)	0/20	0% (0–16)	0/20	0% (0–16)	0/20	0% (0–16)	0/20	0% (0–16)
Herd VI	0/22	0% (0–15)	2/22	9% (3–28)	8/22	36% (20–57)	6/22	27% (13–48)	2/22	9% (3–28)	10/22	45% (27–65)	2/19	11% (3–32)
All herds	6/116	5% (2–11)	5/107	5% (2–10)	35/153	23% (17–30)	16/153	10% (7–16)	15/153	10% (6–15)	53/153	35% (27–41)	28/177	16% (11–22)

* The prevalence of cows within a herd with at least one *S. aureus* colonized extramammary site. This excludes milk.

colonies from each morphology present on the plate were replated individually on Columbia Agar plate supplemented with 5% Sheep blood and incubated for 22–24 h at 37 °C. Bacillus colonies, characterized by an irregular, expanded shape and a grey to light yellowish color, were not replated. The number of colonies replated depended on the number of individual colonies available for sampling, as the number of colonies per morphology were limited and some plates were (partially) overgrown with typical Bacillus colonies.

2.3. Limit of detection of Staphylococcus aureus of a high salt selective culture method

The limit of detection of *S. aureus* of a two-step high salt selective culture method was tested by spiking dilution series of five *S. aureus* strains in duplicate in the first culture step. The strains used are listed in Supplementary Table S2. Strains were cultured on Columbia Agar plate supplemented with 5% Sheep blood for 22–24 h at 37 °C and a colony was resuspended in PBS and the optical density relative to a McFarland standard was measured using a McFarland densitometer (Buch & Holm, Herlev, Denmark). The CFU/mL was estimated based on the McFarland standard, assuming 1 × 10^8 CFU/mL at McFarland 0.5. A dilution series in PBS was made for each strain. The 5 mL MHB supplemented with 15% NaCl was spiked with an estimated 1, 10, 50, 100, 500 and 1000 CFU in 150 μL PBS and subsequently cultured as described above. The true spiked CFU were checked by plating 100 μL of the dilution series on Columbia Agar plate supplemented with 5% Sheep blood and counting the number of colonies after incubation for 22–24 h at 37 °C. After the second high salt culture step, 10 μL of the broth was plated on a Columbia Agar plate supplemented with 5% Sheep blood and incubated for 22–24 h at 37 °C. If there was growth (≥1 colony) after incubation, the spiked number of CFU of *S. aureus* could be detected using this method. Spiking with PBS only was included as a negative control and these samples were negative in all experiments.

2.4. Identification of Staphylococcus aureus

2.4.1. PCR

Single colonies from the replated colonies isolated from milk or extramammary sites after the high salt selective culture were picked for DNA extraction and washed in 500 μL of Tris-EDTA (TE) buffer (Sigma-Aldrich, Saint Louis, Missouri, USA). The samples in TE buffer were boiled for ten minutes at 100 °C and centrifuged for one minute at 16,110 x g at room temperature. Primers for *femA* and *spa* were taken from literature (Table 2) and PCRs were performed separately. The reaction was performed in a total volume of 20 μL containing 5 μL boiled DNA sample, 10 μL GoTaQ G2 hotstart green mix (Promega, Madison, WI, USA), 0.5 μM of each *femA* or *spa* primer (Invitrogen, Carlsbad, CA, USA), and nuclease free water (Promega, Madison, Wisconsin, USA). First a *femA* PCR was performed to confirm the presence of *S. aureus*. After an initial denaturation step at 95 °C for 2 min, 35 cycles (30 s at 95 °C, 30 s at 60 °C and 35 s at 72 °C) and a finalization step at 72 °C for 5 min were performed in a 2720 thermal cycler (Applied Biosystems, Waltham, MA, USA). Electrophoresis on 1% agarose gel was used to visualize PCR products. On *femA* positive colonies a *spa* PCR was performed. After an initial denaturation step at 95 °C for 2 min, 30 cycles (45 s at 95 °C, 45 s at 59.5 °C and 90 s at 72 °C) and a finalization step at 72 °C for 7 min were performed in a 2720 thermal cycler. Electrophoresis on 1% agarose gel was used to visualize PCR products.

