INTERPRETIVE SUMMARY

Prevalence of non-*aureus* staphylococcus species causing intramammary infection in Canadian dairy herds. By Condas et al., page xxxx. Non-*aureus* staphylococci (NAS) are the most frequently isolated microorganisms from bovine udder quarters. Overall, 6.3% of 98,233 milk samples from 5,149 cows and 20,305 quarters in 91 Canadian dairy herds were NAS-positive. The five most common NAS species of the 5,434 samples confirmed as NAS were *Staphylococcus chromogenes* (48.9%), *S. simulans* (16.8%), *S. xylosus* (11.6%), *S. haemolyticus* (7.9%), and *S. epidermidis* (4.1%). Individual NAS species associated with intramammary infections (IMI) were mostly prevalent in heifers after calving, particularly *S. chromogenes*, with similar prevalence among udder quarters for the most common NAS species. Differences across regions were linked to housing type, and NAS species were more prevalent in high BMSCC herds.

RUNNING HEAD: NON-*AUREUS* STAPHYLOCOCCI IN MILK

Prevalence of non-*aureus* staphylococcus species causing intramammary infections in Canadian dairy herds

Larissa A.Z. Condas[*,†], Jeroen De Buck*[,†], Diego B. Nobrega*[,†], Domonique A. Carson*[,†], Sohail Naushad[*,†], Sarne De Vliegher[‡], Ruth N. Zadoks[§], John R. Middleton[#], Simon Dufour[‖,†], John P. Kastelic[*], and Herman W. Barkema*[,†,1]

*Department of Production Animal Health, Faculty of Veterinary Medicine, University of Calgary, Calgary, AB, T2N 4N1, Canada

[†]Canadian Bovine Mastitis and Milk Quality Research Network, St-Hyacinthe, QC, J2S 7C6, Canada

[‡]M-team and Mastitis and Milk Quality Research Unit, Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

[§]Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK

[#]Department of Veterinary Medicine and Surgery, University of Missouri, Columbia, MO, 65211, USA

[‖]Dept. of Pathology and Microbiology, Faculty of Veterinary Medicine, University of Montreal, C.P. 5000, St-Hyacinthe, QC, J2S 7C6, Canada

[1]Corresponding author: Herman W. Barkema, Department of Production Animal Health, Faculty of Veterinary Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, AB, T2N 4N1

Tel.: +1 (403) 220-2659; Fax: +1 (403) 210 6693; E-mail: barkema@ucalgary.ca

ABSTRACT

Non-*aureus* staphylococci (NAS), the microorganisms most frequently isolated from bovine milk worldwide, are a heterogeneous group of numerous species. To establish their importance as a group, distribution of individual species needs to be determined. In the present study, NAS intramammary infection (IMI) was defined as a milk sample containing ≥ 1,000 cfu/mL in pure or mixed culture, obtained from a cohort of cows assembled by the Canadian Bovine Mastitis Research Network (CBMRN). Overall, 6,213 (6.3%) of 98,233 quarter-milk samples from 5,149 cows and 20,305 udder quarters were associated with a NAS IMI. Of the 6,213 phenotypically identified NAS isolates, 5,509 (89%) were stored by the CBMRN Mastitis Pathogen Collection, and characterized using partial sequencing of the *rpoB* housekeeping gene, confirming 5,434 isolates as NAS. Prevalence of each NAS species IMI was estimated using Bayesian models, with presence of a specific NAS species as the outcome. Overall quarter-level NAS IMI prevalence was 26%. The most prevalent species causing IMI were *Staphylococcus chromogenes* (13%), *S. simulans* (4%), *S. haemolyticus* (3%), *S. xylosus* (2%), and *S. epidermidis* (1%). The prevalence of NAS IMI as a group was highest in first-parity heifers, and evenly distributed throughout parities ≥ 2. The IMI prevalence of some species such as *S. chromogenes*, *S. simulans* and *S. epidermidis* differed among parities. Overall prevalence of NAS IMI was 35% at calving, decreased over the next 10 d, and then gradually increased until the end of lactation. Species that had their highest prevalence at calving were *S. chromogenes, S. gallinarum, S. cohnii,* and *S. capitis,* whereas the prevalence of *S. chromogenes, S. haemolyticus, S. xylosus,* and *S. cohnii* increased during lactation. Although the overall prevalence of NAS IMI was similar across barn types, the prevalence of *S. simulans, S. xylosus, S. cohnii, S.*

saprophyticus, *S. capitis*, and *S. arlettae* IMI was higher in tie-stall barns, the prevalence of *S. epidermidis* IMI was lowest, and the prevalence of *S. chromogenes* and *S. sciuri* IMI was highest in bedded-pack barns. *Staphylococcus simulans*, *S. epidermidis*, *S. xylosus*, and *S. cohnii* IMI were more prevalent in intermediate to high BMSCC herds and *S. haemolyticus* IMI was more prevalent in high BMSCC herds, whereas other common NAS species IMI were equally prevalent in all 3 BMSCC categories. Distribution of NAS species IMI differed among the 4 regions of Canada. In conclusion, distribution differed considerably among NAS species IMI; therefore, accurate identification (species level) is essential for studying NAS epidemiology.

Key-words: dairy, mastitis, intramammary infection, coagulase-negative staphylococci, prevalence

INTRODUCTION

Non-*aureus* staphylococci (NAS) have been considered pathogens of minor importance in dairy production, particularly compared to major udder pathogens such as *Staphylococcus aureus*, streptococci, and coliforms. Nevertheless, NAS are the most frequently isolated bacteria from udder quarters in all recent North-American and European subclinical mastitis surveys (Piepers et al., 2007; Pyorala and Taponen, 2009; Sampimon et al., 2009a; Thorberg et al., 2009; Dufour et al., 2012; De Vliegher et al., 2012). Additionally, because NAS intramammary infection (IMI) moderately increases SCC, lower acceptable limits for bulk milk SCC (BMSCC) have increased the relative importance of NAS IMI (Piepers et al., 2007).

Although the high prevalence of NAS is widely recognized, their importance remains a topic of debate (Oliver and Jayarao, 1997; Piepers et al., 2007; Fox, 2009; Nickerson, 2009; Sampimon et al., 2009a; Schukken et al., 2009). Some authors consider NAS a main cause of subclinical and persistent mastitis (Sampimon et al., 2009a; Fry et al., 2014), whereas others suggest NAS have a protective effect against major pathogen IMI (Matthews et al., 1990; De Vliegher et al., 2004). Additionally, although milk production was higher in heifers with NAS IMI compared to uninfected heifers (Schukken et al., 2009; Piepers et al., 2010), in other studies, no effect on milk production (Tomazi et al., 2015) or decreased milk production associated with NAS IMI have also been reported (Taponen and Pyörälä, 2009).

Apparently contrasting findings among studies regarding impact of NAS on udder health and milk production could be the result of regarding the NAS as one group (Woodward et al., 1987; Woodward et al., 1988; Matthews et al., 1990). However, NAS are a large and heterogeneous group (Vanderhaeghen et al., 2015; Zadoks and Watts, 2009), and it is known that

there are differences among NAS species regarding their interactions with the host and the environment; consequently, they have variable effects on udder health and milk production (Piepers et al., 2009; Vanderhaeghen et al., 2014; Piccart et al., 2016). For example, IMI with *S. chromogenes*, *S. simulans* and *S. xylosus* have a greater impact on SCC compared to IMI with other species, e.g. *S. cohnii* and *S. sciuri* (Taponen et al., 2007; Supré et al., 2011; Fry et al., 2014; De Visscher et al., 2016). Some species, such as *S. chromogenes* and *S. epidermidis,* seem to be host-adapted, whereas others, e.g. *S. haemolyticus,* act as opportunists (Piessens et al., 2011).

Most studies have included staphylococci species with variable responses to the coagulase test, such as *Staphylococcus agnetis,* in the group of coagulase-negative staphylococci (CNS; Taponen et al., 2012). This classification was based on the phenotypic characteristic of *S. aureus* to coagulate plasma, often applied at a time when only *S. aureus* was characterized as pathogenic and the other NAS species were considered minor pathogens (Becker et al., 2014). However, this classification grouped species that are not necessarily phylogenetically related and does not accurately reflect the variability within the genus *Staphylococcus* (Becker et al., 2014; Naushad et al., 2016). Therefore, because some NAS species can vary in their response to the coagulase test, we prefer and propose to use the name NAS for this group of species.

Geometric mean BMSCC and housing of lactating cows differ by geographical region (Barkema et al., 2015). Based on the National Cohort of Dairy Farms study conducted in Canada during 2007 and 2008, Dufour et al. (2012) reported no difference in prevalence of NAS IMI among tie-stall, free-stall, and bedded pack barns, when considering NAS as a single group. However, Olde Riekerink et al. (2008) reported a difference between tie- and free-stalls in incidence of clinical mastitis caused by various NAS species. Perhaps differences in

management practices among housing systems impact prevalence of IMI with specific NAS species.

Within-herd prevalence of IMI with various NAS species is influenced by parity and lactation stage (Sampimon et al., 2009a; De Visscher et al., 2016). *Staphylococcus simulans* and *S. epidermidis* are most commonly isolated from multiparous cows (Taponen and Pyörälä, 2009; Mork et al., 2012), whereas *S. chromogenes* more frequently causes IMI in heifers. In the latter, the prevalence is usually higher close to calving, but persistency is also reported (Taponen et al., 2007). In that regard, *S. simulans* can persist in the udder for long intervals throughout lactation, whereas prevalence of *S. chromogenes* IMI decreases shortly after calving (Piessens et al., 2011). Comparison in quarter distribution between front and rear quarters provides information about the source of the IMI due to a particular species. Although Barkema et al. (1997) isolated NAS more frequently in rear versus front quarters, De Visscher et al. (2016) did not report a species-specific NAS quarter distribution. There are apparently no North-American data on parity and DIM distribution of NAS causing IMI in bovine udder quarters.

A large field cohort study was conducted during 2007 and 2008 by the Canadian Bovine Mastitis Research Network (CBMRN). Data and isolates from this prospective study has enabled further investigation on relevance of NAS species for the dairy industry. The first objective of the current study was to determine the prevalence and distribution of NAS species causing bovine IMI on Canadian dairy farms. The second objective was to evaluate potential associations of species-specific NAS IMI with herd characteristics (region, BMSCC, and barn type), as well as cow characteristics (parity, DIM, and quarter location).

MATERIALS AND METHODS

144

145 **Herds and Cows**

146 Data and samples were collected in the National Cohort of Dairy Farms (NCDF) study
147 conducted in Canada during 2007 and 2008, as described (Reyher et al., 2011). Briefly, the study
148 included 91 farms allocated into four regions: Atlantic Canada (represented by Prince Edward
149 Island, New Brunswick, and Nova Scotia), Québec, Ontario, and the Western provinces
150 (represented by Alberta). Eligibility was primarily based on BMSCC and housing system. Farms
151 were randomly selected based on 3 strata of 12-mo geometric mean average BMSCC and
152 classified as low, intermediate and high (<150,000, 150,000 to 300,000 and >300,000 cells/mL,
153 respectively). Herds enrolled had to match (within 15 percentage points) the proportion of free-
154 stall systems of their respective regional free-stall percentages. Additionally, each herd was
155 comprised of at least 80% lactating Holstein-Friesian cows milked twice a day and participated
156 in a DHI recording system. A total of 91 herds were sampled, 17 in Alberta, 27 in Ontario, 29 in
157 Québec, and 18 in the Atlantic Provinces.

