Teat apex colonization with coagulase-negative *Staphylococcus* species before parturition: Distribution and species-specific risk factors

A. De Visscher,^{*1} S. Piepers,^{*} F. Haesebrouck,[†] and S. De Vliegher^{*}

^{*}M-team and Mastitis and Milk Quality Research Unit, Department of Reproduction, Obstetrics, and Herd Health, and

[†]Department of Pathology, Bacteriology, and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, 9820 Merelbeke, Belgium

ABSTRACT

Coagulase-negative staphylococci (CNS) are the main cause of bovine intramammary infections and are also abundantly present in extramammary habitats such as teat apices. Teat apex colonization (TAC) with CNS has already been explored in lactating dairy cows at the species level, whereas this is not true for dry cows and end-term heifers. Therefore, the aim of this observational study was to describe CNS TAC in non-lactating dairy cows and end-term heifers in Flemish dairy herds and to identify associated risk factors at the herd, cow, and quarter level. All CNS were molecularly identified to the species level using transfer RNA intergenic spacer PCR (tDNA-PCR) and sequencing of the 16S rRNA gene, allowing for species-specific statistical analyses using multivariable, multilevel logistic regression. *Staphylococcus devriesei*, *Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, and *Staphylococcus equorum* were the most frequently isolated species. *Staphylococcus chromogenes* was the sole species colonizing teat apices of cows and heifers in all herds, whereas large between-herd differences were observed for the other species. Teat apices of red and white Holstein Friesians, of quarters dried off without an internal teat sealer, and swabbed in months with lower precipitation and higher ambient temperature were significantly more likely to be colonized by *S. devriesei*. Slightly dirty teat apices and teat apices swabbed in months with lower precipitation had higher odds of being colonized by *S. chromogenes*, whereas teat apices sampled in months with lower precipitation and higher ambient temperature were more likely to be colonized by *S. haemolyticus*. Dirty teat apices and teat apices swabbed in months with lower ambient temperature in combination with low precipitation had higher odds of being colonized by *S. equorum*. Diverse factors explaining CNS TAC, yet mostly related to humidity, ambient

temperature, and hygiene, substantiate differences in epidemiological behavior and ecology between species.

Key words: teat apex, coagulase-negative *Staphylococcus* species

INTRODUCTION

Besides being the most common cause of intramammary infections in dairy cows in many regions and countries (Vanderhaeghen et al., 2015), CNS are abundantly present in extramammary habitats such as sawdust and air (Piessens et al., 2011) and also colonize dairy cows' teat apices (Taponen et al., 2008; Braem et al., 2012; De Visscher et al., 2014). In fact, the majority of teat apices colonizing microbiota belong to the group of the CNS (Braem et al., 2013). Differences in ecological and epidemiological characteristics among bovine-associated CNS species have been revealed (Vanderhaeghen et al., 2015), yet many aspects remain undetermined. The presence of CNS on teat apices of lactating dairy cows and heifers has already been explored at the species level (Taponen et al., 2008; Braem et al., 2013; De Visscher et al., 2014; Vanderhaeghen et al., 2015) but little is known about CNS teat apex colonization (**TAC**) in dry cows and end-term heifers. A high prevalence of CNS colonized teat apices in pregnant dairy heifers before calving has been described (De Vliegher et al., 2003; Piepers et al., 2011), but those studies either solely concerned heifers, studied CNS as a group, or focused specifically on *Staphylococcus chromogenes* only using nonmolecular identification. Large observational studies describing the species distribution on teat apices in nonlactating dairy cows and end-term heifers and identifying factors associated with their presence are needed to add to our understanding of the role of CNS species in bovine udder health (Taponen et al., 2008; De Visscher et al., 2014; Vanderhaeghen et al., 2015). Although we have started to learn about the relation between CNS TAC and IMI and associations seem to be present (Leroy et al., 2015), potential protective aspects of TAC remain to be studied (Vanderhaeghen et al., 2014).

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¹Corresponding author: Anneleen.Devisscher@UGent.be

Therefore, this study aimed to determine and to describe (1) the species-specific prevalence of CNS colonization of teat apices of dry cows and end-term heifers before calving; (2) the CNS distribution; (3) associated herd-, cow-, and quarter-level risk factors; and (4) the variance components of CNS TAC.

MATERIALS AND METHODS

Herds and Cows

Thirteen commercial Flemish dairy herds were included, all of them participating in the DHI program (CRV, Arnhem, the Netherlands), being a first selection criterion. Other inclusion criteria were no prepartum antibiotic treatment of heifers and the use of AI as accurate expected calving dates were demanded. The majority of farmers ($n = 8$) only housed dairy cattle, whereas the others also farmed pigs ($n = 1$) or beef cattle ($n = 4$). The DHI records and bulk milk quality data of the Milk Control Centre Flanders (Lier, Belgium) allowed for calculating the herd size and the bulk milk SCC, respectively, at the start of the sampling period in July 2012. The entire sampling period lasted until February 2013. Before the start of the study, an average of 57 cows and heifers were in lactation (range = 30–95) per herd (arithmetic mean of the 6 last test-day samples). The geometric mean bulk milk SCC was 201,000 cells/mL (range = 79,000–310,000 cells/mL). On 7 of the farms, dry cows were housed on concrete slatted floor with cubicles with mats ($n = 4$), mattresses ($n = 2$), or without bedding ($n = 1$). The other 6 farms kept the dry cows on straw, either in a deep litter barn ($n = 5$) or in deep litter boxes with a bottom layer of sand and a full concrete floor ($n = 1$). Pregnant heifers were typically housed on a concrete slatted floor in cubicles ($n = 11$) with mats ($n = 5$), with mattresses ($n = 5$), or without bedding ($n = 1$). The other 2 farms housed the pregnant heifers either in a deep litter barn with straw ($n = 1$) or in deep litter boxes with a bottom layer of sand and straw ($n = 1$). On the majority of farms, dry cows and pregnant heifers ($n = 12$ and $n = 11$, respectively) were kept on pasture between May and September. Seven of the herds housed the dry cows separated from the lactating cows before calving, whereas in the majority of herds ($n = 10$) the pregnant heifers were housed together with the lactating cows.