2.4.2. Spa typing

The polymorphic X-region of the Staphylococcal Protein A (*spa*) gene of all *spa* PCR positive *S. aureus* isolates was amplified according to the RidomStaphType standard protocol (https://www.*spa*server.ridom.de/). PCR amplicons from the *spa* PCR were purified using ExoSAP-it PCR-clean up (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions and sequenced using Sanger sequencing (Baseclear, Leiden, The Netherlands). Bionumerics v7.1 software (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyze sequence data and assign *spa* genotypes. Isolates that were positive in the *femA* PCR, but negative in the *spa* PCR were defined as *spa* genotype 'untypeable'.

2.4.3. Data and statistical analyses

Prevalence of colonization and prevalence of IMI were calculated using Eq. (1) and the 95% confidence interval was calculated according to Wilson (https://epitools.ausvet.com.au/ciproportion).

$$Prevalence\ of\ colonization = \frac{Number\ of\ cows\ with\ S.aureus\ positive\ culture\ from\ body\ site\ x}{Total\ number\ of\ cows\ sampled\ on\ body\ site\ x} * 100 \quad (1)$$

To test whether *S. aureus spa* genotype was associated with IMI or extramammary site colonization a Fisher's exact test (2× table) or Fisher-Freeman-Halton exact test (n x m table) was performed using SPSS version 27.0.1.0. *P*-values ≤0.05 were considered significant.

3. Results

3.1. Limit of detection of Staphylococcus aureus of a high salt selective culture method

If approximately one CFU (0.8–2 CFU) was spiked in the high salt selective broth there was detection of *S. aureus* in 50% of the samples and when 16 CFU's or more were spiked, there was detection of *S. aureus* in all samples (Supplementary Table S2).

Prevalence of *Staphylococcus aureus* in milk and extramammary sites.

In total, 207 cows were sampled and data from 180 cows were included in the study. From 153 cows both milk and extramammary sites were sampled, from 27 cows only a milk sample was collected. From three cows in Herd VI data of the milk was excluded as the sample was contaminated. Of the 177 cows with uncontaminated milk samples, 28 cows had a *S. aureus* IMI (Table 3). The extramammary sites of 153 cows were sampled and in 53 cows at least one extramammary site was colonized, in 18 cows two sites were colonized and in three cows three sites were colonized. Table 3 provides a more detailed overview of the number of cows sampled and extramammary sites colonized for the different herds. The cow level prevalence of colonization of extramammary sites (the prevalence of cows within a herd with at least one *S. aureus* colonized extramammary site) was 35% across all herds. In herd V, milk and extramammary sites of 20 cows were sampled, all of these cows were negative for *S. aureus* on both the extramammary sites and in the milk. This was the only herd in which *S. aureus* colonization or *S. aureus* IMI were not detected. In contrast, the highest cow level prevalence of colonization was 73% in herd I. For most herds, the colonization of the extramammary sites was more frequent compared to the number of cows with *S. aureus* IMI from CMT positive quarters. For example in herd VI, where the cow level prevalence of colonization was 29%, while the prevalence of IMI was 6%. Herd II was an exception as the cow level *S. aureus* prevalence of IMI (61%) was higher than the cow level prevalence of colonization of the extramammary sites (29%). The cow level prevalence of *S. aureus* IMI from CMT positive quarters was

16% when including all cows, but varied from 0% to 61% between herds (Table 3). The cow level prevalence of colonization and prevalence of *S. aureus* IMI were significantly different between herds ($p = 0.002$ and $p < 0.001$ respectively, Fisher's exact test). The most commonly colonized extramammary site was the hock with an overall prevalence of 23%, varying from 0% to 73% between herds (Table 3). Feces was colonized the least with an overall prevalence of 5%, varying from 0% to 10% between herds (Table 3). Prevalence of colonization of the hock, groin, and udder cleft were significantly different between herds ($p < 0.001$, $p = 0.025$, $p = 0.003$ respectively, Fisher's exact test). The prevalence of colonization of the nares ($p = 0.519$) and feces ($p = 0.266$) were not significantly different between herds.