158 Among the 91 herds, 60% housed lactating cows in a tie-stall, 33% in a free-stall, and 6%
159 were housed on a bedded-pack. Two herds were excluded from the analysis to determine the
160 effect of barn type on NAS distribution, as both had a mixed-barn design. However, most herds
161 in Alberta and the Atlantic provinces were housed in free-stalls (12 and 9 herds, respectively),
162 with a few bedded-pack barns, whereas Ontario and Québec herds most often had tie-stalls (20
163 and 25 herds, respectively), followed by free-stall barns (6 and 3 herds). Average herd size was
164 85 cows, mean 305-d milk production was 9,781 kg, and production-weighted arithmetic median
165 SCC was 228,000 cells/mL (Reyher et al., 2011). A total of 29.5, 29.5, 19.2, 11.3, and 10.5% of
166 lactations were from cows in parity 1, 2, 3, 4 and ≥5, respectively.

Sampling

Field sampling and corresponding applied techniques have been described (Reyher et al. (2011). Briefly, over a 2-y interval (2007 and 2008) CBMRN field technicians and producers aseptically collected quarter milk samples and shipped them to the closest CBMRN laboratory. Two schematic samplings of non-clinical mastitis cows were conducted during the 2 y of field collection, divided in 4 periods (March-May 2007, June-August 2007, January-March 2008, and June-August 2008). The first sampling covered 10 random lactating cows and the 5 most recently calved cows in each herd. For these cows, quarter milk samples were collected at 3-wk intervals in March-May 2007. The sampling scheme was repeated in January-March 2008 and June-August 2008. The second set of samples was collected during June to August 2007. Samples were obtained at weekly intervals from 15 cows per herd, sampled 2 to 4 wk before dry-off, at calving, and 2 wk after calving.

Laboratory Analyses

Bacteriological culture was done on sheep blood agar and MacConkey agar. All plates were incubated at 37°C and examined for bacterial growth at 24 and 48 h. Following 48 h of incubation, colonies were enumerated and species were presumptively identified using recommended phenotype-based bacteriologic procedures (Hogan et al., 2009). Milk samples with growth of ≥ 3 different microorganisms were considered contaminated (Reyher et al., 2011), whereas of the samples identified as NAS-positive, NAS presenting ≥ 10 phenotypically identical colonies in pure culture were stored (Reyher et al., 2011).

Definition of intramammary infection

To be stored in the CBMRN Mastitis Pathogen Culture Collection (MPCC) a NAS isolate had to be retrieved from a milk sample containing ≥1000 NAS cfu/mL of milk, in pure culture. Whenever 2 phenotypically different colony types were retrieved (including 2 different NAS), a mixed growth status was attributed to the milk sample, and NAS isolates were not preserved in the MPCC. A quarter was defined as having a NAS IMI using these same criteria.

NAS Isolate Identification

All NAS isolates were shipped in lyophilized form in vials labeled with individual isolate barcodes to the University of Calgary. Bacteria were re-suspended with sterile ultra-pure distilled water, plated on 5% defribinated sheep blood agar plates, and incubated at 35°C for 24 h. Initial screening to confirm that isolates were staphylococci was done using morphology and phenotypic tests (catalase, coagulase, and Gram-stain). Subsequent PCR amplification and sequencing with universal primers 27F-1392R were done to confirm identification of non-NAS isolates previously misclassified during collection (Chakravorty et al., 2007). Extraction of DNA was done as described (Sampimon et al., 2009b). Briefly, a loopful (1 μl) of cells was suspended in 20 μl lysis buffer (0.25% SDS, 0.05 N NaOH), heated for 5 min at 95 °C, and diluted 10-fold in distilled water. After elimination of cell debris by centrifugation, the supernatant was used as PCR template.

Partial sequencing of the *rpoB* gene was performed as described (Mellmann et al., 2006). *Staphylococcus*-specific primer sets used for amplification were staph_*rpoB*_1418F and staph_*rpoB*_3554R, which resulted in an amplicon of 899 bp. Thermal cycling conditions were 5 min at 94°C as the first denaturation step, followed by 35 cycles of denaturating at 94°C for 45 s,

annealing at 52°C for 60 s, and extension at 72°C for 90 s, with a final extension step at 72°C for 10 min. Primers for sequencing were 1418F and 1975R (500 bp; Mellman et al., 2006). Sanger sequencing for all amplicons was performed at University of Calgary Core DNA Services (UCDNA). Pairwise aligned sequence data were compared to sequence data in GenBank using the nucleotide BLAST algorithm of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov). The NAS species were identified with ≥ 97% identity to database sequences, as described (Drancourt and Raoult, 2002; Mellmann et al., 2006), except when the coverage identity was ≤ 50%, or there was high identity (≥ 97%) with 2 species. In these cases, complete *rpoB* gene sequences (full length) were retrieved and BLASTed against whole-genome sequences of 450 bovine intramammary NAS isolates, selected from the current study to include all NAS species present in the MPCC and verified by their full 16S rDNA sequences (Naushad et al., 2016).

Statistical Analyses

Data were analyzed using R (Crawley, 2013) with $P < 0.05$ considered significant. Bulk tank SCC categories were determined using the monthly geometric mean BMSCC from the 2-y duration of the study. Three categories were created: ≤ 150,000, 151,000 to 249,000, and ≥ 250,000 cells/mL. The cut-off for the highest BMSCC category was lower than in the original categories because the BMSCC of a relatively large number of farms decreased during data collection.

Prevalence estimation. The prevalence of each NAS species IMI was estimated using a Bayesian latent class approach. Estimated sensitivity of using a single sample to classify a

quarter as infected by NAS was 24.2%, with specificity equal to 100% (Dohoo et al., 2011). Nevertheless, for unknown reasons, some NAS isolates present in ≥ 1000 cfu/mL of milk and in pure culture in the original study were not available for identification (i.e., not preserved in the MPCC), likely resulting in underestimation of true IMI prevalence, based on traditional frequentist statistical approaches. In this scenario, Bayesian models were expected to be a reliable approach dealing with imperfections in diagnosis and for dealing with unpreserved isolates. The latent class model used was similar to a previous one (McInturff et al., 2004) and was as follows:

$Y_{collection} \sim \text{Bernoulli}\ (P_{collection})$

$P_{collection} = P_{observed} * Se_{collection} + (1 - P_{observed}) * (1 - Sp_{collection})$ (*Measurement error part 1)*

$P_{observed} = P_{population} * Se_{IMI\ def} + (1 - P_{population}) * (1 - Sp_{IMI\ def})$ (*Measurement error part 2)*

$\text{Logit}\ (P_{population}) = \beta_0 + \beta_1 x$ *(Response part)*

Where $Y_{collection}$ is the NAS species-specific status of an isolate available in the MPCC (e.g., is it or not a *S. chromogenes*) and is assumed to followed a Bernoulli distribution with a probability ($P_{collection}$) of having $Y_{collection} = 1$. This probability of observing a given NAS species in the MPCC can be linked to the probability of observing such an IMI among all samples collected during this observational cohort study and identified as NAS IMI with the chosen IMI definition ($P_{observed}$) using the first measurement error part of the model. Thus, this part of the model represented the probability of an isolate that met inclusion criteria of actually being included in the MPCC (or not). These probabilities can be linked together using prior knowledge on probability for an isolate of a given species of being included in the MPCC ($Se_{collection}$) and

probability of an irrelevant isolate of being wrongly included in the MPCC ($Sp_{collection}$). Then, probability of a sample being observed as positive to a given NAS with the used IMI definition ($P_{observed}$) can be linked to the true population prevalence of that NAS species ($P_{population}$) using the second measurement error part (achieved by including prior knowledge on Se and Sp of the IMI definition chosen, i.e. $Se_{IMI\ def}$ and $Sp_{IMI\ def}$, respectively). The response part can be used to model the true prevalence of a given NAS species in the population.

Specificity of IMI definition was deemed to be 100%; therefore, only 3 distinct priors were used for modelling misclassification of NAS species: sensitivity for using a single sample to define IMI ($Se_{IMI\ def} \sim \beta$ (66.97, 211.80), estimated to be ~ 24%); sensitivity for samples that were not obtained / stored ($Se_{collection} \sim \beta$ (136.5, 17.8), estimated to be ~89% (5,509 isolates available out of 6,213 in total), and specificity for samples misclassified by our laboratory ($Sp_{collection} \sim \beta$ (56.99, 1.5) estimated to be ~99% (75 isolates misclassified out of 5,509 evaluated)). The latter specificity prior was used only for estimating prevalence of NAS IMI as a group, using all isolates that met the IMI definition on the original dataset. After initial evaluation using Bayesian models, this value was assumed to be 100% and the specificity prior was excluded from final models. Various strategies were tested according to the NAS species. Initially, mixed-effects logit models with herd and cow specific components for the intercept were fitted for all explanatory variables and for presence or absence of overall NAS IMI and NAS species IMI as a dependent variable in separate models (i.e., one model for NAS in general and for each NAS species, respectively). Initial analyses considered the complete structure of our dataset (repeated measures clustered inside quarter, cow and herd). Random slopes of DIM at the quarter-level introducing autocorrelation (for repeated observation per quarter) were considered, but excluded from final models (lack of convergence). Various explanatory factors were

introduced in the models as fixed effects in separate models (e.g., parity, DIM, barn type, province, BTSCC category and quarter location). For presentation, regression coefficients of interest were converted back to proportions using the invert logit function. For Bayesian models, the Markov Chain Monte Carlo method with Gibbs sampling was used, with 8 chains in parallel (total of 100,000 iterations using the *runjags* package in R; Denwood and Plummer, 2016). Visual inspection of the Markov chain, plots of autocorrelation and effective sample sizes (ESS) were used to evaluate efficacy. Plots of posterior distribution were visualized, and differences in prevalence estimates between explanatory factors for each species were considered significant when its respective 95% credible interval did not include zero. For uncommon species, visual inspection of the Markov chains, poor ESS (defined as <1,000) and clear autocorrelation of the chains indicated failures in estimation of one or more population parameters. In this instance, models were fitted again considering the observations to be independent, but introducing the imperfection in sensitivities and specificity as priors. Two important assumptions were that misclassification of NAS species was assumed to be: 1) independent of sample storage; and 2) common for all NAS species.

Intraclass Correlation Coefficients. Level of clustering at herd, cow and quarter (repeated samplings) levels was assessed using intraclass correlation coefficients (ICC) and their respective 95% credible intervals were estimated for each species using the Bayesian latent class approach as described previously. Models containing only the intercept and quarter, cow and herd random effects were fitted and the proportion of variance in each level was estimated considering the variance at the measurement level as described by Dohoo et al. (2010).

Multilevel Multivariable Analysis. The association of quarter-level prevalence of NAS species (*S. chromogenes, S. simulans, S. xylosus* and *S. haemolyticus*) IMI and overall NAS IMI

with potential explanatory independent herd-level variables including barn type, herd size (recorded monthly) and average monthly milk production, and cow-level variable parity (binary, heifers vs older cows), observation-level variables DIM and DIM^2 as independent variables was estimated using multilevel logistic regression using maximum likelihood and Gaussian quadrature estimation. Models included intercept-specific components for region, herd, cow and quarter. Two-way interactions were considered based on biological plausibility and possible modification effects (involving barn type, average milk production, and parity) but excluded from final models. No 3-term interaction was evaluated. Non-significant associations (based on likelihood-ratio tests) were excluded from final models if no confounding of remaining estimates was observed (change by 15% or more in the remaining regression coefficients). For predictors with more than 2 categories, overall *P*-values were obtained and adjustments for multiple comparisons were made using the Bonferroni approach. Final models assumed communality of the interpretations of the regression coefficients in terms of other variables presents in the models. For this analysis, an important assumption was that samples identified and not identified did not differ with regards to any association evaluated.

RESULTS

Dataset

Overall, 115,294 milk samples were obtained by the CBMRN. Those samples were obtained from 30,398 lactations (range 1-15 samples per lactation, mean 3.7 samples per lactation), 20,571 udder quarters (range 1-22 samples per quarter, mean 5.5 samples per quarter), and 5,157 cows (range 1-85 samples per cow, mean 21.9 samples per cow). From this total,

17,061 samples were contaminated or unavailable for analysis. The remaining 98,233 samples were from 91 herds, 5,149 cows and 20,305 quarters.

