Short communication: Species group-specific predictors at the cow and quarter level for intramammary infection with coagulase-negative staphylococci in dairy cattle throughout lactation

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ABSTRACT

Coagulase-negative staphylococci (CNS) are frequently isolated from quarters with subclinical mastitis, teat apices, and the cows' environment. Virulence, ecology, epidemiological behavior, and effect on udder health vary between different CNS species. *Staphylococcus chromogenes*, *Staph. simulans*, and *Staph. xylosus* are frequently present in milk and have a more substantial effect on quarter milk somatic cell count than other species. Therefore, these species are considered the "more relevant" CNS. As species-specific factors associated with CNS intramammary infection (IMI) have not yet been identified and susceptibility for IMI differs between cows and quarters, this study aimed to identify predictors for CNS IMI at the cow and quarter level (some of them changing over time) with a specific focus on the aforementioned more relevant CNS. Precise data were available from a longitudinal study (3,052 observations from 344 quarters from 86 dairy cows belonging to 3 commercial dairy herds). All CNS were molecularly identified to the species level, and multivariable, multilevel logistic regression models taking into account the longitudinal nature of the data, were fit to study the likelihood of infection. *Staphylococcus chromogenes*, *Staph. xylosus*, and *Staph. cohnii* were the most frequently isolated species from CNS IMI in older cows, whereas *Staph. chromogenes*, *Staph. xylosus*, and *Staph. simulans* were the main species found in IMI in heifers. Quarters from heifers (as opposed to multiparous cows), from heifers and multiparous cows in third or fourth month in lactation (as opposed to early lactation, <60 d in milk), and with an increasing quarter milk SCC were more likely to be infected with the more relevant CNS species. Quarter milk SCC was identified as the sole statistically significant predictor for IMI with other CNS species, although the size of the effect

was lower [odds ratio of 1.6 (1.4–1.9) vs. 2.1 (1.8–2.5)] than the effect for IMI with the more relevant CNS. As a strong herd effect was present, studying herd-level predictors is warranted.

Key words: predictors, coagulase-negative *Staphylococcus* species, intramammary infection

Short Communication

Coagulase-negative staphylococci are the most frequently isolated bacteria from subclinical mastitis cases in dairy cows (Makovec and Ruegg, 2003; Piepers et al., 2007; Sampimon et al., 2009a). In addition, they are abundantly present in the cows' environment (Piessens et al., 2011) and on their teat apices (Taponen et al., 2008; Braem et al., 2012). Several factors associated with CNS-group IMI have been identified (Sampimon et al., 2009a; Piepers et al., 2011) but in none of the studies were CNS accurately identified to the species level and one study included only heifers. As species-specific differences in protective effects, (presumed) virulence, antimicrobial resistance, persistence, and effect on udder health have been revealed (Sampimon et al., 2011; Vanderhaeghen et al., 2014, 2015), studying CNS as a group is no longer recommended. Rather, accurate species-level studies or studies at the subgroup level, when analyses at the species level are hindered by small numbers, are needed.

Intramammary infection with *Staphylococcus chromogenes*, *Staph. simulans*, and *Staph. xylosus* is associated with a more pronounced increase in SCC compared with other species (Supré et al., 2011; Fry et al., 2014), suggesting that these species have a more substantial effect on udder health and are, for that reason considered more relevant than others. Differences in susceptibility for CNS IMI between cows and quarters exist as some cows in a specific herd have no CNS infected quarters whereas others have multiple quarters infected with CNS (Piessens et al., 2011; Supré et al., 2011), indicating that the likelihood of infections is explained not only by CNS traits but also by host factors.

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The aim of this study was to identify cow- and quarter-level predictors for IMI throughout lactation caused by the more relevant CNS species for udder health, *Staph. chromogenes*, *Staph. simulans*, and *Staph. xylosus*, as well as other species.