3.2. Distribution of *Staphylococcus aureus spa* genotypes between milk and extramammary sites

An overview of the different *spa* genotypes found within herds and their distribution between extramammary sites and milk is presented in Fig. 1. As multiple *spa* genotypes were observed in herds, the association between *spa* type of the *S. aureus* isolates and milk or extramammary site colonization was analyzed within the herd. Each herd had one or two highly abundant *spa* types either associated with *S. aureus* IMI, colonization of extramammary sites or both (Fig. 1, Supplementary Tables S3-S7). In herd III most *S. aureus* IMI cases and extramammary site colonization were associated with *spa* types t529 and t543 (Supplementary Table S5). In this herd both *spa* types were found in equal proportion on both extramammary sites and in milk and no association was found between *spa* type and colonization site ($p = 0.650$, Fisher-Freeman-Halton exact test). Similar results were found in herd VI, with the dominant *spa* type (t529) detected both in *S. aureus* IMI cases and on extramammary sites, no association was found between *spa* type and colonization site ($p = 0.833$, Fisher-Freeman-Halton exact test) (Supplementary Table S7). In herd I there was also no association between *spa* type and either milk or extramammary site colonization ($p = 1.000$, Fisher-Freeman-Halton exact test), with t529 being the dominant *spa* type as well (Supplementary Table S3). However, in two herds there was an association between *spa* type and milk or extramammary site colonization. *Staphylococcus aureus* IMI in herd IV belonged to *spa* type t1028, but this type was not isolated from the extramammary sites ($p = 0.004$, Fisher-Freeman-Halton exact test) (Supplementary Table S6). In herd II, the *S. aureus* isolates found on the extramammary sites were negative for the *spa* PCR (untypeable).Whereas *spa* type t3614 was the dominant *spa* type in milk and was not found on extramammary sites ($p < 0.001$, Fisher-Freeman-Halton exact test) (Supplementary Table S4). The untypeable and t3614 *spa* types were significantly associated with extramammary sites and milk, respectively.

As mentioned above, only one third of the colonized cows were colonized on more than one extramammary site. If multiple extramammary sites were colonized, 76% of these cows had the same *spa* type colonizing all sites (16 of 21 cows). If a cow had both *S. aureus* IMI and was colonized on at least one extramammary site, the *spa* type was different in all cows. However, only a small number of cows had both *S. aureus* IMI and colonization of an extramammary site (nine of 28 cows with *S. aureus* IMI).

To determine whether an extramammary site can be colonized by multiple genotypes, several colonies cultured from each swab were *spa* typed. From all extramammary site swabs where multiple colonies could be tested only 1 out of 48 extramammary sites showed to be colonized with two *spa* types (Supplementary Tables S8 and S9). To further characterize the strain variation within extramammary sites an additional 13 cows were sampled consecutively with three swabs at the exact same site (Supplementary Table S1). *Staphylococcus aureus* was detected in six of the 36 sites sampled. In 32 of the sampled sites the *S. aureus* result for the three swabs was identical (either all swabs were negative or all swabs were positive), while in four other sites one or two of the three swabs were positive for *S. aureus* (Supplementary Table S1). From one swab of an extramammary site, colonies with two different *spa* types were obtained, from another extramammary site, colonies with different *spa* types were present in two different swabs. For four of the extramammary sites one *spa* type was isolated from all positive swabs (Supplementary Table S1).