On each farm, 12 pregnant heifers and dry cows (total $n = 156$) were selected according to the proportion of lactating cows and heifers present in the herd at the start of the study, reflecting the parity distribution in the herd, resulting in a total of 53 pregnant heifers and 103 dry cows (range = start of second lactation to start of tenth lactation). The majority of the selected

animals were black and white Holstein Friesian (HF; 85%, $n = 132$), and 15% were red and white HF ($n = 24$). Eighty-five percent ($n = 132$) of the animals were supplemented with minerals and vitamins before parturition. On all farms, blanket dry cow treatment was applied using either cloxacillin benzathine (47% of the 103 dry cows, $n = 48$ cows) or cephalosporins with a broad or gram-positive spectrum (40%, $n = 41$ cows and 10%, $n = 10$ cows, respectively). Dry cow treatment information was lacking for a few cows only (3%, $n = 3$) as these cows were purchased when dry. One cow did not receive antimicrobials at drying-off due to a sudden drop in milk production. Only a minority of the dry cows received an internal teat sealer (39% of the 103 dry cows, $n = 40$) at drying off. Iodine teat dip was applied to 38 dry cows and end-term heifers (24%) before parturition.

Samples and Data Collection

To determine TAC before parturition, swabs of all teat apices of the 153 cows and heifers (total $n = 624$) were collected 14 d before expected calving date. Visible soil and manure were first removed. Further, a dry cotton swab (Copan, Novolab, Belgium) was rotated gently on the teat apex as described by De Vlieghe et al. (2003). Swabs were transported under cooled condition (4°C) to the Mastitis and Milk Quality Research Lab (Faculty of Veterinary Medicine, Ghent University, Mellebeke, Belgium).

Several herd- and cow-level factors, potentially associated with CNS (species-specific) TAC, were either calculated based on DHI records or on data of the Milk Control Centre Flanders, or collected before the onset of the study via a questionnaire (Table 1). Other potential cow- and quarter-level factors were recorded at sampling (teat swabbing; Table 1).

Laboratory Analyses

All swabs were plated on mannitol salt agar (Oxoid, Erembodegem, Aalst, Belgium; one plate per swab; De Visscher et al., 2013). Plates were aerobically incubated at 37°C and examined after 24 and 48 h. One colony of all phenotypically different colony types was picked up and subcultured on esculin blood agar (Oxoid; one quadrant per isolate) to obtain pure cultures for subsequent analysis. All potential CNS isolates were stored at –80°C or immediately identified to the species level using transfer RNA intergenic spacer PCR (tDNA-PCR). If no identification could be obtained, sequencing of the 16S rRNA gene was performed (Supré et al., 2009).

Table 1. Overview of all herd-, cow-, and quarter-level risk factors potentially associated with (species-specific) teat apex colonization with CNS

Risk factor	Recording method	Description	Categories considered in the final model
Herd level			
Herd type	Questionnaire	Dairy cattle only or with pigs or beef cattle	Dairy cattle only vs. dairy cattle with other animals
Herd size	DHI records	Mean number of animals in lactation at onset of sampling period ¹	Smaller (<60 lactating cows) vs. larger (≥60 lactating cows) herd ²
Bulk milk SCC	MCC records	Bulk milk SCC at onset of sampling period ³	Lower bulk milk SCC (<196 × 10 ³ cells/mL) vs. higher bulk milk SCC (≥196 × 10 ³ cells/mL) ²
Cow level			
Housing	Questionnaire	Housing of dry cows and pregnant heifers	Cubicles vs. deep litter
Pasture access	Questionnaire	Pasture access during outdoor season	No pasture vs. pasture
Contact ⁴	Questionnaire	Contact with lactation cows before parturition	No contact vs. contact
Breed	DHI records	Breed	Black and white HF ⁵ vs. red and white HF
Parity	DHI records	Parity starting at calving	First lactation vs. second lactation or older
Vitamins	Questionnaire	Supplementation with minerals and vitamins before parturition	No supplementation vs. supplementation ⁶
Antimicrobials	Questionnaire	Antimicrobials used at drying-off	No vs. narrow-spectrum vs. broad-spectrum ⁶
Teat sealer	Questionnaire	Application of an internal teat sealer at drying-off	No teat sealer vs. teat sealer
Teat dip	Questionnaire	Teat dipping/spraying before parturition	No dipping/spraying vs. dipping/spraying
BCS	Visual	Five-point scale ⁷ at sampling	<2.5 vs. 2.5–3 vs. >3
Hygiene	Visual	Hygiene of mammary gland and teats at sampling ⁸	Very clean vs. slightly dirty vs. dirty
Temperature	RMI ⁹	Monthly ambient temperature (°C) at sampling	Low vs. high ¹⁰
Precipitation	RMI	Monthly precipitation (L/m ²) at sampling	Low vs. high ¹⁰
Quarter level			
Quarter position	Visual	Position of the quarter	Front vs. hind quarter
Teat apex condition	Visual	Teat apex condition at sampling	Good condition vs. protuberant teat end vs. invaginated teat end
Teat skin condition	Visual	Teat skin condition at sampling	Little grooves vs. many grooves

¹Arithmetic mean based on 6 last DHI records before the onset of the study (July 2012).²Categorization based on median value of all mean values of all herds.³Geometric mean based on 6 last records of the Milk Control Centre Flanders before the onset of the study.⁴Housing of dry cows and end-term heifers together with lactating animals.⁵Holstein Friesian.⁶Antimicrobials with a narrow spectrum include cloxacillin benzathine, cefalexine, and cefalonium, whereas cefquinome is a broad-spectrum antibiotic.⁷Edmonson et al. (1989).⁸Categorization based on a 5-point scale (Reneau et al., 2005).⁹Royal Meteorological Institute of Belgium.¹⁰Categorization based on median value of all monthly records during 1 yr (March 2012 until February 2013).