Out of the 98,233 milk samples, a total of 6,213 quarter observations from 91 herds, 2,091 cows, and 3,159 quarters met our NAS IMI definition criteria. A total of 704 isolates were not stored, leaving 5,509 NAS isolates for identification. From these NAS isolates, 75 isolates were identified as another bacterial genus / species (32 *S. aureus*, 19 *Corynebacterium* spp., 2 *Bacillus* spp., 3 *Brachybacterium* spp., 7 *Enterococcus* spp., 3 *Micrococcus* spp., 1 *Moraxella* spp., and 1 yeast) or did not grow from storage media (7 isolates); therefore, 5,434 NAS isolates were available to be included in the analyses. These isolates were obtained from 1,901 cattle and 2,805 quarters.

Distribution of NAS species IMI

The 10 most frequently isolated NAS species causing IMI were *S. chromogenes, S. simulans, S. xylosus, S. haemolyticus, S. epidermidis, S. cohnii, S. sciuri, S. gallinarum, S. capitis*, and *S. arlettae*, with *S. chromogenes* accounting for 49% of isolates (Table 1).

Clustering

Quarter-level ICC, measuring correlation in the same quarter for repeated sampling, was high for all NAS species, but particularly for *S. haemolyticus*, *S. chromogenes*, and overall NAS (Table 1). At the cow-level, ICC ranged from 0.21 to 0.37 (lowest for *S. cohnii* and highest for *S. simulans)*. Notably, for *S. cohnii, S. sciuri* and *S. xylosus* the proportion of variance at the herd level was higher in comparison to NAS grouped as a single species (Table 1).

Prevalence of NAS IMI

Overall prevalence. Quarter-level prevalence of NAS IMI was 25.9% (Table 1). Except for *S. sciuri* being less prevalent in left front quarters, prevalence of overall NAS IMI and species-specific IMI was not different among udder quarters (Table 2). Considering parity, quarter-level prevalence of NAS IMI in first lactation heifers (35.9%) was higher than in parity 2, 3, 4, ≥ 5 (ranging from 23.2 for parity ≥ 5 to 24.2% for parity 3; Figure 1). Prevalence of overall NAS IMI was high at calving (35%), decreased until 10 DIM, and then increased throughout lactation to 40% in quarters with ≥ 12 mo of lactation (Figures 2 and 3).

Specific-specific prevalence. Quarter-level prevalence of *S. chromogenes* IMI was highest in first-lactation heifers (23.2%; 95% CR: 18.8 – 27.4), compared to 9.6% (95% CR: 7.7 – 11.5) in second parity, 9.2% (95% CR: 7.5 – 11.4) in third parity, 8.2% (95% CR: 6.4 – 10.3) in fourth parity, and 8.0% (95% CR: 6.2 – 10.0) for fifth or higher parities (Figure 1). Prevalence of *S. simulans* IMI was also highest in first parity cows (5.9%; 95% CR: 4.8 – 7.3) when compared to second (3.5%; 95% CR: 2.8 – 4.4), and fourth (3.4%; 95% CR: 2.5 – 4.5) parity, whereas prevalence of *S. epidermidis* IMI increased with increasing parity from 0.5% (95% CR: 0.4 – 0.8) in heifers to 2.2% (95% CR: 1.5 – 3.0) in parity 4 (Figure 1). Prevalence of *Staphylococcus sciuri* and *S. gallinarum* IMI was lowest in first-lactation heifers (0.4%; 95% CR: 0.3 – 0.6 and 0.1%; 95% CR: 0.03 – 0.2, respectively) (Figure 4).

Staphylococcus chromogenes was the most frequently isolated species throughout the entire lactation. Its prevalence was particularly high immediately after calving, then decreased in prevalence until 5 to 10 DIM, whereas thereafter its prevalence increased again (Figures 2, 3). *Staphylococcus simulans* had the next highest prevalence at calving and maintained that over the first 5 mo of lactation. After month 4 of lactation, the prevalence of *S. simulans* IMI decreased

(Figure 2, 3). Prevalence of *S. haemolyticus* and *S. gallinarum* was relatively high at calving, but decreased at 5-10 DIM (Figures 2, 5). As observed for overall NAS IMI prevalence, *S. chromogenes*, *S. xylosus*, and *S. haemolyticus* IMI prevalence increased over lactation (Figures 3, 6).

Prevalence as a function of region, barn type and bulk milk SCC. Overall NAS IMI prevalence differed among regions, with highest prevalence in Ontario and the lowest prevalence in Quebec and Alberta (Table 3). Similarly, IMI prevalence of most of the 14 most prevalent NAS species, except *S. haemolyticus, S. gallinarum, S. warneri*, and *S. agnetis,* differed among regions. Prevalence of *S. chromogenes* IMI was lowest in Québec, whereas the prevalence of *S. simulans* and *S. capitis* IMI was lowest in Alberta, and the prevalence of *S. xylosus* IMI was lowest in Alberta and the Maritimes (Table 3). Prevalence of *S. sciuri* was highest in the Maritimes and Alberta, whereas the prevalence of *S. cohnii* and *S. arlettae* IMI was highest in Ontario. Prevalence of *S. equorum* IMI was highest in Alberta.

Although bedded-pack herds had numerically higher NAS IMI prevalence, the difference was not significant (Table 4). However, IMI prevalence of the 12 most prevalent NAS species, except *S. haemolyticus, S. gallinarum,* and *S. warneri* differed between barn types (Table 4). Tie-stall herds had higher prevalence of *S. simulans, S. xylosus, S. cohnii, S. capitis, S. arlettae,* and *S. saprophyticus* IMI compared to free-stall herds, whereas the prevalence of *S. simulans, S. xylosus, S. epidermidis,* and *S. cohnii* IMI was higher in tie-stalls compared to bedded-packs. Prevalence of *S. chromogenes, S. epidermidis,* and *S. sciuri* IMI was highest and the prevalence of *S. epidermidis* IMI was lowest in cows housed on bedded-packs.

Prevalence of NAS IMI decreased numerically with decreasing BMSCC, but the difference was not significant (Table 5). Prevalence of *S. simulans, S. epidermidis,* and *S. cohnii*

IMI were lowest in low BMSCC herds, and prevalence of *S. haemolyticus* IMI was lower in low than in high BMSCC herds (Table 5). Prevalence of the other NAS species, including *S. chromogenes, S. xylosus,* and *S. sciuri*, was not different among the 3 BMSCC categories.

Multivariable Analysis

Mixed-effect logistic regression models were fitted for overall NAS IMI and the IMI prevalence of the 4 most frequently isolated NAS species (Table 6). Associations between species-specific NAS IMI and the independent variables were different for the 4 NAS species. The prevalence of overall NAS IMI was associated with DIM and parity, but the direction and shape of the association differed among NAS species, e.g. prevalence of *S. chromogenes* and *S. simulans* IMI was higher in heifers than in multiparous animals, whereas the prevalence of *S. xylosus* and *S. haemolyticus* IMI was lowest in heifers (Table 6). Also, prevalence of *S. chromogenes, S. xylosus* and *S. haemolyticus* IMI increased with increasing DIM.

Free-stall herds had a lower prevalence of overall NAS IMI compared to tie-stall herds, and low BMSCC herds (< 150,000 cells/mL) had a lower prevalence of overall NAS IMI compared to intermediate (151,000 to 249,000 cells/mL) and high BMSCC herds (Table 6).

Prevalence of *S. chromogenes* IMI was higher in cows housed on a bedded pack than in tie-stalls and was not different among BMSCC categories. Prevalence of *S. xylosus* IMI was higher in tiestalls than in free-stall or bedded pack herds (Table 6). Prevalence of *S. simulans* IMI was higher in tie-stalls than in free-stalls (OR = 3.0). Barn type and BMSCC category were not associated with prevalence of *S. haemolyticus* IMI, but they remained in the model due to confounding issues.

DISCUSSION

The data and large number of samples collected in 91 dairy herds provided a unique opportunity to study distribution of NAS IMI across Canada. The herds included closely represented the Canadian commercial dairy farm population regarding distribution of BMSCC and barn type, average milk production and distribution of parities (Reyher et al., 2011). A total of 25 NAS species were isolated from quarter milk samples. The 5 most prevalent NAS species in this large cohort study were *S. chromogenes, S. simulans, S. xylosus, S. haemolyticus,* and *S. epidermidis,* consistent with findings of the most common NAS species isolated from bovine milk in a study using a subset of the isolates by Fry et al. (2014). The order of frequency, however, differed from studies done in other regions, and prevalence of *S. hyicus* IMI was very low; this was attributed to numerous factors influencing pathogen distribution, such as study design, schematic milk sampling, definition of IMI, and not using molecular methods for species identification (Zadoks and Watts, 2009), as well as differences in management conditions and housing (Taponen et al., 2006; Taponen et al., 2007; Sampimon et al., 2009a; Thorberg et al., 2009; Piessens et al., 2011; Koop et al., 2012; Piepers et al., 2013).

In our study, NAS species were identified using partial sequencing of the *rpoB* gene. Identification of a subset of 441 NAS isolates was confirmed using whole-genome sequencing (Naushad et al., 2016). In earlier studies involving NAS species identification, phenotypic methods and methods with low accuracy (e.g. API Staph and Staph-Zym) were used; however, these methods are no longer recommended (Sampimon et al., 2009b; Vanderhaeghen et al., 2015). Sequencing of housekeeping genes and tDNA-PCR are the molecular techniques considered the most reliable for identification of NAS species; however, there is a lack of

standardization among studies in bovine mastitis (Zadoks et al., 2011; Vanderhaeghen et al., 2015). Whole-genome sequencing is now being used to characterize NAS species and in the future, may replace other techniques currently used (Vanderhaeghen et al., 2015). A recent example by Calcutt et al. (2014) mentioned that a proportion of isolates identified as *S. hyicus* in previous studies would likely now be identified as *S. agnetis*. *Staphylococcus hyicus* was reported as one of the most frequently isolated NAS species in earlier studies, not only because of misclassification, but also because *S. chromogenes* was considered a subspecies of *S. hyicus* (Zadoks and Watts, 2009). In the current study and others using molecular identification techniques confirmed by whole-genome-sequencing (Naushad et al., 2016), *S. hyicus* was infrequently isolated. Therefore, differences among studies in methods used to characterize NAS species likely contributed to apparent discrepancies among studies (Vanderhaeghen et al., 2015).

The prevalence of NAS as a group was 26% at quarter-level, similar to some studies conducted in Europe (Chaffer et al., 1999; Pitkälä et al., 2004; Taponen et al., 2007; Supré et al., 2011). By contrast, Schukken et al. (2009) and Gillespie et al. (2009) in the USA, Tenhagen et al. (2006) in Germany, Piessens et al. (2011) and Piessens et al. (2012) in Belgium, and Sampimon et al. (2009a) in The Netherlands reported a much lower NAS IMI prevalence of 6-12% at the quarter-level. However, as mentioned, comparisons of NAS IMI prevalence in the present study to previous studies should be done with caution, due to variations in definition of IMI and methodology used to analyze data. In our study, the apparent NAS prevalence (total number of NAS observed out of total number of eligible milk samples) was 6.3 per 100 samples (6,213/98,233), similar to the estimated prevalence reported in studies that ignored misclassification of NAS species. Østerås et al. (2006) reported a very low prevalence of 3.3% in Norwegian herds, probably the result of a high cut-off for IMI (>4,000 cfu/mL). In studies that

reported a NAS IMI prevalence at 6 to 12% (Tenhagen et al., 2006; Gillespie et al., 2009; Sampimon et al., 2009a; Schukken et al., 2009; Piessens et al., 2011; Piessens et al., 2012), IMI was defined as quarters with consecutive samplings presenting with a range of colony counts (>500 cfu/mL to >1000 cfu/mL). In the present cohort study, NAS IMI was defined as quarter milk samples with ≥ 10 colonies in microbiologic culture of 10 μL of milk (i.e. ≥ 1,000 cfu/mL), at a single-milk sampling (a method with an estimated sensitivity of 24.4% to diagnose a NAS infection, based on previously defined criteria; Dohoo et al., 2011). Ignoring test sensitivity in our study dramatically underestimated NAS prevalence. Therefore, the Bayesian latent class approach should be used more consistently when dealing with estimation of prevalence or incidence when data regarding sensitivity and specificity of the method(s) used are readily available.