4. Discussion

The objectives of this study were to determine the prevalence of colonization of extramammary body sites with *S. aureus* in Dutch dairy cattle with a composite milk somatic cell count ≥200,000 cells/mL and to compare *S. aureus spa* genotypes of isolates from IMI to *spa* genotypes of isolates from extramammary sites. We showed that there was a high cow level prevalence of colonization of extramammary sites with

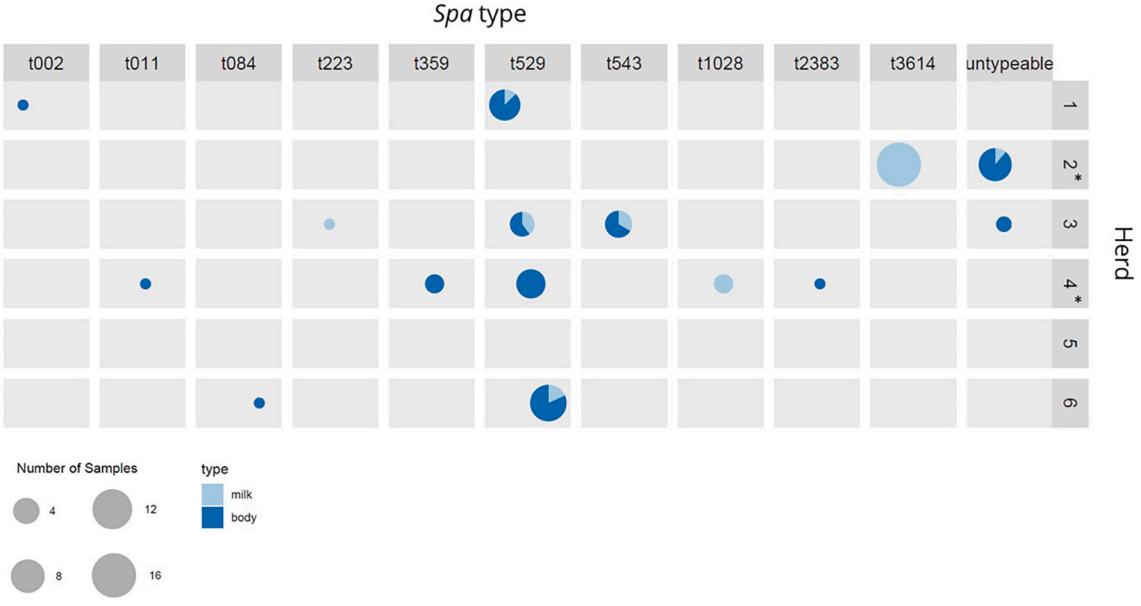

Fig. 1. Overview of the distribution of *spa* genotypes in isolates from milk and extramammary sites in the different herds. *For these farms there was a significant association between *spa* genotypes with either milk or extramammary site colonization.

S. aureus in the different herds sampled and that there were differences in prevalence of colonization between extramammary sites and between herds. Furthermore, *spa* typing showed that in two herds there was an association between *S. aureus spa* genotypes and either milk (i.e. an IMI) or extramammary site colonization.