Descriptive and Statistical Analyses

Observations were checked for unlikely values before analyses were performed. Complete data were available from 596 teat apices from 96 dry cows and 53 end-term heifers.

Logistic multilevel regression models were fit using MLwiN 2.16 (Centre for Multilevel Modeling, University of Bristol, Bristol, UK) applying reweighted iterative generalized least squares and first-order penalized quasi-likelihood. As quarters were spatially clustered within cows and cows within herds, analysis were run with cow and herd as random effects.

Five different binary outcome variables at the quarter level were used: (1) teat apex colonized with CNS (whatever species) versus teat apex not colonized with CNS, and (2 to 5) teat apex colonized with one of the 4 most frequently isolated CNS species: (2) *Staphylococcus devriesei*, (3) *S. chromogenes*, (4) *Staphylococcus haemolyticus*, and (5) *Staphylococcus equorum*, versus teat apex not colonized with CNS or colonized with (an)other CNS species.

First, univariable associations were examined between the outcome variables and the independent factors (Table 1) with statistical significance assessed at $P < 0.15$. Second, Spearman rank correlation coefficients were calculated among the significant independent variables to avoid multicollinearity in the next steps. A correlation coefficient $\geq |0.6|$ among 2 factors led to the selection of 1 of the 2 variables for further analysis. In a third step, multivariable models were fit for all outcome variables using backward stepwise elimination. Statistical significance was assessed at $P < 0.05$. Biological relevant interaction terms were tested between all remaining statistically significant factors and kept in the final multivariable model when significant ($P < 0.05$). A Bonferroni correction was applied to adjust for multiple comparisons. The observational-level standardized residuals were plotted against the observational-level predicted values to test the adequacy of all final models. In addition, the Hosmer-Lemeshow goodness-of-fit test and 2 residual log pseudo-likelihood statistics were assessed on the fixed effect models only (SAS 9.3, SAS Institute Inc., Cary, NC; Dohoo et al., 2009). The test was for none of the final models statistically significant indicating good fit of our models. Data are presented as odds ratios (OR) and 95% confidence intervals. The proportion of variation for the outcome variables at the herd, cow, and quarter level was estimated based on the null and final models by assuming that the variance at the quarter level was $\pi^2/3$ (Dohoo et al., 2001) as described by Piepers et al. (2011).

RESULTS

Descriptive Analyses

The majority of teat apices (87%, $n = 540$) yielded growth on mannitol salt agar and per plate 0 to 4 phenotypically different colony types were present. Different colony types sometimes appeared to belong to the same CNS species after tDNA-PCR or 16S rRNA gene sequencing, eventually resulting in 619 CNS isolates available for further analysis. Seventy-two percent ($n = 448$) of all teat apices were colonized with CNS and harbored 1 up to 3 different CNS species. Twenty-four different CNS species were identified.

The overall most frequently isolated species was *S. devriesei* (19% of all teat apices colonized, $n = 120$ teat apices), followed by *S. chromogenes* (17%, $n = 107$), *S. haemolyticus* (17%, $n = 107$), *S. equorum* (13%, $n = 81$), *Staphylococcus auricularis* (8%, $n = 49$), *Staphylococcus sciuri* (7%, $n = 41$), *Staphylococcus cohnii* (4%, $n = 25$), and *Staphylococcus xylosus* (4%, $n = 25$; Table 2). Isolates ($n = 390$) other than CNS were phenotypically or genotypically (i.e., using tDNA-PCR or 16S rRNA gene sequencing) identified as *Staphylococcus aureus* ($n = 4$), *Arthrobacter* spp. ($n = 1$), *Bacillus* spp. ($n = 307$), *Jeitgalicoccus* spp. ($n = 28$), other gram-positive cocci such as *Aerococcus* spp. ($n = 12$), *Enterococcus* spp. ($n = 1$), or *Streptococcus* spp. ($n = 5$), gram-negative bacteria such as *Pseudomonas* spp. ($n = 16$), *Psychrobacter* spp. ($n = 4$) or other ($n = 7$), and fungi ($n = 5$).

On each herd, between 6 (herd 12) and 12 (herd 7) different CNS species were isolated from the teat apices. *Staphylococcus chromogenes* was the sole species colonizing teat apices in all 13 herds whereas *S. devriesei*, *S. haemolyticus*, and *S. auricularis*, were colonizing teat apices on all farms except one. *Staphylococcus equorum*, *S. xylosus*, *S. sciuri*, and *S. cohnii* could not be isolated from teat apices in 3, 4, 5, and 6 herds, respectively (Table 2, Figure 1). Species other than the aforementioned ones were only found in a minority of herds (Table 2).

The majority of the dry cows and pregnant heifers (95%, $n = 98$ out of 103 dry cows and 94%, $n = 50$ out of 53 heifers, respectively) had at least one teat apex colonized with CNS. Seventy-four percent ($n = 156$) of all teat apices of the end-term heifers ($n = 212$) harbored CNS, whereas 71% ($n = 292$) out of 412 teat apices of the dry cows were colonized with CNS. Teat apices of the pregnant heifers were mainly colonized by *S. chromogenes* (55% heifers, $n = 29$ out of 53 heifers and 21% teat apices, $n = 45$ out of 212 teat apices), followed by *S. haemolyticus* (42% heifers,

Table 2. Species distribution of CNS colonizing teat apices (n = 624) from 103 dry cows and 53 end-term heifers on 13 Flemish dairy herds