Clustering within cow was high for most NAS species IMI, particularly when compared to herd-level clustering, possibly the result of a high rate of quarter-to-quarter transmission, which indicates a possible contagious characteristic of some NAS species (Barkema et al., 1997). Additionally, relatively high cow-level clustering can also be due to unknown factors that confer higher susceptibility for some cows. The relatively high quarter-level clustering indicates persistence of species-specific IMI or re-infection with the same species between samplings (Reyher et al., 2013; Zadoks et al., 2001). Non-*aureus* staphylococci species might be spread among quarters from direct contact with other teats or indirectly via fomites (e.g. milking machines; Reyher et al., 2013).

In agreement with other reports, heifers had the highest prevalence of overall NAS IMI and in all studies, NAS was the most frequently identified group of bacteria isolated from 22 to 55% of infected quarters (White et al., 1989; Fox, 2009; Mork et al., 2012; De Vliegher et al.,

2012). The species-specific NAS IMI prevalence differed considerably among parities. Although the 10 most common NAS species in the present study were all present in heifers, *S. chromogenes* and *S. simulans* were particularly prevalent in this category. Other reports of common NAS species in heifers include *S. hyicus* (Trinidad et al., 1990), *S. simulans* (Myllys, 1995), *S. chromogenes*, *S. epidermidis* and *S. xylosus* (Aarestrup et al., 1995; Myllys, 1995). However, studies in which *S. hyicus* has been identified frequently predate molecular typing (Zadoks and Watts, 2009), whereas this NAS species has been infrequently identified in the 'molecular era' (e.g., Naushad et al., 2016). *Staphylococcus chromogenes* and *S. simulans* are described as the most udder-adapted NAS species, which may contribute to their high prevalence in heifers (Piepers et al., 2011; De Visscher et al., 2016). *Staphylococcus chromogenes* is, however, also part of the commensal bovine udder microbiota, albeit with capacity to act as an opportunistic pathogen (De Vliegher et al., 2003; Braem et al., 2012). Equally, *S. simulans* might act as an udder-adapted opportunist, since it was not detected from any source in the milking parlour, but was isolated from teat apices (Taponen et al., 2008).

Prevalence of *S. sciuri,* and *S. gallinarum* IMI increased with increasing parity, and prevalence of *S. epidermidis* IMI was higher in cows with parities 2 to 4 compared to first-parity heifers. Previous studies did not focus on prevalence of NAS species IMI according to parity, even though Thorberg et al. (2009) reported persistence of IMI by *S. chromogenes, S. simulans, S. xylosus, S. epidermidis,* and *S. cohnii* in multiparous cows. These pathogens are ubiquitous in the environment and have the potential to be transmitted cow-to-cow; therefore, increasing prevalence with age may be related to a combination of persistence and increased susceptibility for infection (Mork et al., 2012; De Visscher et al., 2014).

Similar to parity, prevalence of IMI with NAS species during lactation differed among species. In several studies, the prevalence of NAS IMI was highest soon after calving, particularly in heifers, and prevalence of NAS IMI subsequently decreased (Matthews et al., 1992; Aarestrup et al., 1995; Piepers et al., 2010). Those studies were similar to the present study with regards to overall NAS IMI, which was attributed to the high prevalence of *S. chromogenes* and to a lesser extent *S. simulans*. According to Piepers et al. (2010), 72% of subclinical mastitis cases in fresh heifers were caused by NAS, although 1 wk later, those isolates were no longer isolated.

In contrast to a report by Piepers et al. (2010), prevalence of NAS IMI increased over lactation, for NAS as a group and for most individual species. The prevalence of IMI with the most common NAS species, S. *chromogenes*, increased, as well as the prevalence of *S. sciuri* (until 4 mo of lactation), *S. cohnii*, and *S. arlettae*. According to Taponen et al. (2007), 55% of *S. chromogenes* and 67% of *S. simulans* persisted throughout lactation, and Supré et al. (2011) reported an average duration of 150 d for *S. chromogenes, S. simulans* and *S. xylosus* IMI. Additionally, perhaps continuous endemic transmission of certain NAS species occurs throughout lactation (Reksen et al., 2012). Further investigation would require a longitudinal study, including strain typing of NAS isolates within cows and quarters.

In most studies, the prevalence of NAS species IMI was highest in herds with a higher BMSCC (Taponen et al., 2006; Taponen et al., 2008; Capurro et al., 2009; Sampimon et al., 2009a; Piessens et al., 2011; Supré et al., 2011). The distribution of NAS species IMI differed among the 3 BMSCC categories in the present study, with *S. simulans, S. haemolyticus, S. epidermidis,* and *S. cohnii* IMI less prevalent in low BMSCC herds (Table 5), whereas *S. chromogenes* and *S. xylosus* IMI was equally prevalent in all 3 BMSCC categories. The

proportion of BMSCC associated with NAS IMI has not been frequently evaluated. In a French study, NAS IMI contributed to 18% of the somatic cells in BMSCC (Rainard et al. 1990). In some large US herds, although the prevalence of NAS IMI increased with increasing BMSCC, the relative impact of NAS IMI on BMSCC decreased with an increasing BMSCC (Schukken et al. 2009). Recently Reyher et al. (2013), using the same cohort data reported herein, reported a quadratic relationship between prevalence of NAS IMI and herd SCC that increased proportionally until prevalence reached 20%, decreasing in SCC after that threshold. Although this association might appear paradoxical, it might be due to not only the prevalence of NAS IMI in herds with a higher BMSCC, but also the higher prevalence of major pathogen IMI in the same herds (Napel et al., 2009; Schukken et al., 2009). In general, IMI with NAS species is associated with a low to moderate increase in quarter milk SCC (Vanderhaeghen et al., 2014; Condas et al., submitted). On farms with BMSCC > 250,000 cells/mL recommended mastitis management practices (e.g. blanket dry cow therapy, post-milking disinfection, treatment of clinical cases, and other hygiene practices) are less common (Dufour et al., 2011). Other practices may also have a role. For example, housing dry cows and pregnant heifers together and contamination of stalls with milk increases the risk of NAS IMI (Sampimon et al., 2009a). Therefore, factors not included in our prevalence models might have had important effects.

The distribution of barn types in this study was similar to that in the regions represented (Canadian Dairy Information Centre, 2016) and also to a previous Canadian study on clinical mastitis (Olde Riekerink et al., 2006). Farms in Alberta and the Atlantic provinces mostly had free-stall and bedded-pack barns, whereas herds in Ontario and particularly Québec had a higher proportion of tie-stall barns, with variations in BMSCC among the 4 regions. We corrected for these differences in the multivariable analysis, although some differences among the 4 regions

remained, likely due to differences among regions in management practices. The distribution of NAS species IMI was different in herds with different housing types (Tables 4 and 6). Before molecular species identification of NAS was available, White et al. (1989) reported that *S. epidermidis* in non-lactating heifers was more frequently isolated from cows housed in free-stalls, whereas *S. chromogenes* and *S. sciuri* were more prevalent in heifers housed in bedded-pack barns than in free-stall herds, consistent with our study. *Staphylococcus simulans, S. xylosus, S. cohnii, S. capitis, S. arlettae,* and *S. saprophyticus* IMI were most prevalent in tie-stall barns. Different housing systems and management practices within each barn type partially explained the present results. For example, the high prevalence of *S. xylosus* and *S. simulans* IMI could be linked to sawdust, which is mostly used in tie- and free-stall herds for bedding stalls (Matos et al., 1991; Pyörälä and Taponen, 2009). Additionally, De Visscher et al. (2017) reported that environmental aspects such as bedding cleanliness, cow pen grouping, and sources of drinking water were associated with high NAS IMI prevalence. Effects of these management practices on the prevalence of NAS IMI warrant further research.

Various factors evaluated resulted in different associations according to bacterial species. Regardless, unmeasured factors associated with provinces, housing systems and/or BMSCC categories could have accounted for part of the residual variance in our analysis. Although other factors may be responsible for the observed effects (i.e. the association in question does not exist once we introduce possible confounders, or the factor in hand was a confounder of a non-evaluated association), the present study represented a first step in identifying specific risk factors for each NAS species.

When evaluating results for samples from bedded-pack herds, any associations should be evaluated carefully, due to the low number of herds under this housing system in the present

study. Whenever an interaction was present involving housing system, stratification of bedded pack herds into smaller categories may reflect an individual herd factor as deemed responsible for the result (or absence of significant result). Further studies are needed to evaluate prevalence of various NAS species in bedded-pack herds.

CONCLUSIONS

This large dataset facilitated determination of the disparate association of quarters infected by species-specific NAS as predictors at herd- and cow-levels, when compared to NAS as an overall pathogen and demonstrated clear differences between species. Therefore, each species should be evaluated independently. Quarter-level prevalence of NAS IMI as a group was 26%. Consistent with other studies, *S. chromogenes* was the most prevalent species; however, order of prevalence of *S. simulans*, *S. xylosus*, *S. epidermidis*, and *S. haemolyticus* IMI differed from other countries. Prevalence of IMI with most NAS species was relatively high at calving, particularly in heifers. Prevalence thereafter decreased for a short interval, but increased again during lactation. From a herd perspective, management related to different barn types and BMSCC affected NAS species IMI distribution and influenced distribution among regions.

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Table 1. Distribution of non-*aureus* staphylococci (NAS) species intramammary infection from bovine milk in Canadian dairy herds and intraclass correlation (ICC) of quarters within cow, cows within herd, and among herds.