Interventions against *S. aureus* IMI are aimed at preventing transmission from infected to susceptible quarters (Barkema et al., 2006; Keefe, 2012). However, *S. aureus* can also colonize extramammary sites and this may constitute a reservoir for infection that is not targeted by interventions aimed at preventing transmission from infected quarters. Therefore, the first aim of this study was to determine the prevalence of extramammary site colonization by *S. aureus* in dairy cattle in the Netherlands. The prevalence of colonization of extramammary sites at cow level varied from 0% to 73% between herds. These results are comparable to those by Roberson et al. (1994), who observed a herd specific prevalence of colonization varying from 0% to 78%. However, in the current study a different combination of extramammary sites was sampled and teat skin was not included. Other studies determining the prevalence of colonization of various extramammary sites (Capurro et al., 2010; Leuenberger et al., 2019) determined a lower prevalence of colonization varying from 4% to 37%, compared to this study and Roberson et al. (1994). Prevalence differences may be caused by various factors, such as farm characteristics (tie stall or free stall (Leuenberger et al., 2019)), breed of cows, sampling and culturing method, and characteristics of specific *S. aureus* genotypes present (e.g. higher prevalence, or ability to colonize extramammary sites) (Leuenberger et al., 2019). Prevalence estimates of individual extramammary sites were significantly different, with the feces (5%) and nares (5%) having the lowest and groin (10%) and hock (23%) having the highest prevalence. Between herds the prevalence of colonization of extramammary sites also varied significantly. In addition, prevalence estimates of different extramammary sites vary between studies (Capurro et al., 2010; Leuenberger et al., 2019; Matos et al., 1991; Mørk et al., 2012; Roberson et al., 1994) (Supplementary Table S10). For example, the prevalence estimate of the hock skin was higher in this study (23%) than in Leuenberger et al. (2019) (7%). This difference could be a result of the culture method used to detect *S. aureus*, as Leuenberger et al. (2019) directly cultured samples on agar and in this study a selective culture method was used. In contrast, Capurro et al. (2010) described a much higher prevalence of colonization of the hock of 37%. However they only sampled cattle housed in tie stalls, which could explain the high prevalence of colonization as Leuenberger et al. (2019) determined previously that the hock was colonized more in tie stall than in free stalls. The estimated prevalence of nasal colonization of 5% was similar to the prevalences reported by Capurro et al. (2010) (5%) and Roberson et al. (1994) (9%). However, it was lower than Anderson et al. (2012) (28.4%) and Matos et al. (1991) (18.3%) reported. Nasal sampling methods were similar in these studies, but there were differences in *S. aureus* culture methods, husbandry, and animal characteristics (e.g. including heifers or lactating cattle only, breeds). In summary, *S. aureus* frequently colonizes different extramammary sites, but there is a large variation in prevalence of colonization between sampled extramammary sites and herds.

Staphylococcus aureus does not cause disease when colonizing extramammary body sites and is considered to be part of the commensal microbiome. However, it is unknown if there is transmission of *S. aureus* from extramammary sites to the udder or other cows resulting in IMI. To address this question, all *S. aureus* isolates were *spa* typed to determine the association between *spa* type and presence in milk or extramammary site. Between herds there were considerable differences in the detected *spa* types. In most herds one or two types were dominant and several *spa* types were only present in a single herd. This is in line with results from Capurro et al. (2010), Leuenberger et al. (2019), and Sommerhäuser et al. (2003). The distribution of *S. aureus spa* genotypes between milk or extramammary sites could be categorized into three groups: i) predominantly milk associated, ii) associated with both milk and extramammary sites, and iii) associated with extramammary sites (Fig. 1). These findings corroborate the results of Leuenberger et al. (2019) who showed that certain *S. aureus* genotypes are milk associated and highly contagious and other genotypes are associated with extramammary sites and only sporadically cause IMI. Leuenberger et al. (2019) found that *S. aureus* isolates that belong to 16S—23S ribosomal *spa*cer PCR genotype B were contagious and associated with milk rather than extramammary sites. Genotype B contains isolates that belong to *spa* types t2953, t2915, and t3802 and genotype B positive isolates have not been identified in the Netherlands (Cosandey et al., 2016; Hoekstra et al., 2020). However, both genotype B isolates and the *spa* type t3614 isolates as found in this study were contagious (high prevalence of IMI) and associated with milk rather than extramammary sites. In contrast we found *spa* types t529 and t543 both in milk and extramammary sites in different herds. Leuenberger et al. (2019) identified *spa* type t529 within isolates of genotype C and genotype C isolates were associated with extramammary sites and occasionally caused IMI. Hoekstra et al. (2018) described that *spa* types t529 and t543 were highly prevalent in *S. aureus* IMI in the Netherlands. Our findings indicate that extramammary sites potentially are reservoirs for intramammary infections and this can have implications for control measures against *S. aureus* IMI. This may explain the observation of Sommerhäuser et al. (2003) that in some herds control measures aimed at preventing contagious transmission were not effective in eradicating all *S. aureus* genotypes. In addition, in a bio-economic simulation model, we (Exel et al., 2022) previously demonstrated that for spill-over *S. aureus* strain (strains with an extramammary site reservoir that occasionally "spill-over" to milk) the economic and epidemiologic outcome of control measures differ compared to contagious *S. aureus* strains.