<i>Staphylococcus</i> species	Herd 1		Herd 2		Herd 3		Herd 4		Herd 5		Herd 6		Herd 7		Herd 8		Herd 9		Herd 10		Herd 11		Herd 12		Herd 13	
	% _{DC} ¹	% _H ²	% _{DC}	% _H	% _{DC}	% _H	% _{DC}	% _H	% _{DC}	% _H	% _{DC}	% _H	% _{DC}	% _H	% _{DC}	% _H	% _{DC}	% _H	% _{DC}	% _H	% _{DC}	% _H	% _{DC}	% _H	% _{DC}	% _H
<i>S. deuresei</i>	25.0	6.3	28.6	45.0	20.8	37.5	31.3	18.8	21.4		19.4	25.0	18.8	25.0	21.9	12.5	38.9	50.0	14.3	10.0	6.3	37.5	6.3	5.0		
<i>S. chromogenes</i>	9.4		10.7	25.0		8.3	34.4	62.5	7.1	10.0			21.9	18.8	6.3	37.5	5.6	33.3	21.4	10.0	9.4	31.3	28.1	6.3	17.5	25.0
<i>S. haemolyticus</i>			21.4	25.0	12.5	12.5	18.8	56.3	28.6		8.3	25.0	34.4	50.0	9.4	6.3	38.9		35.7	20.0	3.1	12.5	6.3	50.0		
<i>S. equorum</i>	31.3	68.8			20.8	20.8			3.6	45.0	22.2		3.1	6.3	18.8				10.7	30.0	12.5	6.3	6.3	17.5		
<i>S. auricularis</i>	6.3				4.2	8.3	3.1	12.5	7.1		5.6	16.7	18.8	56.3	15.6	12.5	5.6			5.0	3.1		7.5	0.0		
<i>S. sciuri</i>			7.1								30.6		3.1	6.3	9.4		13.9	25.0	3.6	10.0	3.1		22.5	37.5		
<i>S. cohnii</i>	31.3				16.7	25.0	6.3		10.7		8.3		15.6	6.3			13.9	25.0		5.0	3.1		2.5	12.5		
<i>S. xylosus</i>			3.6			25.0					8.3		3.1	6.3							3.1			2.5		
<i>S. saprophyticus</i>					16.7	4.2							18.8								3.1					
<i>S. vitulinus</i>			3.6								11.1			6.3			5.6	8.3								
<i>S. arlettae</i>										5.0			12.5													
<i>S. succinus</i>			3.6				3.1								3.1	6.3	2.8	8.3			6.3			2.5		
<i>S. caseolyticus</i>	3.1	6.3									2.8															
<i>S. capitis</i>									3.6						3.1		8.3		7.1	3.6			6.3			
<i>S. simulans</i>																										
<i>S. hyicus</i>				10.0		4.2																				
<i>S. epidermidis</i>									7.1								2.8									
<i>S. agnetis</i>	6.3																								12.5	
<i>S. kloosii</i>																										
<i>S. fleuretii</i>						4.2							6.3													
<i>S. warneri</i>							3.1				5.6															
<i>S. pasteurii</i>																										
<i>S.</i>																										
<i>S. pseudintermedius</i>																										
<i>S. gallinarum</i>																										

¹Percentage of teat apices of dry cows colonized with a specific species within each herd.²Percentage of teat apices of end-term heifers colonized with a specific species within each herd.

Figure 1. Distribution of CNS species on teat apices in 13 Flemish dairy herds in accordance with the proportion of teat apices from end-term heifers (black) and dry dairy cows (gray) respectively, per herd, colonized with a certain species relative to the total number of isolates over all herds per species.

$n = 22$ and 18% teat apices, $n = 38$), *S. devriesei* (40% heifers, $n = 21$ and 18% teat apices, $n = 38$), and *S. equorum* (32% heifers, $n = 17$ and 16% teat apices, $n = 34$). *Staphylococcus epidermidis*, *Staphylococcus fleurettii*, *Staphylococcus gallinarum*, *Staphylococcus pasteurii*, *Staphylococcus simulans*, and *Staphylococcus*

warneri were not observed on teat apices from these young animals. *Staphylococcus devriesei* was the most frequently isolated species from the teat apices of the dry cows (44% dry cows, $n = 45$ out of 103 dry cows and 19% teat apices, $n = 82$ out of 412 teat apices), but the dominance of one species was less pronounced

as observed for the heifers as other species were also often identified (*S. haemolyticus*: 39% dry cows, $n = 40$ and 17% teat apices, $n = 69$, *S. chromogenes*: 38% dry cows, $n = 39$ and 15% teat apices, $n = 62$, *S. equorum*: 28% dry cows, $n = 29$ and 11% teat apices, $n = 47$). Three species recovered from the heifers, *Staphylococcus hyicus*, *Staphylococcus kloosii*, and *Staphylococcus pseudintermedius*, could not be isolated from the teat apices of the dry cows.

Risk Factors

A first reduction based on unconditional associations revealed 4, 7, 6, 4, and 5 herd-, cow-, and quarter-level factors to be associated with TAC with CNS, *S. devriesei*, *S. chromogenes*, *S. haemolyticus*, and *S. equorum*, respectively (Table 3). No significant associations were identified for bulk milk SCC, housing, teat dip, BCS, and quarter position. Strong correlations between 2 or more factors were not observed. The final multilevel, multivariable logistic regression models are shown in Table 4.