Data were obtained from a longitudinal study conducted between September 2007 and January 2009 in 3 commercial dairy herds in Flanders (Belgium). On all farms, cows were housed in freestall barns with cubicles with sawdust bedding and a concrete slatted floor. Postmilking teat disinfection and blanket dry cow therapy combined with application of an internal teat sealant were applied. Detailed herd information can be found elsewhere (Supré et al., 2011; De Visscher et al., 2014). Single quarter milk samples were aseptically collected every month for determining quarter milk SCC (qSCC) and for bacteriological culturing. Several potential cow- and quarter-level predictors were recorded at each sampling occasion, some of them changing over time (observation level, lowest level of the data set; Table 1). Animals with a test-day milk yield ≥ 33.35 kg/d (i.e., the median value of all test-day records of all animals) were defined as high producing, whereas animals with a test-day milk yield < 33.35 kg/d were defined as low producing. Stage in lactation was categorized as described by Zadoks et al. (2001): 1 = 1–60 DIM; 2 = 61–120 DIM; 3 = 121–180 DIM; 4 = > 180 DIM. Body condition score of each animal was scored on a 5-point scale divided into quarter-point increments as described by Edmonson et al. (1989). Recoding was applied for further analyses (1 = ≤ 2.5 ; 2 = 2.5–3.5; 3 = ≥ 3.5). The teat skin and teat apex condition were determined based on a 9-point scale visual scoring system (De Vliegher et al., 2003) and recoded (0 = without cracks; 1 = with cracks). Quarter milk SCC was determined at the Flanders Milk Control Centre (Lier, Belgium) using a Fossomatic 5000/FC (Foss Electric, Hillerød, Denmark). Bacteriological culturing of all milk samples was performed following National Mastitis Council guidelines (Hogan et al., 1999). All CNS were identified to the species level using transfer RNA intergenic spacer PCR (tDNA-PCR; Supré et al., 2009) and IMI status was assigned based on subsequent samplings as described by Supré et al. (2011). *Staphylococcus chromogenes*, *Staph. simulans*, and *Staph. xylosus* were considered CNS species with a more substantial effect on udder health (Supré et al., 2011; Fry et al., 2014) and referred to as “more relevant” species throughout the paper; all other CNS species originating from milk in this study are referred to as “less relevant” species. Before statistical analysis was conducted, observations were revised and checked for unlikely values. Complete data were available from 344 quarters from 86 cows, making 3,052 observations in total. Logistic mixed

regression models were fit in SAS 9.3 (SAS Institute Inc., Cary, NC). Clustering of observations occurred at multiple levels. Repeated observations were clustered in time within quarters, whereas quarters within cows and cows within herds were spatially clustered. For those reasons, analyses were run with herd and sampling forced into all models as fixed effects and quarter as random effect (REPEATED statement). Cow as random effect was omitted because of nonconvergence of the models when combined with a quarter random effect. The contribution of each of the random effects was estimated (VARCOMP procedure) and revealed that quarter accounted for more variability than cow (Zadoks et al., 2001). Compound symmetry was used as the covariance structure for within-quarter correlation (Barkema et al., 1997; Zadoks et al., 2001). Two different binary outcome variables at the quarter level were used: (1) IMI with the more relevant CNS, and (2) IMI with the less relevant CNS, both versus being noninfected. Initially, univariable associations were tested between the 2 binary outcome variables and all predictor variables at the cow, quarter, and observation level. Statistical significance in this step was assessed at $P < 0.15$. Second, Spearman correlation coefficients were calculated among all statistically significant predictor variables to check for multicollinearity. If 2 predictor variables had a correlation coefficient ≥ 0.60 , only one was selected for further analyses. In the third step, multivariable models were fit for the 2 outcome variables using backward stepwise elimination. Statistical significance in this step was assessed at $P < 0.05$. Interaction terms were tested between all remaining statistically significant predictors. The goodness-of-fit of the final models was evaluated by plotting the observational-level standardized residuals against the observational-level predicted values.