Several studies found an association between *S. aureus* genotypes from teat skin and IMI (Anderson et al., 2012; Capurro et al., 2010; Haveri et al., 2008). However, teats can be contaminated by milk and milk liners, potentially leading to transient colonization. It is unknown whether teat skin colonization is a risk factor for IMI or whether teat skin is colonized following an IMI. Therefore, in this study extramammary sites unlikely to be contaminated by milk were selected. Additionally, feces was tested since many skin surfaces could be contaminated with feces, but *S. aureus* prevalence in feces was much lower than for other extramammary sites. Together, this makes it likely that the *S. aureus* positive extramammary sites in this study reflect true colonization and not contamination of the site by spill-over from milk. Spill-over of *S. aureus* from extramammary sites to the teat and from there to the milk could occur, for example, by migrating colonization, rubbing skin against the udder/teats, licking or lying in feces. In the present study, only a limited number of cows had both *S. aureus* IMI and colonization of extramammary sites (nine of 28 cows with *S. aureus* IMI) and in these animals different *spa* types were found in milk and extramammary sites. The exact spill-over transmission routes from extramammary sites to milk are unknown and could occur both between and within cows. Spill-over from extramammary sites to milk may be very rare (explaining the low prevalence of IMI with these isolates), but once an IMI is established, contagious quarter-to-quarter transmission could also occur. To study how the transmission from extramammary reservoirs occurs, detailed longitudinal studies are necessary.

In one case of 157 sampled extramammary sites, two different *S. aureus spa* types colonized the same extramammary site. To our knowledge, this is the first study showing that in cattle the same extramammary site can be colonized by two different *S. aureus spa* genotypes. The repeated swabbing of the same extramammary sites gave consistent results in most cases. However, in 11% of the repeatedly swabbed extramammary sites the results varied (either one or two swabs positive for *S. aureus*). Variation between swabs was expected since the superficial and deep microbiome of the skin are different (Kong et al., 2017; Zeeuwen et al., 2012) and it is impossible to sample the exact same site twice. Nevertheless, these results show that *S. aureus* colonization is probably underestimated and that there might be more

variation in *S. aureus* colonization, both in exact location and in genotype, than previously recognized. Little is known about the *S. aureus* bacterial load and whether specific sites may be colonized transiently or for longer periods. Both these aspects might affect the likelihood of transmission from colonized sites to other cattle and milk. Follow-up studies should focus on these unknown aspects of *S. aureus* colonization in cattle. To increase the chance of sampling cattle with an *S. aureus* IMI only cows with a composite milk SCC $\geq$ 200,000 cells/mL were included and this should be taken into account when extrapolating these results to dairy cattle in general. However, from other studies it is known that cattle with a composite milk SCC $<$ 200,000 cells/mL and heifers before their first lactation are also colonized by *S. aureus* (Anderson et al., 2012; Leuenberger et al., 2019; Matos et al., 1991; Svennesen et al., 2019) and as hypothesized above, the transmission from extramammary sites to milk is likely to occur between cows. Therefore this selection criterium is unlikely to affect our overall conclusion that colonization of extramammary sites with *S. aureus* can act as a reservoir for IMI in dairy cattle.

In conclusion, our results showed that different *S. aureus spa* genotypes have different colonization characteristics, and that extramammary *S. aureus* reservoirs could be a source for spill-over IMI infections that cannot be eradicated by intervention measures aimed at contagious mastitis pathogens.

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Declaration of Competing Interest

All authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2022.12.010.

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