Teat apices of red and white HF dry cows and pregnant heifers were more likely to be colonized with *Staphylococcus devriesei* (OR = 2.22; 95% CI: 1.02–4.84) as opposed to teat apices from black and white HF. The application of an internal teat sealer significantly decreased the odds of being colonized with *S. devriesei* (OR = 0.45; 95% CI: 0.21–0.95). Dirty teat apices were significantly more likely to be colonized with CNS in general (OR = 2.91; 95% CI: 1.47–5.77) and *S. equorum* in particular (OR = 5.24; 95% CI: 1.87–14.66) than very clean teat apices. Slightly dirty teat apices had higher odds of being colonized with *S. chromogenes* (OR = 2.39; 95% CI: 1.30–4.40) as opposed to very clean teat apices. A higher ambient temperature significantly increased the odds of being colonized with *S. devriesei* (OR = 6.63; 95% CI: 1.37–32.13) or *S. haemolyticus* (OR = 10.89; 95% CI: 1.27–93.52), whereas the opposite was true for *S. equorum* (OR = 0.30; 95% CI: 0.12–0.76) with the association even being more pronounced in case of low precipitation than in case of high precipitation (significant interaction term, see Figure 2). Teat apex colonization with *Staphylococcus chromogenes* was not associated with ambient temperature. Teat apices swabbed in months with high precipitation were less likely to be colonized with CNS in general (OR = 0.45; 95% CI: 0.24–0.84), and with *S. devriesei* (OR = 0.35; 95% CI: 0.14–0.86), *S. chromogenes* (OR = 0.44; 95% CI: 0.21, 0.94), and *S. haemolyticus* (OR = 0.23; 95% CI: 0.08–0.67) in particular than teat apices swabbed in months with low precipitation. For *S. equorum*, the latter was only true in case of low ambient temperature and when no Bonferroni correction was applied (OR

= 0.21; 95% CI: 0.05–0.93; Table 4, Figure 2). In case of high ambient temperatures, teat apices swabbed in months with high precipitation were even more likely to be colonized with *S. equorum* than teat apices swabbed in months with low precipitation (Figure 2).

The highest variability was observed at the quarter level for all dependent variables. For the outcome variable *Staphylococcus chromogenes* TAC, the variation at the cow level in the null model was much more pronounced as opposed to the herd level, whereas this discrepancy was less pronounced for *S. haemolyticus* or *S. devriesei* and even absent for *S. equorum* (Table 5). For the latter species, the variation occurred in an almost equal amount at the cow and herd level.

DISCUSSION

This is the first large species-specific observational study that describes the prevalence and distribution of CNS colonizing teat apices from dry cows and end-term heifers and simultaneously identifies risk factors for TAC before calving at different levels of the data hierarchy. A sufficient number of herds, reflecting different management strategies, and the use of molecular speciation allowed us to precisely picture the species-specific CNS distribution on the teat apices. Similar to what was reported in lactating cows (Braem et al., 2013; De Visscher et al., 2014), the species distribution strongly varied among the different herds (Figure 1). None of the species was found on all farms except for *S. chromogenes*, which corresponds well with an earlier study reporting a herd-level prevalence of *S. chromogenes* colonized heifers of at least 10% (De Vlieghe et al., 2003). The prevalence of teat apices colonized with CNS as a group and with *S. chromogenes* in particular was higher than those observed in previous studies focusing on prepartum TAC with CNS as a group (Piepers et al., 2011) and *S. chromogenes* (De Vlieghe et al., 2003), respectively. These differences might partially be explained by using a more suitable and appropriate medium in our study (De Visscher et al., 2013).

A lower percentage of teat apices was colonized with CNS in our previous work (De Visscher et al., 2014) than in the current study in which only teat apices before calving were swabbed. Routinely cleaning of the teats before cluster attachment, a flushing effect of the milking process (De Visscher et al., 2014), and the application of postmilking teat disinfection (Piessens et al., 2012; Quirk et al., 2012) may at least partly explain this discrepancy. Further, a higher number of species and some remarkable diversity in species distribution were found in our prepartum study compared with the studies concerning lactating cows (Braem et al., 2013; De Visscher et al., 2014). *Staphylococcus devriesei*, the

Table 3. Univariable multilevel logistic regression models¹ for test apex colonization with CNS as a group or with 1 of the 4 most frequently isolated CNS species, respectively