	Distribution					Herds (n=91)			Cows (n=5,149)			Quarters (n=20,305)		
Species	N	%[1]	Prev[2]	95% CR[3]		N	%[4]	ICC (95% CR[3])	N	%[5]	ICC (95% CR[3])	N	%[6]	ICC (95% CR[3])
S. chromogenes	2,660	48.9	13.04	10.60	15.52	91	100	0.04 (0.03-0.06)	980	51.55	0.33 (0.28-0.37)	1,301	0.46	0.59 (0.54-0.63)
S. simulans	913	16.8	4.33	3.63	5.38	81	89.01	0.06 (0.04-0.09)	347	18.25	0.37 (0.30-0.43)	433	0.15	0.49 (0.43-0.57)
S. xylosus	628	11.6	3.03	2.48	3.71	71	78.02	0.11 (0.08-0.16)	353	18.57	0.27 (0.20-0.33)	422	0.15	0.47 (0.39-0.54)
S. haemolyticus	428	7.9	2.06	1.67	2.54	78	85.71	0.05 (0.03-0.09)	277	14.57	0.24 (0.14-0.32)	304	0.11	0.56 (0.48-0.66)
S. epidermidis	225	4.1	1.06	0.86	1.36	50	54.95	0.11 (0.06-0.17)	130	6.84	0.35 (0.25-0.45)	151	0.05	0.36 (0.26-0.47)
S. cohnii	139	2.6	0.66	0.52	0.86	33	36.26	0.20 (0.14-0.29)	102	5.37	0.21 (0.11-0.33)	112	0.04	0.38 (0.22-0.52)
S. sciuri	121	2.23	0.58	0.45	0.75	44	48.35	0.15 (0.09-0.23)	87	4.58	0.36 (0.24-0.48)	102	0.04	0.22 (0.08-0.35)
S. gallinarum	50	0.92	0.24	0.17	0.33	23	25.27	-	35	1.84	-	37	0.013	-
S. capitis	45	0.83	0.21	0.15	0.31	18	19.78	-	28	1.47	-	30	0.011	-
S. arlettae	44	0.81	0.21	0.15	0.30	20	21.98	-	36	1.89	-	39	0.014	-
S. warneri	31	0.57	0.15	0.10	0.22	14	15.38	-	21	1.10	-	25	0.009	-
S. saprophyticus	30	0.55	0.14	0.09	0.21	20	21.98	-	28	1.47	-	30	0.011	-
S. agnetis	24	0.44	0.11	0.07	0.18	14	15.38	-	16	0.84	-	17	0.006	-
S. equorum	24	0.44	0.11	0.07	0.18	15	16.48	-	24	1.26	-	24	0.009	-
S. succinus	17	0.31	0.08	0.05	0.13	13	14.29	-	15	0.79	-	17	0.006	-
S. hominis	12	0.22	0.06	0.03	0.10	9	9.89	-	11	0.58	-	11	0.004	-
S. devriesei	9	0.17	0.04	0.02	0.08	8	8.79	-	9	0.47	-	9	0.003	-
S. pasteuri	8	0.15	0.04	0.02	0.07	6	6.59	-	8	0.42	-	8	0.003	-
S. nepalensis	7	0.13	0.03	0.01	0.07	2	2.20	-	3	0.16	-	4	0.001	-
S. vitulinus	6	0.11	0.03	0.01	0.06	6	6.59	-	6	0.32	-	6	0.002	-
S. auricularis	4	0.07	0.02	0.01	0.05	4	4.40	-	4	0.21	-	4	0.001	-
S. hyicus	4	0.07	0.02	0.01	0.05	4	4.40	-	4	0.21	-	4	0.001	-
S. caprae	2	0.04	0.01	0.00	0.03	2	2.20	-	2	0.11	-	2	0.001	-
S. fleuretti	2	0.04	0.01	0.00	0.03	1	1.10	-	2	0.11	-	2	0.001	-
S. kloosii	1	0.02	0.00	0.00	0.02	1	1.10	-	1	0.05	-	1	0.0004	-
Total NAS[8]	6,213	87.46	25.89	22.08	31.64	91	100	0.05 (0.04-0.07)	1,901	36.93	0.29 (0.25-0.32)	2,805	13.81	0.60 (0.57-0.63)

848 [1]Percentage of NAS species from the 5,434 characterized NAS isolates at species-level in the study; [2]True quarter prevalence
849 estimated using a Bayesian latent class model accounting for IMI misclassification; [3]95% credible region; [4]Percentage of herds with at
850 least 1 quarter positive for a species; [6]Percentage of cows with at least 1 quarter positive for a species; [7]Percentage of quarters positive
851 for a species; [8]Total NAS isolates that were phenotypically identified as NAS including the NAS isolates that were not stored.

Table 2. Quarter-level prevalence of non-*aureus* staphylococci (NAS) species intramammary infection, according to quarter location.

	Left front				Right front				Left rear				Right rear			
Species	N	Prevalence[1]	95% CR[2]		N	Prevalence	95% CR		N	Prevalence	95% CR		N	Prevalence	95% CR	
S. chromogenes	720	14.01	11.56	17.07	702	13.80	11.40	16.86	632	12.79	10.41	15.46	606	12.35	10.02	14.90
S. simulans	262	5.07	4.07	6.31	223	4.41	3.49	5.48	225	4.53	3.59	5.64	203	4.02	3.22	5.11
S. xylosus	153	2.97	2.32	3.78	145	2.88	2.23	3.66	146	2.93	2.27	3.73	184	3.66	2.89	4.65
S. haemolyticus	117	2.27	1.75	2.94	115	2.27	1.73	2.93	103	2.05	1.57	2.69	93	1.89	1.41	2.45
S. epidermidis	54	1.05	0.75	1.44	49	0.95	0.68	1.33	56	1.11	0.81	1.54	66	1.33	0.97	1.79
S. cohnii	19	0.38	0.22	0.59	39	0.76	0.52	1.09	42	0.86	0.58	1.19	39	0.78	0.54	1.12
S. sciuri	15	0.30[4]	0.16	0.48	34	0.67	0.44	0.97	32	0.65	0.42	0.94	40	0.79	0.54	1.13
S. gallinarum	13	0.26	0.13	0.43	11	0.22	0.11	0.39	18	0.36	0.21	0.58	8	0.16	0.07	0.31
S. capitis	15	0.28	0.16	0.48	14	0.27	0.15	0.46	6	0.11	0.05	0.25	10	0.21	0.10	0.37
S. arlettae	6	0.12	0.05	0.25	7	0.14	0.06	0.28	18	0.36	0.21	0.58	13	0.26	0.14	0.44
S. warneri	7	0.14	0.06	0.27	10	0.19	0.09	0.36	7	0.13	0.06	0.28	7	0.14	0.06	0.28
S. saprophyticus	11	0.21	0.11	0.38	5	0.10	0.03	0.22	6	0.12	0.05	0.25	8	0.16	0.07	0.31
S. agnetis	5	0.09	0.03	0.22	6	0.12	0.04	0.25	6	0.12	0.05	0.25	7	0.14	0.06	0.28
S. equorum	2	0.04	0.01	0.13	7	0.14	0.06	0.27	7	0.14	0.06	0.28	8	0.16	0.07	0.31
S. succinus	3	0.06	0.01	0.16	4	0.08	0.02	0.19	6	0.12	0.05	0.25	4	0.08	0.02	0.19
S. hominis	4	0.08	0.02	0.19	2	0.04	0.01	0.13	2	0.04	0.01	0.13	4	0.08	0.02	0.19
S. devriesei	3	0.06	0.01	0.16	2	0.04	0.01	0.13	1	0.02	0.001	0.10	3	0.06	0.01	0.16
S. pasteuri	4	0.08	0.02	0.19	2	0.04	0.01	0.13	0	0.003	0	0.06	2	0.04	0.01	0.13
S. nepalensis	1	0.02	0.001	0.10	2	0.04	0.01	0.13	4	0.08	0.02	0.19	0	0.003	0	0.06
S. vitulinus	0	0.003	0	0.06	1	0.02	0.001	0.10	3	0.06	0.01	0.16	2	0.04	0.01	0.13
S. auricularis	0	0.003	0	0.06	2	0.04	0.01	0.13	2	0.04	0.01	0.13	0	0.003	0	0.06
S. hyicus	1	0.02	0.001	0.09	0	0.003	0	0.06	1	0.02	0.001	0.10	2	0.04	0.01	0.13
S. caprae	0	0.003	0	0.06	1	0.02	0.001	0.10	1	0.02	0.001	0.10	0	0.003	0	0.06
S. fleuretti	1	0.02	0.001	0.09	0	0.003	0	0.06	0	0.003	0	0.06	1	0.02	0.001	0.10
S. kloosii	0	0.003	0	0.06	1	0.02	0.001	0.10	0	0.003	0	0.06	0	0.003	0	0.06
Total NAS[3]	1,600	26.93	22.98	32.98	1,605	27.66	23.32	33.41	1,519	26.77	22.53	32.29	1,489	25.98	22.12	31.71

852 [1]True quarter prevalence estimated using a Bayesian latent class model accounting for IMI misclassification; [2]95% credible region; [3]Total NAS
853 isolates phenotypically identified as NAS, including NAS isolates that were not stored.

Table 3. Quarter-level prevalence of non-*aureus* staphylococci (NAS) species intramammary infection within region of 91 Canadian dairy herds.

Species	Alberta (17 herds)				Maritimes (18 herds)				Ontario (27 herds)				Québec (29 herds)			
	N	Prevalence[1]	95% CR[2]		N	Prevalence	95% CR		N	Prevalence	95% CR		N	Prevalence	95% CR	
S. chromogenes	545	14.87[5]	12.12	18.09	607	14.04	11.51	17.11	885	15.10[5]	12.54	18.44	623	9.73	7.87	11.71
S. simulans	64	1.75[3,4,5]	1.28	2.36	165	3.85[4]	3.01	4.86	408	7.16[5]	5.72	8.66	276	4.32	3.43	5.33
S. xylosus	65	1.77[4,5]	1.29	2.39	67	1.52[4,5]	1.14	2.10	287	5.05	3.98	6.16	209	3.22	2.56	4.05
S. haemolyticus	61	1.67	1.20	2.25	91	2.10	1.59	2.78	135	2.31	1.81	2.99	141	2.21	1.71	2.81
S. epidermidis	19	0.52[4,5]	0.31	0.82	47	1.09	0.77	1.52	74	1.27	0.95	1.71	85	1.30	0.99	1.74
S. cohnii	4	0.11[4,5]	0.03	0.27	13	0.30[4]	0.16	0.52	94	1.63[5]	1.23	2.13	28	0.44	0.28	0.64
S. sciuri	30	0.81[5]	0.52	1.20	55	1.28[4,5]	0.90	1.74	20	0.34	0.20	0.54	16	0.25	0.14	0.41
S. gallinarum	3	0.08	0.02	0.22	10	0.23	0.11	0.42	15	0.26	0.15	0.43	22	0.33	0.21	0.53
S. capitis	0	0.004[3,4,5]	0	0.08	10	0.23	0.11	0.43	19	0.32	0.19	0.52	16	0.24	0.14	0.41
S. arlettae	4	0.11[3,4]	0.03	0.26	1	0.02[4]	0.00	0.11	36	0.62[5]	0.41	0.89	3	0.05	0.01	0.13
S. warneri	2	0.05	0.01	0.18	10	0.24	0.11	0.42	8	0.14	0.06	0.27	11	0.17	0.09	0.30
S. saprophyticus	0	0.004[3]	0	0.08	11	0.27	0.13	0.45	9	0.16	0.07	0.29	10	0.15	0.08	0.28
S. agnetis	2	0.05	0.01	0.18	3	0.07	0.02	0.19	10	0.17	0.08	0.32	9	0.14	0.06	0.26
S. equorum	15	0.41[3,4,5]	0.23	0.68	1	0.02	0.00	0.11	5	0.08	0.03	0.19	3	0.04	0.01	0.13
S. succinus	3	0.08	0.02	0.22	6	0.14	0.05	0.29	7	0.12	0.05	0.24	1	0.02	0.001	0.08
S. hominis	0	0.004	0	0.08	2	0.05	0.01	0.15	2	0.03	0.01	0.11	8	0.12	0.06	0.24
S. devriesei	2	0.05	0.01	0.18	3	0.07	0.02	0.19	4	0.07	0.02	0.17	0	0.002	0	0.05
S. pasteuri	1	0.03	0.001	0.13	1	0.02	0.00	0.11	0	0.00	0.00	0.05	6	0.09	0.04	0.20
S. nepalensis	0	0.004	0	0.08	0	0.003	0.00	0.07	7	0.12	0.05	0.24	0	0.002	0	0.05
S. vitulinus	2	0.06	0.01	0.18	3	0.07	0.02	0.19	1	0.02	0.001	0.08	0	0.002	0	0.05
S. auricularis	1	0.03	0.001	0.13	2	0.05	0.01	0.15	1	0.02	0.001	0.08	0	0.002	0	0.05
S. hyicus	0	0.004	0	0.08	1	0.02	0.001	0.11	1	0.02	0.001	0.08	2	0.03	0.005	0.10
S. caprae	0	0.004	0	0.08	1	0.02	0.001	0.11	1	0.02	0.001	0.08	0	0.002	0	0.05
S. fleuretti	2	0.05	0.01	0.18	0	0.004	0	0.07	0	0.003	0	0.052	0	0.002	0	0.048
S. kloosii	0	0.004	0	0.083	0	0.004	0	0.071	1	0.018	0.001	0.084	0	0.002	0	0.048
Total NAS[6]	986	23.45[4]	20.01	28.61	1,240	25.22	21.48	30.56	2,294	35.05[5]	29.72	41.88	1,693	23.25	19.66	27.84

854 [1]True quarter prevalence estimated using a Bayesian latent class model accounting for IMI misclassification; [2]95% credible region;

855 [3]Different from Maritimes (Prince Edward Island, New Brunswick, and Nova Scotia); [4]Different from Ontario; [5]Different from

856 Québec; [6]Total NAS isolates phenotypically identified as NAS, including NAS isolates that were not stored.

Table 4. Quarter-level prevalence of non-*aureus* staphylococci (NAS) species intramammary infection in 3 barn types.