On average, 53 lactating cows were present in the herds (range = 39–70) with an average of 29 animals per herd being included in the study, resulting in a total of 3,052 analyzed milk samples. One hundred twenty-eight IMI with the more relevant CNS and 48 IMI with the less relevant CNS were available for analysis. Overall, *Staph. chromogenes* caused most IMI ($n = 83$; 47% of all IMI) followed by *Staph. xylosus* ($n = 28$; 16%), *Staph. cohnii* ($n = 19$; 11%), *Staph. simulans* ($n = 17$; 10%), *Staph. haemolyticus* ($n = 11$; 6%), and *Staph. fleurettii* ($n = 6$; 3%). Sixty-nine percent ($n = 121$) of all CNS IMI ($n = 176$) originated from quarters of cows in second or higher lactation. However, only 0.07% of all quarters of older cows were infected with CNS, whereas 16.4% of all quarters of heifers were infected. The majority of CNS IMI from quarters of heifers (93%, $n = 50$) and multiparous cows (64%, $n = 77$) were caused by the more relevant species. *Staphylococcus simulans*

Table 1. Overview of all potential cow-, quarter-, and observation-level predictor variables (independent variables)

Independent variable	Recording method	Description	Breakdown of categories in final model
Cow level			
Breed	DHI records	Two breeds	Black and white HF ¹ vs. red and white HF
Quarter level			
Quarter position	Visual	Position of the quarter	Front vs. hind quarter
Observation level			
Parity ²	DHI records	Parity at test-day	First lactation vs. second lactation or older
Milk yield ³	DHI records	Milk yield at test-day	Low ⁴ (<33.35 kg) vs. high (≥33.35 kg)
Stage of lactation ³	DHI records	Stage in lactation at test-day	1–60 DIM vs. 61–120 DIM vs. 121–180 DIM vs. >180 DIM
BCS ³	Visual and palpation	Five-point scale ⁵	<2.5 vs. 2.5–3.5 vs. >3.5
Quarter milk SCC ⁶	Fossomatic 5000/FC ⁷	Quarter SCC at test-day	Continuous
Teat skin condition ⁶	Visual and palpation	Nine-point scale ⁸	Without cracks vs. with cracks
Teat apex condition ⁶	Visual and palpation	Nine-point scale ⁸	Without cracks vs. with cracks

¹Holstein Friesian.
²Cow-level variable potentially changing during the study.
³Cow-level variable changing during the study.
⁴Categorization based on median value of all test-day records of all cows.
⁵Edmonson et al. (1989).
⁶Quarter-level variable changing during the study.
⁷Foss, Hillerød, Denmark.
⁸De Vliegher et al. (2003).

was mainly isolated from IMI in quarters of heifers (82%, n = 14). Coagulase-negative staphylococci IMI in quarters of multiparous cows were most frequently caused by *Staph. chromogenes* (43%, n = 52), *Staph. xylosus* (18%, n = 22), and *Staph. cohnii* (15%, n = 18). *Staphylococcus chromogenes* (56%, n = 31), *Staph. simulans* (26%, n = 14) and *Staph. xylosus* (11%, n = 6) were the majority of CNS IMI in quarters of heifers. Overall, CNS species were equally collected in any stage of lactation. *Staphylococcus chromogenes* (71%, n = 12), *Staph. haemolyticus* (12%, n = 2), *Staph. xylosus* (12%, n = 2), and *Staph. sciuri* (6%, n = 1) were the only species causing IMI in the first 2 mo after parturition (1 to 60 DIM). The more relevant CNS species were mostly isolated from IMI in quarters of heifers in a later stage of lactation (>180 DIM), whereas IMI in quarters of older cows caused by those relevant species were equally present in any stage in lactation. Most of the less relevant CNS (n = 36, 77%) were isolated from IMI in quarters with a qSCC <200,000 cells/mL, whereas for the more relevant CNS, this was only 45%.

A first reduction based on the univariable models revealed 5 and 4 predictor variables associated with IMI ($P < 0.15$) with more relevant CNS and less relevant CNS, respectively (Table 2). As no correlation coefficient ≥ 0.6 between the statistically significant predictors was calculated, all independent variables were added in the multivariable models.