Risk factor ²	CNS (n = 448)					Staphylococcus devriesei (n = 120)					Staphylococcus chromogenes (n = 107)					Staphylococcus haemolyticus (n = 107)					Staphylococcus equorum (n = 81)				
	N _n	% _n	N _{CNS} ³	% _{CNS} ³	P ⁴	N _{no} ⁵	% _{no} ⁵	N _s ⁶	% _s ⁶	P	N _{no}	% _{no}	N _s	% _s	P	N _{no}	% _{no}	N _s	% _s	P	N _{no}	% _{no}	N _s	% _s	P
Herd level																									
Herd type	105	59.7	279	62.3	0.84	297	58.9	87	72.5	0.15	310	60.0	74	69.2	0.33	303	58.6	81	75.7	0.12	347	63.9	37	45.7	0.31
Dairy cattle	71	40.3	169	37.7	Ref. ⁸	207	41.1	33	27.5	Ref.	207	40.0	33	30.8	Ref.	214	41.4	26	24.3	Ref.	196	36.1	44	54.3	Ref.
With other ⁷					0.70					0.43					0.32					0.12					0.44
Herd size	88	50.0	200	44.6	Ref.	239	47.4	49	40.8	Ref.	247	47.8	41	38.3	Ref.	254	49.1	34	31.8	Ref.	241	44.4	47	58.0	Ref.
Smaller	88	50.0	248	55.4		265	52.6	71	59.2		270	52.2	66	61.7		263	50.9	73	68.2		302	55.6	34	42.0	
Larger					0.73					0.48					0.33					0.49					0.94
Bulk milk																									
SCC																									
Lower	85	48.3	203	45.3	Ref.	242	48.0	46	38.3	Ref.	230	44.5	58	54.2	Ref.	229	44.3	59	55.1	Ref.	255	47.0	33	40.7	Ref.
Higher	91	51.7	245	54.7		262	52.0	74	61.7		287	55.5	49	45.8		288	55.7	48	44.9		288	53.0	48	59.3	
Cow level																									
Housing					0.85					0.74					0.31					0.57					0.69
Cubicles	115	65.3	285	63.6	Ref.	326	64.7	74	61.7	Ref.	325	62.9	75	70.1	Ref.	326	63.1	74	69.2	Ref.	343	63.2	57	70.4	Ref.
Deep litter	61	34.7	163	36.4		178	35.3	46	38.3		192	37.1	32	29.9		191	36.9	33	30.8		200	36.8	24	29.6	
Pasture access					0.21					0.09					0.29					1.00					0.73
No	12	6.8	48	10.7	Ref.	59	11.7	1	0.8	Ref.	46	8.9	14	13.1	Ref.	53	10.3	7	6.5	Ref.	51	9.4	9	11.1	Ref.
Yes	164	93.2	400	89.3		445	88.3	119	99.2		471	91.1	93	86.9		464	89.7	100	93.5		492	90.6	72	88.9	
Contact					0.15					0.14					0.28					0.03					0.06
No	74	42.0	202	45.1	Ref.	226	44.8	50	41.7	Ref.	236	45.6	40	37.4	Ref.	235	45.5	41	38.3	Ref.	240	44.2	36	44.4	Ref.
Yes	102	58.0	246	54.9		278	55.2	70	58.3		281	54.4	67	62.6		282	54.5	66	61.7		303	55.8	45	55.6	
Breed					0.86					0.12					0.22					0.41					0.39
Black and white	153	86.9	375	83.7	Ref.	438	86.9	90	75.0	Ref.	432	83.6	96	89.7	Ref.	443	85.7	85	79.4	Ref.	453	83.4	75	92.6	Ref.
HF ⁹																									
Red and white	23	13.1	73	16.3		66	13.1	30	25.0		85	16.4	11	10.3		74	14.3	22	20.6		90	16.6	6	7.4	
HF																									
Parity					0.43					0.45					0.06					0.84					0.33
1st lactation	56	31.8	156	34.8	Ref.	174	34.5	38	31.7	Ref.	167	47.7	45	42.1	Ref.	174	33.7	38	35.5	Ref.	178	32.8	34	42.0	Ref.
≥2nd	120	68.2	292	65.2		330	65.5	82	68.3		350	67.7	62	57.9		343	66.3	69	64.5		365	67.2	47	58.0	
lactation																									
Vitamins					0.14					0.24					0.16					0.51					0.75
No	15	8.5	81	18.1	Ref.	67	13.3	29	24.2	Ref.	87	16.8	9	8.4	Ref.	82	15.9	14	13.1	Ref.	75	13.8	21	25.9	Ref.
Yes	161	91.5	367	81.9		437	86.7	91	75.8		430	83.2	98	91.6		435	84.1	93	86.9		468	86.2	60	74.1	
Antimicrobials ¹⁰					0.39					0.06					0.20					0.76					0.46
No	58	34.3	158	35.7	Ref.	178	36.2	38	31.7	Ref.	171	33.7	45	42.9	Ref.	178	35.2	38	35.5	Ref.	180	33.9	36	44.4	Ref.
Small	70	41.4	162	36.6		200	40.7	32	26.7		198	39.1	34	32.4		194	38.4	38	35.5		205	38.61	27	33.3	
Broad	41	24.3	123	27.8		114	23.2	50	41.7		138	27.2	26	24.8		133	26.3	31	29.0		146	27.5	18	22.2	
Teat sealer ¹⁰					0.26					0.13					0.96					0.31					0.56
No	127	75.1	325	73.4	Ref.	349	70.9	103	85.8	Ref.	374	73.8	78	74.3	Ref.	372	73.7	80	74.8	Ref.	395	74.4	57	70.4	Ref.
Yes	42	24.9	118	26.6		143	29.1	17	14.2		133	26.2	27	25.7		133	26.3	27	25.2		136	25.6	24	29.6	
Teat dip					0.59					0.33					0.31					0.82					0.44
No	133	75.6	339	75.7	Ref.	385	76.4	87	72.5	Ref.	394	76.2	78	72.9	Ref.	385	74.5	87	81.3	Ref.	406	74.8	66	81.5	Ref.
Yes	43	24.4	109	24.3		119	23.6	33	27.5		123	23.8	29	27.1		132	25.5	20	18.7		137	25.2	15	18.5	
BCS ¹¹					0.22					0.87					0.57					0.60					/
<2.5	8	4.7	12	2.7	Ref.	18	3.6	2	1.7	Ref.	16	3.1	4	3.7	Ref.	19	3.7	1	0.9	Ref.	20	3.7			
2.5-3	158	92.4	406	91.2		455	91.5	109	91.6		464	91.2	100	93.5		465	91.4	99	92.5		490	91.2	74	93.7	
>3	5	2.9	27	6.1		24	4.8	8	6.7		29	5.7	3	2.8		25	4.9	7	6.5		27	5.0	5	6.3	