	Tiestall (54 herds)				Freestall (30 herds)				Bedded-pack (5 herds)			
Species	N	Prevalence[1]	95% CR[2]		N	Prevalence	95% CR		N	Prevalence	95% CR	
S. chromogenes	1,462	11.97[4]	9.82	14.38	930	13.63[4]	11.34	16.74	238	22.88	17.90	28.02
S. simulans	694	5.62[3,4]	4.63	6.88	187	2.82	2.19	3.50	19	1.79	1.05	2.84
S. xylosus	515	4.20[3,4]	3.42	5.13	89	1.31	0.99	1.74	11	1.01	0.54	1.87
S. haemolyticus	269	2.15	1.74	2.71	133	1.94	1.53	2.54	25	2.27	1.50	3.58
S. epidermidis	117	0.94[4]	0.72	1.22	100	1.47[4]	1.13	1.94	2	0.19	0.03	0.62
S. cohnii	118	0.97[3,4]	0.73	1.24	19	0.27	0.17	0.45	0	0.01	0	0.29
S. sciuri	47	0.37[4]	0.27	0.53	44	0.64[4]	0.45	0.91	27	2.54	1.64	3.82
S. gallinarum	34	0.28	0.19	0.40	12	0.17	0.09	0.31	3	0.29	0.07	0.77
S. capitis	36	0.29[3]	0.20	0.42	5	0.07	0.03	0.17	0	0.01	0	0.29
S. arlettae	40	0.33[3]	0.22	0.46	1	0.01	0.001	0.07	1	0.09	0.003	0.46
S. warneri	25	0.20	0.13	0.31	5	0.07	0.03	0.17	0	0.01	0	0.29
S. saprophyticus	28	0.22[3]	0.15	0.34	2	0.03	0.004	0.10	0	0.01	0	0.29
S. agnetis	19	0.16	0.09	0.25	4	0.06	0.02	0.14	1	0.09	0.004	0.46
S. equorum	8	0.07	0.03	0.13	16	0.25	0.13	0.39	0	0.01	0	0.29
S. succinus	8	0.06	0.03	0.13	8	0.12	0.05	0.23	1	0.10	0.003	0.46
S. hominis	9	0.07	0.03	0.14	2	0.03	0.005	0.10	0	0.01	0	0.29
S. devriesei	8	0.06	0.03	0.13	1	0.01	0.001	0.07	0	0.01	0	0.28
S. pasteuri	6	0.05	0.02	0.1	2	0.03	0.004	0.10	0	0.01	0	0.29
S. nepalensis	7	0.06	0.02	0.11	0	0.002	0	0.04	0	0.01	0	0.29
S. vitulinus	2	0.02	0.002	0.05	4	0.06	0.02	0.14	0	0.01	0	0.29
S. auricularis	3	0.03	0.006	0.066	1	0.02	0.001	0.072	0	0.01	0	0.29
S. hyicus	2	0.02	0.002	0.053	2	0.03	0.005	0.097	0	0.01	0	0.28
S. caprae	2	0.02	0.002	0.053	0	0.002	0	0.045	0	0.01	0	0.29
S. fleuretti	0	0.001	0	0.025	2	0.03	0.004	0.097	0	0.01	0	0.29
S. kloosii	1	0.01	0	0.039	0	0.002	0	0.045	0	0.01	0	0.29
Total NAS[5]	3,947	27.59	23.79	34.09	1,808	23.62	19.74	28.47	373	31.40	25.17	38.03

857 [1]True quarter prevalence estimated using a Bayesian latent class model accounting for IMI misclassification; [2]95% credible region;

858 [3]Different from free-stalls; [4]Different from bedded-pack; [5]Total NAS isolates phenotypically identified as NAS, including NAS

859 isolates that were not stored.

Table 5. Quarter-level prevalence of non-*aureus* staphylococci (NAS) species intramammary infection according to bulk tank somatic cell count category.

	Bulk tank somatic cell count (cells/mL)											
	≤ 150,000 (13 herds)				151,000 - 249,000 (47 herds)				≥ 250,000 (31 herds)			
Species	N	Prevalence[1]	95% CR[2]		N	Prevalence	95% CR		N	Prevalence	95% CR	
S. chromogenes	351	12.03	9.75	14.89	1,392	12.55	10.36	15.17	917	14.24	11.86	17.44
S. simulans	71	2.44[3,4]	1.79	3.29	570	5.15	4.21	6.30	272	4.13	3.34	5.19
S. xylosus	71	2.42	1.79	3.27	359	3.21	2.61	3.99	198	3.15	2.47	3.93
S. haemolyticus	41	1.40[4]	0.96	1.99	219	1.97	1.57	2.47	168	2.62	2.08	3.36
S. epidermidis	7	0.24[3,4]	0.10	0.48	124	1.10	0.85	1.44	94	1.45	1.11	1.94
S. cohnii	6	0.21[3,4]	0.08	0.44	88	0.78	0.59	1.04	45	0.70	0.49	1.00
S. sciuri	18	0.62	0.37	1.00	72	0.64	0.48	0.87	31	0.49	0.32	0.72
S. gallinarum	7	0.24	0.10	0.49	27	0.25	0.16	0.36	16	0.25	0.14	0.41
S. capitis	4	0.14	0.04	0.33	28	0.25	0.16	0.38	13	0.20	0.11	0.35
S. arlettae	1	0.03	0.002	0.17	26	0.23	0.15	0.36	17	0.26	0.15	0.43
S. warneri	7	0.24	0.10	0.49	8	0.07	0.03	0.14	16	0.25	0.14	0.41
S. saprophyticus	4	0.14	0.04	0.33	18	0.16	0.09	0.26	8	0.13	0.06	0.25
S. agnetis	1	0.03	0.001	0.17	16	0.14	0.08	0.24	7	0.11	0.05	0.22
S. equorum	5	0.17	0.06	0.38	14	0.13	0.07	0.21	5	0.08	0.03	0.18
S. succinus	4	0.14	0.04	0.33	7	0.06	0.03	0.13	6	0.10	0.04	0.20
S. hominis	1	0.03	0.001	0.17	6	0.05	0.02	0.11	5	0.07	0.02	0.15
S. devriesei	5	0.18	0.06	0.39	2	0.02	0.003	0.06	2	0.03	0.005	0.10
S. pasteuri	1	0.04	0.001	0.17	4	0.04	0.01	0.09	3	0.05	0.01	0.13
S. nepalensis	0	0.005	0	0.11	7	0.06	0.03	0.13	0	0.002	0	0.05
S. vitulinus	0	0.005	0	0.11	4	0.04	0.01	0.09	2	0.03	0.005	0.10
S. auricularis	2	0.07	0.01	0.23	1	0.01	0	0.04	1	0.02	0.001	0.08
S. hyicus	1	0.03	0.001	0.17	1	0.01	0	0.04	2	0.03	0.004	0.10
S. caprae	0	0.01	0	0.11	2	0.02	0.003	0.06	0	0.002	0	0.05
S. fleuretti	2	0.08	0.01	0.23	0	0.001	0	0.03	0	0.002	0	0.05
S. kloosii	0	0.01	0	0.11	1	0.01	0	0.04	0	0.002	0	0.05
Total NAS[5]	694	21.08	17.37	25.61	3,433	27.18	22.66	32.70	2,086	28.40	23.98	34.60

860 [1]True quarter prevalence estimated using a Bayesian latent class model accounting for IMI misclassification; [2]95% credible region;

861 [3]Different from 151,000 to 249,000 cells/mL; [4]Different from ≥ 250,000 cells/mL; [5]Total NAS isolates phenotypically identified as

862 NAS, including NAS isolates that were not stored.

863 **Table 6**. Final multilevel logistic regression model for the quarter-level prevalence of overall non-*aureus* staphylococci (NAS) and the

864 4 most prevalent NAS species intramammary infections in 91 Canadian dairy herds.

	Overall NAS[1]			*S. chromogenes*			*S. xylosus*			*S. simulans*			*S. haemolyticus*		
	β	OR	*P*	β	OR	*P*	β	OR	*P*	β	OR	*P*	β	OR	*P*
Intercept	-4.2	-	<0.001	-6.36	-	<0.001	-6.43	-	<0.001	-7.05	-	<0.001	-8.57	-	<0.001
Herd-level factors															
Housing															
Free-stall	-0.22	0.80	0.04	0.02	1.02	0.45	-1.42	0.24	<0.001	-1.11	0.33	0.01	-0.10	0.90	0.71
Bedded pack	-0.18	0.83	0.22	0.52	1.68	<0.001	-1.84	0.16	<0.001	-1.09	0.34	0.12	0.14	1.15	0.77
Tie-stall	ref	-	-	ref	-	-	ref	-	-	ref	-	-	ref	-	-
Bulk Milk SCC (cells/mL)[2]															
≤ 150,000	ref	-	-	-	-	-	-	-	-	-	-	-	ref	-	-
151,000 - 249,000	0.34	1.40	0.001	-	-	-	-	-	-	-	-	-	0.48	1.62	0.16
≥ 250,000	0.47	1.60	0.001	-	-	-	-	-	-	-	-	-	0.73	2.08	0.06
Cow-level factors															
Parity															
Heifer	0.42	1.52	<0.001	1.14	3.13	<0.001	-0.43	0.65	<0.001	0.44	1.55	<0.001	-0.29	0.75	0.03
Multiparous	ref	-	-	ref	-	-	ref	-	-	ref	-	-	ref	-	-
Days in Milk (DIM)	0.001	-	<0.001	0.001	-	<0.001	<0.001	-	0.75	-0.002	-	0.04	0.003	-	<0.001
DIM*DIM	-	-	-	-	-	-	7.4E-06	-	0.002	7.50E-06	-	0.04	-	-	-

865 [1]NAS intramammary infection grouped as a single category; [2]2-y average bulk milk somatic cell count.