The final multivariable, multilevel models are presented in Table 3. Quarters from heifers (as opposed to multiparous cows), from heifers and multiparous cows in third or fourth month in lactation (as opposed

to early lactation, <60 DIM), and with an increasing qSCC were more likely to be infected with the more relevant CNS. Quarter SCC was identified as the sole statistically significant predictor for IMI with other CNS species, although the size of the effect was lower [odds ratio of 1.6 (1.4–1.9) compared with 2.1 (1.8–2.5)] than for IMI with the relevant species. None of the tested interaction terms was significant. Statistically significant herd effects (herd forced in all models to correct for clustering of cows within herds) for both IMI with the more and less relevant CNS species, respectively, were present.

This is to our opinion, the first study identifying CNS group-specific predictor variables in a longitudinal setting using precise molecular techniques for CNS speciation. Before the current study, past investigations examined factors associated with CNS IMI, but researchers either did not differentiate CNS species or used inaccurate phenotypic identification methods (Sampimon et al., 2009b). Several studies demonstrated a high prevalence of CNS around calving. Still, to our knowledge, in none of the other studies was the prevalence of CNS IMI in early lactation compared with the prevalence of CNS IMI later in lactation.

We confirmed that heifers were more likely to be infected with (relevant) CNS compared with multiparous cows (Sampimon et al., 2009a). Quarters from heifers and cows in peak lactation (61–120 DIM) were more likely to be infected with the more relevant species despite applying postmilking teat disinfection and using dry cow antibiotics in all 3 herds. This might support the assumption of CNS being able to develop resistance

Table 2. Univariable mixed logistic regression models¹ for IMI with CNS considered more² or less relevant for udder health

Predictor variable	Noninfected		IMI with more relevant CNS			IMI with less relevant CNS		
	No.	%	No.	%	P-value	No.	%	P-value
Quarter position					0.266			0.087
Front quarters	1,007	50.0	56	44.1	Ref. ³	18	38.3	Ref.
Hind quarters	1,005	50.0	71	55.9		29	61.7	
Breed					0.959			0.434
Black and white HF ⁴	1,915	95.2	124	97.6	Ref.	46	97.9	Ref.
Red and white HF	97	4.8	3	2.4		1	2.1	
Parity					<0.001			0.353
First lactation	329	16.4	50	39.4	Ref.	4	8.5	Ref.
Second lactation or older	1,683	83.6	77	60.6		43	91.5	
Stage of lactation					0.023			0.116
1–60 DIM	393	19.5	14	11	Ref.	3	6.4	Ref.
61–120 DIM	375	18.6	26	20.5		10	21.3	
121–180 DIM	370	18.4	23	18.1		8	17.0	
>180 DIM	874	43.4	64	50.4		26	55.3	
BCS					0.281			0.878
<2.5	560	27.8	31	24.4	Ref.	11	23.4	Ref.
2.5–3.5	1,300	64.6	74	58.3		31	66.0	
>3.5	152	7.6	22	17.3		5	10.6	
Milk yield					<0.001			0.735
Low (<33,35 kg)	995	49.5	85	66.9	Ref.	26	55.3	Ref.
High (≥33,35 kg)	1,017	50.5	42	30.1		21	44.7	
Quarter milk SCC	2,012	—	127	—	<0.001	47	—	0.005
Teat skin condition					0.014			0.129
Without cracks	1,813	90.1	118	92.9	Ref.	38	80.9	Ref.
With cracks	199	9.9	9	7.1		9	19.1	
Teat apex condition					0.256			0.787
Without cracks	1,378	68.5	97	76.4	Ref.	29	61.7	Ref.
With cracks	634	31.5	30	23.6		18	38.3	

¹Herd and sampling were forced into all models as fixed effects to correct for potential clustering of cows within herds and for multiple observations per quarter, respectively.

²*Staphylococcus chromogenes*, *Staph. simulans*, and *Staph. xylosus*.

³Reference category per predictor variable.

⁴Holstein Friesian.

to antibiotics and teat disinfectants (Sampimon et al., 2011; Piessens et al., 2012).