Continued

	CNS (n = 448)						<i>Staphylococcus devriesei</i> (n = 120)						<i>Staphylococcus chromogenes</i> (n = 107)						<i>Staphylococcus haemolyticus</i> (n = 107)						<i>Staphylococcus equorum</i> (n = 81)						
Risk factor ²	N _n ²	% _n ²	N _{CNS} ³	% _{CNS} ³	P ⁴		N _{no} ⁵	% _{no} ⁵	N _s ⁶	% _s ⁶	P	N _{no}	% _{no}	N _s	% _s	P	N _{no}	% _{no}	N _s	% _s	P	N _{no}	% _{no}	N _s	% _s	P	N _{no}	% _{no}	N _s	% _s	
Hygiene ¹²	67	40.6	133	30.0	Ref.	0.04	149	30.5	51	42.9	Ref.	175	34.8	25	23.8	0.01	168	33.5	32	29.9	Ref.	193	36.5	7	8.9	<0.001	218	41.2	30	38.0	Ref.
Very clean	69	41.8	179	40.4			202	41.3	46	38.7		186	37.0	62	59.0		204	40.7	44	41.1		218	41.2	30	38.0		218	41.2	30	38.0	
Slightly dirty	29	17.6	131	29.6			138	28.2	22	18.5		142	28.2	18	17.1		129	25.7	31	29.0		118	22.3	42	53.2		118	22.3	42	53.2	
Dirty						0.05										0.36					0.03										
Temperature																															
Low	15	8.5	53	11.8	Ref.		66	13.1	2	1.7	Ref.	61	11.8	7	6.5	Ref.	67	13.0	1	0.9	Ref.	36	6.6	32	39.5	Ref.	36	6.6	32	39.5	
High	161	91.5	395	88.2			438	86.9	118	98.3		456	88.2	100	93.5		450	87.0	106	99.1		507	93.4	49	60.5		507	93.4	49	60.5	
Precipitation						0.06										0.03					0.01										
Low	127	72.2	365	81.5	Ref.		381	75.6	111	92.5	Ref.	397	76.8	95	88.8	Ref.	391	75.6	101	94.4	Ref.	447	82.3	45	55.6	Ref.	447	82.3	45	55.6	
High	49	27.8	83	18.5			123	24.4	9	7.5		120	23.2	12	11.2		126	24.4	6	5.6		96	17.7	36	44.4		96	17.7	36	44.4	
Quarter level																															
Quarter position						0.26					0.51					0.74					0.31										
Front	94	53.5	218	48.7	Ref.		249	49.4	63	52.5	Ref.	257	49.7	55	51.4	Ref.	263	50.9	49	45.8	Ref.	274	50.5	38	46.9	Ref.	274	50.5	38	46.9	
Hind	82	46.6	230	51.3		0.55	255	50.6	57	47.5		260	50.3	52	48.6		254	49.1	58	54.2		269	49.5	43	53.1		269	49.5	43	53.1	
Teat end											0.64					0.11					0.83										
condition ¹¹																															
Good	149	87.1	373	83.8	Ref.		423	85.1	99	83.2	Ref.	437	85.9	85	79.4	Ref.	432	84.9	90	84.1	Ref.	458									

¹²Missing data from 4 dry cows.

Table 4. Final multivariable multilevel logistic regression models¹ describing potential herd-, cow-, and quarter-level risk factors associated with teat apex colonization with CNS as a group or with 1 of the 4 most frequently isolated species, respectively

Risk factor ²	CNS (n = 448)					<i>Staphylococcus devriesei</i> (n = 120)					<i>Staphylococcus chromogenes</i> (n = 107)				
	β^3	SE	OR ⁴	95% CI	P ⁵	β	SE	OR	95% CI	P	β	SE	OR	95% CI	P
Intercept	0.84	0.27	2.32	1.37	3.94	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Breed															
Black and white HF ⁶															
Red and white HF															
Teat sealer															
No															
Yes															
Hygiene															
Very clean															
Slightly dirty															
Dirty															
Temperature															
Low															
High															
Precipitation															
Low															
High															
Temperature × precipitation ⁸															
Intercept															
Cow level															
Breed															
Black and white HF															
Red and white HF															
Internal teat sealer															
No															
Yes															
Hygiene															
Very clean															
Slightly dirty															
Dirty															
Temperature															
Low															
High															
Precipitation															
Low															
High															
Temperature × precipitation															
Intercept															
Cow level															
Breed															
Black and white HF															
Red and white HF															
Internal teat sealer															
No															
Yes															
Hygiene															
Very clean															
Slightly dirty															
Dirty															
Temperature															
Low															
High															
Precipitation															
Low															
High															
Temperature × precipitation															

¹Cow and herd included in all models as random effects to correct for potential clustering of quarters within cows and cows within herds.

²Only cow-level factors were identified.

³Regression coefficient.

⁴Odds ratio.

⁵P-value for the overall effect.

⁶Holstein Friesian.

⁷Reference category per risk factor.

⁸Interaction term between temperature and precipitation.

Table 5. Variance components at the herd, cow, and quarter level of the null and final models for teat apex colonization with CNS as a group or with 1 of the 4 most frequently isolated species, respectively

Item	CNS (n = 448)				<i>Staphylococcus devriesei</i> (n = 120)				<i>Staphylococcus chromogenes</i> (n = 107)				<i>Staphylococcus haemolyticus</i> (n = 107)				<i>Staphylococcus equorum</i> (n = 81)			
	Var. est. ¹	±SE	%		Var. est.	±SE	%		Var. est.	±SE	%		Var. est.	±SE	%		Var. est.	±SE	%	
Null model																				
Herd	0.38	0.22	8.6		0.81	0.43	15.9		0.24	0.18	5.8		0.73	0.40	14.6		1.20	0.63	21.4	
Cow	0.74	0.25	16.8		0.98	0.34	19.3		0.64	0.30	15.4		0.97	0.35	19.4		1.11	0.44	19.8	
Quarter	3.29		74.6		3.29		64.8		3.29		78.9		3.29		65.9		3.29		58.8	
Total variance	4.41		100		5.08		100		4.17		100		4.99		100		5.60		100	
Final model																				
Herd	0.41	0.23	9.5		0.06	0.13	1.4		0.19	0.16	4.8		0.40	0.27	8.7		0.56	0.37	12.7	
Cow	0.62	0.25	14.4		1.02	0.34	23.3		0.51	0.29	12.8		0.91	0.35	19.8		0.57	0.39	12.9	
Quarter	3.29		76.2		3.29		75.3		3.29		82.5		3.29		71.5		3.29		74.4	
Total variance	4.32		100		4.37		100		3.99		100		4.60		100		4.42		100	

¹Variance estimate.