866 **FIGURE LEGENDS**

867

868 **Figure 1**. Quarter-level prevalence of intramammary infection with non-*aureus* staphylococci
869 (NAS) and the 5 most frequently isolated NAS species across parities.[1]

870

871 **Figure 2**. Quarter-level prevalence of intramammary infection with non-*aureus* staphylococci
872 (NAS) and the 5 most frequently isolated NAS species in the first 30 DIM.[1]

873

874 **Figure 3**. Quarter-level prevalence of intramammary infection with non-*aureus* staphylococci
875 (NAS) and the 5 most frequently isolated NAS species throughout lactation.[1]

876

877 **Figure 4**. Quarter-level prevalence of the 6th to 10th most frequently isolated species of non-
878 *aureus* staphylococci intramammary infection across parities.[1]

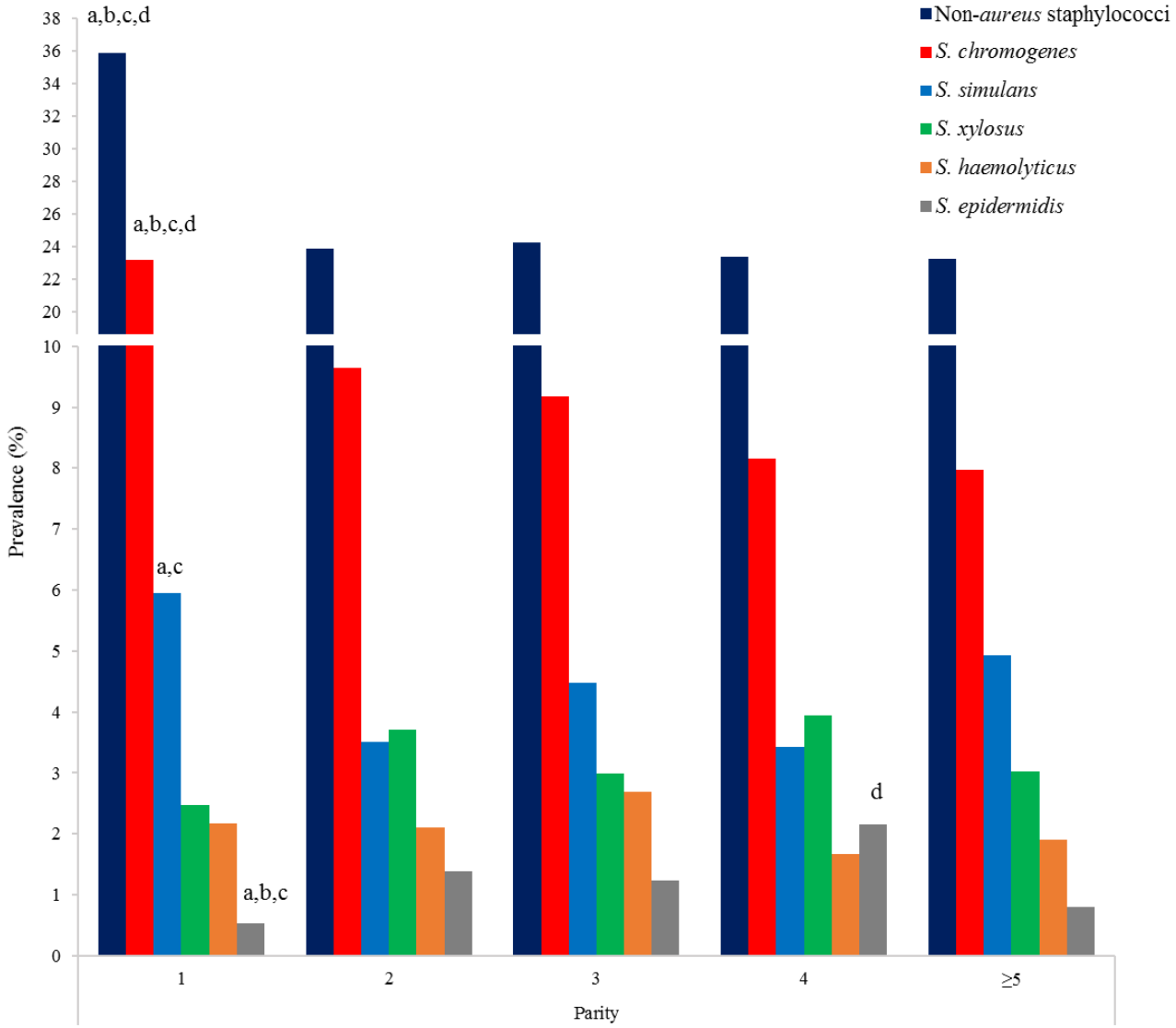
879

880 **Figure 5**. Quarter-level prevalence of the 6th to 10th most frequently isolated species of non-
881 *aureus* staphylococci intramammary infection in the first 30 DIM.[1]

882

883 **Figure 6**. Quarter-level prevalence of the 6th to 10th most frequently isolated species of non-
884 *aureus* staphylococci intramammary infection throughout lactation.[1]

885 **Figure 1**

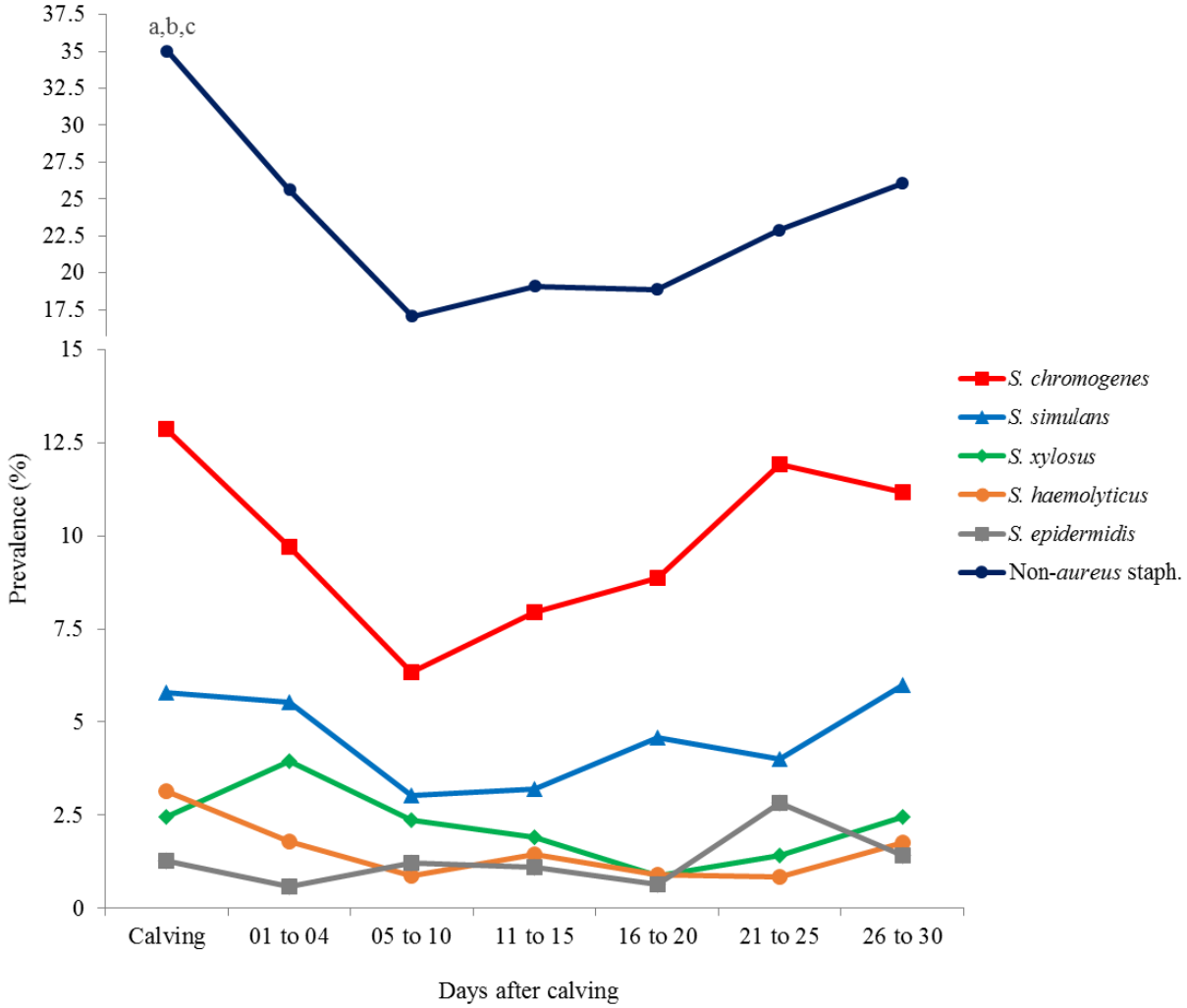


886

887 [1]True quarter prevalence estimated using a Bayesian latent class model accounting for IMI misclassification; [a]Different from second

888 parity; [b]Different from third parity; [c]Different from fourth parity; [d]Different from parity ≥ 5.