A high proportion of the variation in the likelihood of CNS IMI was observed at the quarter level, which is in agreement with the findings of another study conducted by our group (Passchyn et al., 2014). This probably relates to the fact that cows are more likely to be infected with CNS than with major pathogens and that some cows are infected with CNS in 1 quarter, whereas others harbor CNS in up to 4 quarters at the same time (Makovec and Ruegg, 2003; Piepers et al., 2007; Sampimon et al., 2009a). In contrast, other studies (also) dealing with major pathogens such as *Staphylococcus aureus* and *Streptococcus uberis* observed that a higher proportion of the variation in the outcome resided at the cow level (Zadoks et al., 2001; Passchyn et al., 2014).

More statistically significant predictors for IMI caused by the more relevant CNS were identified than for IMI with the less relevant CNS. The greater number of isolates of relevant CNS species in the dataset likely

explains this, although we hypothesize that it also reflects differences in virulence and host adaptation.

On farms with excellent udder health, reflected by a bulk milk SCC <200,000 cells/mL, CNS infections may be an important contributor to the total number of somatic cells in the bulk milk (Schukken et al., 2009). In addition, a high proportion of cows infected with the more relevant CNS might result in an unwarranted increase in the bulk milk SCC in such a herd, also because IMI with the more relevant CNS last longer, on average, than IMI with the less relevant CNS (Supré et al., 2011). We hypothesize that our data are useful for selecting quarters and cows infected with the more relevant CNS in herds in which major pathogens are not an issue. The results suggest that focus should be on quarters with an elevated qSCC from heifers and on heifers and multiparous cows in peak lactation (61–120 DIM). In addition, molecular techniques able to identify CNS at the species level or at least able to distinguish the more relevant from the less relevant CNS would be very valuable when making individual cow-level decisions in

Table 3. Final multivariable models describing cow-, quarter-, and observation-level predictor variables associated with IMI with CNS considered more¹ or less relevant for udder health

Predictor variable	IMI with more relevant CNS (n = 128)					IMI with less relevant CNS (n = 48)				
	β ²	SE	OR ³	95% CI	P-value	β	SE	OR	95% CI	P-value
Intercept	−4.373	0.652	0.013	0.004	0.045	−5.752	0.763	0.003	0.001	0.014
Sampling ⁴	—	—	—	—	0.097	—	—	—	—	0.006
Herd ⁵	—	—	—	—	<0.001	—	—	—	—	0.002
Herd 1	Ref. ⁶	—	—	—	—	Ref.	—	—	—	—
Herd 2	0.048	0.329	1.049	0.550	1.998	−0.914	0.379	0.401	0.191	0.842
Herd 3	−2.299	0.586	0.100	0.032	0.316	−1.663	0.511	0.190	0.070	0.516
Parity	—	—	—	—	—	—	—	—	—	—
First lactation	Ref.	—	—	—	<0.001	—	—	—	—	—
Second lactation or older	−2.266	0.315	0.104	0.056	0.192	—	—	—	—	—
Stage of lactation	—	—	—	—	0.020	—	—	—	—	—
1–60 DIM	Ref.	—	—	—	—	—	—	—	—	—
61–120 DIM	0.679	0.322	1.971	1.049	3.704	—	—	—	—	—
121–180 DIM	0.296	0.339	1.345	0.692	2.613	—	—	—	—	—
>180 DIM	−0.061	0.301	0.941	0.522	1.698	—	—	—	—	—
Quarter milk SCC	0.758	0.078	2.134	1.832	2.486	0.489	0.088	1.631	1.371	1.939

¹*Staphylococcus chromogenes*, *Staph. simulans*, and *Staph. xylosus*.

²Regression coefficient.

³Odds ratio.

⁴Forced into the model to correct for multiple observations per quarter.

⁵Forced into the model to correct for potential clustering within herds.

⁶Reference category per predictor variable.

those herds. As statistically significant differences were observed between herds in likelihood of CNS infection, species-specific analysis at the herd level is required. This implies setting up studies that include large numbers of herds based on quarter milk samples from an even larger number of cows. Using bulk milk samples to identify herd-level predictor variables for IMI with the more relevant CNS could be helpful to circumvent this inconvenience and should be investigated.

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