predominant species in the current study, was only rarely isolated in one (Braem et al., 2013) and even absent in another study (De Visscher et al., 2014), both focusing on TAC in lactating dairy cows. Although less pronounced as for *S. devriesei*, the same was true for *S. auricularis* (Braem et al., 2013; De Visscher et al., 2014). In turn, *S. cohnii* (Braem et al., 2013; De Visscher et al., 2014), *Staphylococcus saprophyticus*, *S. simulans* (Braem et al., 2013), *S. xylosus* (Taponen et al., 2008), and *S. fleurettii* (De Visscher et al., 2014) were less commonly isolated from teat apices from non-lactating cows and heifers as opposed to teat apices from lactating dairy cows. The differences between lactating and nonlactating animals are less consistent for *S. chromogenes*, the second most frequently isolated species. *Staphylococcus chromogenes* could not be or could hardly be isolated from teat apices from lactating dairy cows in some studies (Braem et al., 2013; De Visscher et al., 2014), but was often isolated from bovine teat skin in some other single-dairy-herd studies (White et al., 1989; Taponen et al., 2008). *Staphylococcus haemolyticus*, *S. equorum* (Braem et al., 2013; De Visscher et al., 2014; Vanderhaeghen et al., 2015), and to a lesser extent also *S. sciuri* (Braem et al., 2013; De Visscher et al., 2014) were frequently isolated species from teat apices from both lactating dairy cows and nonlactating cows and heifers. Again, our findings suggest that herds have various CNS microbiota colonizing different habitats implying the potential role of management strategies. Although we have included herd-level factors

Figure 2. Interaction term between temperature and precipitation visualized using odds ratios and 95% confidence intervals for teat apices colonized with *Staphylococcus equorum* versus not colonized or colonized with (an)other coagulase-negative *Staphylococcus* species (as shown in Table 4).

in the current study, the number of herds was too small to identify important ones. Other studies, specifically designed for analyzing herd-level variables, are needed.

Teat apices of red and white HF as opposed to teat apices of black and white HF dry cows and pregnant heifers were more likely to be colonized with *Staphylococcus devriesei*. Also, the application of an internal teat sealer, a management tool useful in preventing environmental pathogens invading quarters during the dry period (Huxley et al., 2002), significantly decreased the odds of being colonized with *S. devriesei* in this study. No well-defined interpretation can explain these observed associations, yet they merit further study. *Staphylococcus devriesei* was also significantly more present on teat apices in months with low precipitation and high ambient temperatures comparable to what was observed for *S. haemolyticus*. Because hygiene did not influence TAC with *Staphylococcus devriesei* or with *S. haemolyticus* in the current study, an environmental nature of *S. devriesei* and *S. haemolyticus* could be questioned as previously reported for *S. haemolyticus* by Leroy et al. (2015). Strain typing is necessary to come to better conclusions. Because staphylococci prefer a higher temperature to grow (Prescott et al., 2002) and dryness might cause imperceptible teat end lesions, forming a good matrix for expanding the present CNS microbiota, warmth, and low precipitation could provide *S. devriesei* and *S. haemolyticus* with good growth conditions.

On the other hand, TAC with *Staphylococcus equorum* was significantly associated with hygiene, with dirty teat apices being more colonized as opposed to very clean teat apices. In addition, teat apices were more likely to be colonized with *S. equorum* when swabbed under humid conditions in combination with a higher ambient temperature. The latter climatic conditions could support the bacterial load from the environment and thus could increase the exposure of teat apices to *S. equorum* (Smith et al., 1985). Earlier studies also observed a more pronounced presence of *S. equorum* in extramammary habitats (Piessens et al., 2011; De Visscher et al., 2014) as opposed to milk (Supré et al., 2011; Piessens et al., 2011; Fry et al., 2014) and our current findings seem to confirm the environmental nature of *S. equorum*.

Staphylococcus chromogenes was rarely isolated from the bovine environment (Piessens et al., 2011), but was predominantly present in bovine IMI (Piessens et al., 2011; Supré et al., 2011; Fry et al., 2014). An earlier study (De Vlieghe et al., 2003) and the current prepartum study observed *S. chromogenes* TAC in all included herds. The latter observations all indicate a host-adapted nature of *S. chromogenes*. On the other hand, teat apices from lactating dairy cows and heifers

were hardly colonized with *S. chromogenes* (Taponen et al., 2008; Braem et al., 2013; De Visscher et al., 2014). This can partly be explained by a smaller number of included herds, different genotypic methods, or both, yet needs further study.

Most of variation of the outcome variables in the null models resided at the quarter level as reported before for CNS IMI (Piepers et al., 2011; Passchyn et al., 2014). As no quarter-level factors were identified and the variation explained in the final models was low, as seen before (Piepers et al., 2011; Verbeke et al., 2012; Passchyn et al., 2014), factors others than the studied ones play a key role in explaining the likelihood of colonization (or infection). For example, focusing on teat dimensions might be useful in improving udder health (Zwertvaegher et al., 2013) and a potential pathway is diversity among cows and heifers due to their distinct genetic background (Detilleux, 2009; Verbeke et al., 2012). Differences in susceptibility for CNS IMI among cows and quarters could also rely on differences in immune status (Piepers et al., 2009), which is also partially genetically determined. Still, calculation of the variance components for binary outcome variables is not as straightforward as it is for continuous outcomes (Dohoo et al., 2009), making comparisons with that level not appropriate. Still, we hypothesize that factors at the quarter level rather than at the cow and herd level play a more important role for *S. chromogenes* TAC than for TAC with the other 3 species. For TAC with *S. chromogenes*, more variation was present between cows than between herds when compared with the other species, which is most likely related to the fact that *S. chromogenes* was the sole species colonizing teat apices in all included herds. Regarding the proportion of variation being present at the herd or cow level, analogous patterns could be observed for *S. devriesei* and *S. haemolyticus*. Remarkably, for TAC with *S. equorum* an almost equal amount of the total variation occurred at the herd and cow level despite being an environmental species.

CONCLUSIONS

Staphylococcus devriesei, *S. chromogenes*, *S. haemolyticus*, and *S. equorum* were the predominant species colonizing teat apices from dry dairy cows and end-term heifers before calving. *Staphylococcus chromogenes* was present on teat apices in all herds whereas large herd differences were observed for other species. Diverse factors explaining CNS species-specific TAC, yet mostly related to humidity, ambient temperature, and hygiene, substantiate differences in epidemiological behavior and ecology between species. An environmental nature for *S. equorum* is suggested, whereas *S. haemolyticus*

and *S. devriesei* seem to act as cow-adapted bacteria, as *S. chromogenes* does.

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