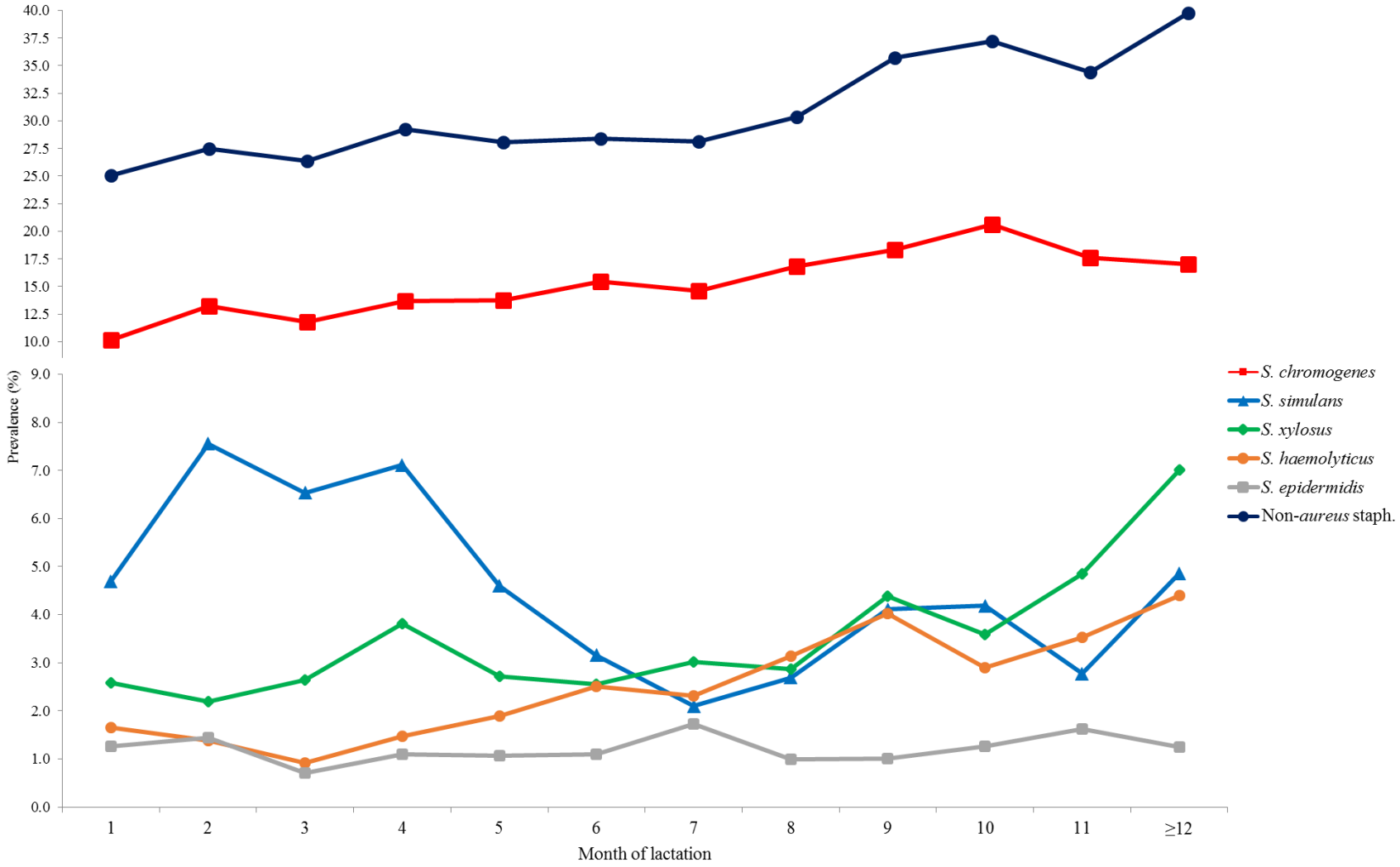
889 **Figure 2**



890

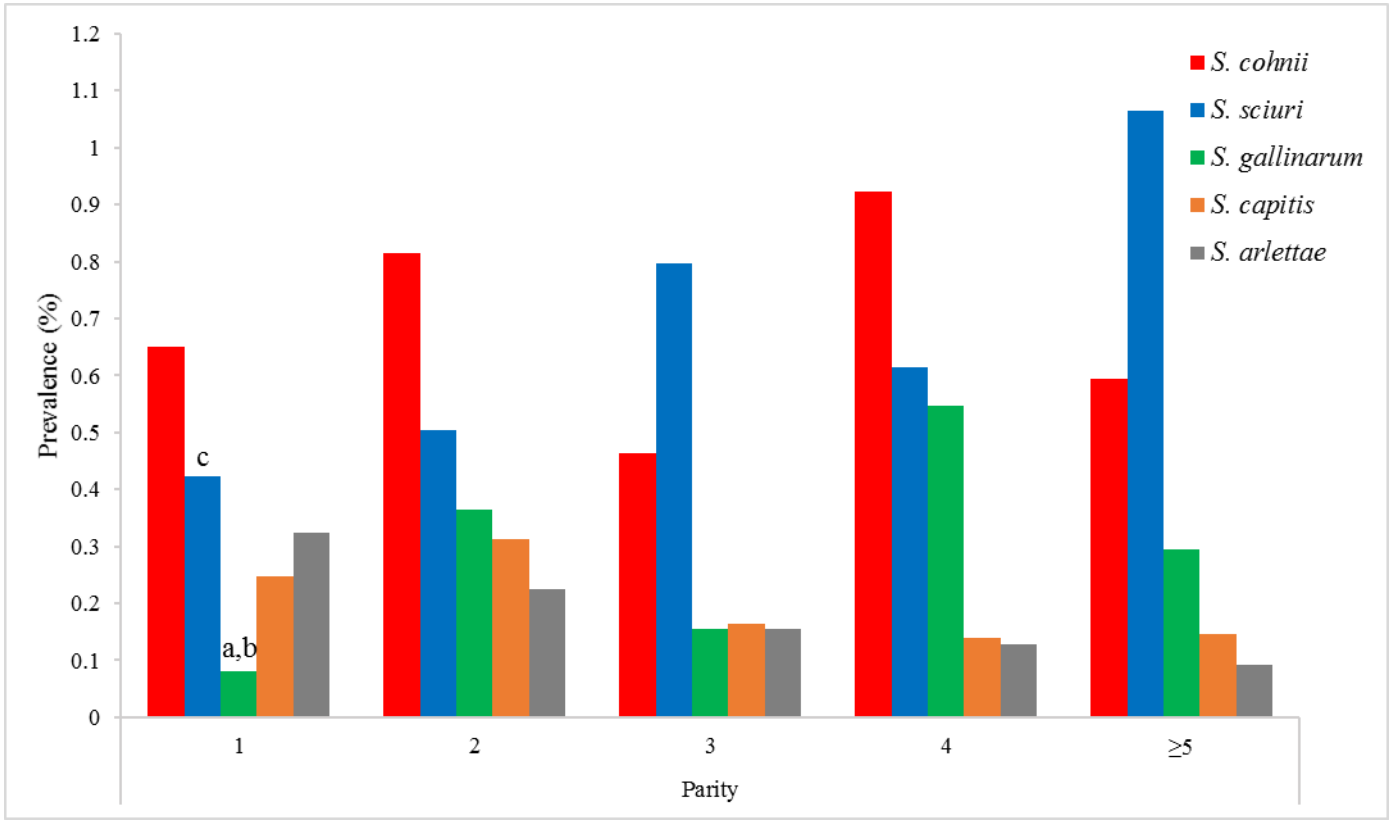
[1]True quarter prevalence estimated using a Bayesian latent class model accounting for IMI misclassification; [a]Different from 5 to 10 DIM; [b]Different from 11 to 15 DIM; [c]Different from 16 to 20 DIM

Figure 3



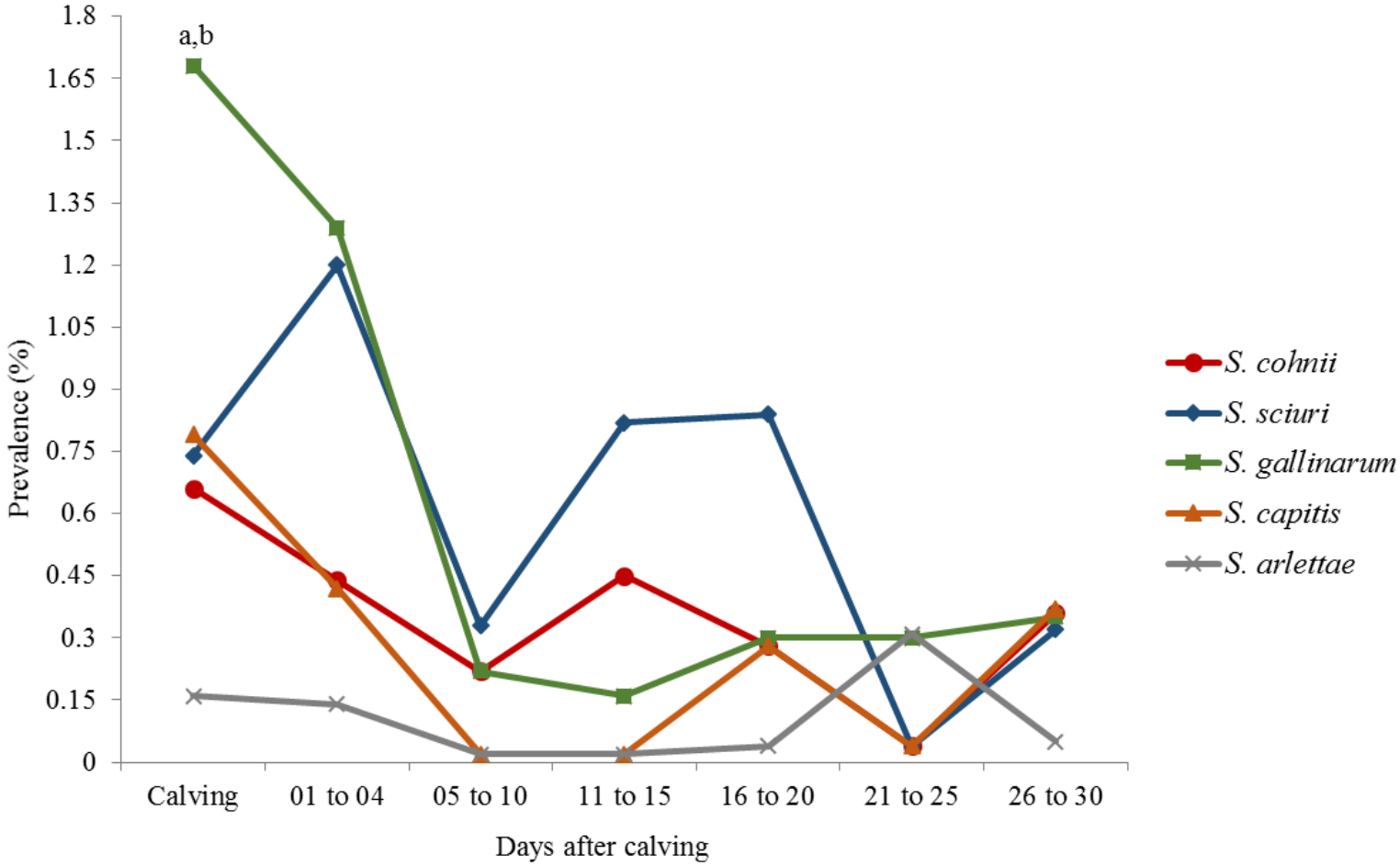
[1]True quarter prevalence estimated using a Bayesian latent class model accounting for IMI misclassification.

Figure 4



[1]True quarter prevalence estimated using a Bayesian latent class model accounting for IMI misclassification; [a]Different from parity 2; [b]Different from parity 4; [c]Different from parity ≥ 5.

900 **Figure 5**

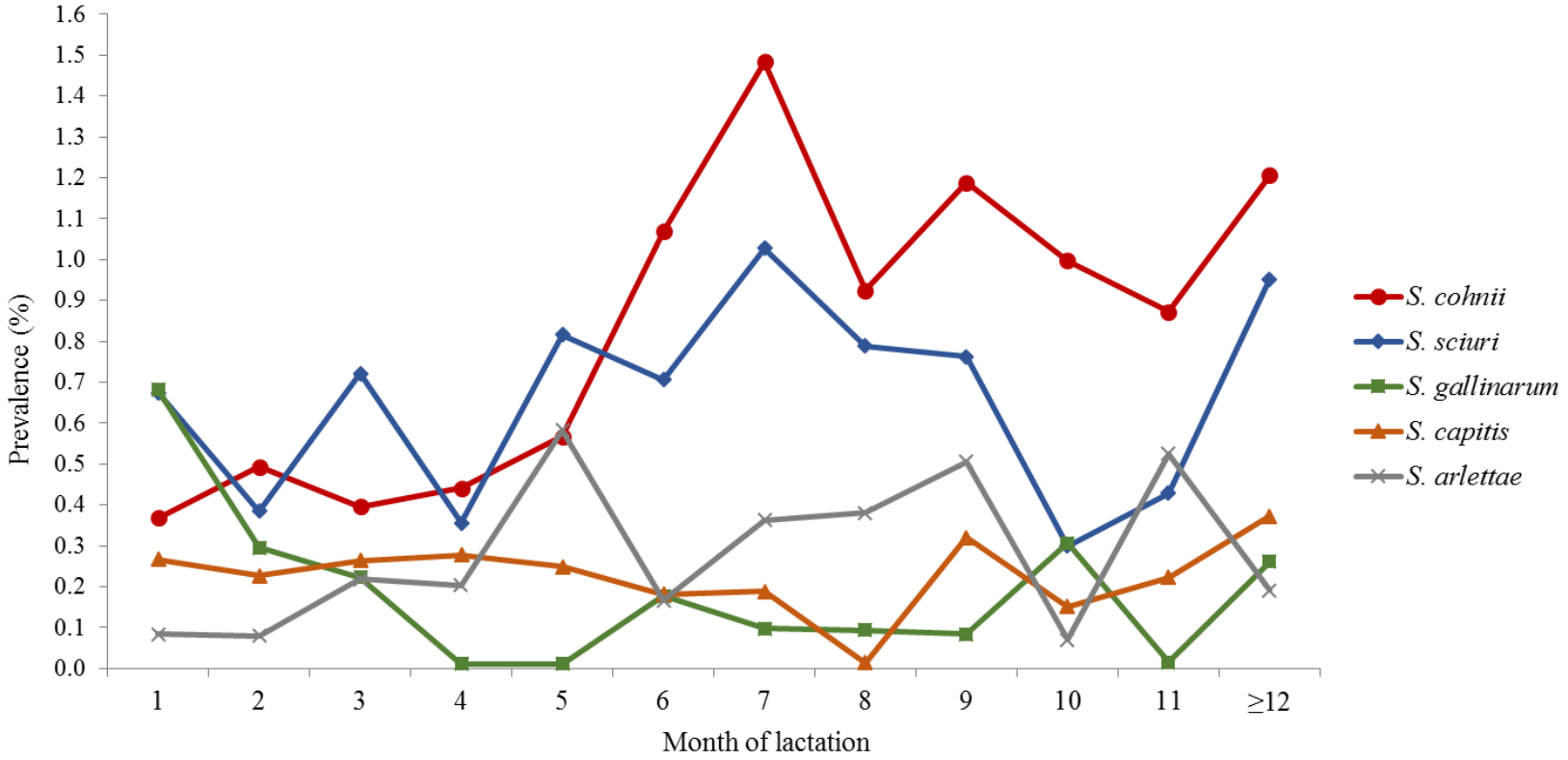


901

902 [1]True quarter prevalence estimated using a Bayesian latent class model accounting for IMI misclassification; [a]Different from 5 to 10 d

903 after calving; [b]Different from 11 to 15 d after calving

Figure 6



[1]True quarter prevalence estimated using a Bayesian latent class model accounting for IMI misclassification.