Veterinary Microbiology 228 (2019) 188–195

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Partial prediction of the duration and the clinical status of *Staphylococcus aureus* bovine intramammary infections based on the phenotypic and genotypic analysis of isolates

S. Pichette-Jolette[a], G. Millette[a], E. Demontier[a], D. Bran-Barrera[a], M. Cyrenne[a], C. Ster[a], D. Haine[b], G. Keefe[c], F. Malouin[a,*], J.P. Roy[b,*]

[a] Centre d'Étude et de Valorisation de la Diversité Microbienne (CEVDM), Département de Biologie, Faculté des Sciences, Université de Sherbrooke, Sherbrooke, QC, J1K 2R1, Canada

[b] Département de Pathologie et Microbiologie and Département de Sciences Cliniques, Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, QC, J2S 2M2, Canada

[c] Department of Health Management, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Prince Edward Island, C1A 4P3, Canada

ARTICLE INFO

Keywords:
S. aureus
Bovine mastitis
Severity of infection
Duration of infection
Persistency

ABSTRACT

Staphylococcus aureus is a bacterial pathogen causing bovine intramammary infections (IMIs) often leading to chronic clinical or subclinical mastitis. Predicting the outcome of *S. aureus* IMIs (duration and clinical vs subclinical) based on the characterization of isolates would help to make better case management decisions. For this purpose, 583 *S. aureus* isolates from series of quarter milk samples were characterized by genotypic tests (detection of virulence genes *seg*, *tst*, *lukM*), epidemiological typing (*spa* type) and by a phenotypic test (biofilm production). VNTR typing (variable number of tandem repeats) was used to establish persistence of the same *S. aureus* strain in each series of sequential isolates. This allowed to associate each strain to a clinical/subclinical status and to validate the duration of infection. We found differences in the distribution of *spa* types between the strains from clinical and subclinical cases. Prevalence of *lukM* was also higher in strains from clinical cases than in strains from subclinical cases. A Kaplan-Meier analysis was then used to determine factors influencing the duration of the infection. Considering a multivariable model of the logistic regression, time to elimination was shorter with the strains of the subclinical lactation series compared to the clinical series (series with at least one clinical case). Strains from the *spa* type t359 and t529 were less likely to persist compared to those of *spa* type t13410. In sum, strain characterization including determination of the *spa* type helps to predict duration of infection and the clinical or subclinical outcome of *S. aureus* IMIs.

1. Introduction

Intramammary infections (IMIs) leading to subclinical or clinical mastitis represent a recurring problem with important economic and animal welfare impacts on the dairy industry worldwide (Halasa et al., 2007). In Canada, *S. aureus* is the most commonly isolated pathogen in the context of both clinical and subclinical IMIs (Reyher et al., 2011). This pathogen is also well known to cause chronic IMIs that are refractory to treatment (Melchior et al., 2006). *S. aureus* infected quarters are the main reservoir that allows the infection to propagate during milking (Zadoks et al., 2002). Better management practices are needed to prevent or control *S. aureus* IMIs.

One way to improve the efficacy of *S. aureus* control programs would be to identify the strains that are either prone to cause clinical symptoms or subclinical infections as well as those able to persist in the mammary gland and to cause chronic infections. Identification of problematic strains could help make better management decisions at the farm.

The persistence of the infection or the severity of the mastitis symptoms is determined by multiple factors from the environment, host and pathogen including the genotype and phenotype of the *S. aureus* strain involved (Veh et al., 2015). Determining the clonal origin of *S. aureus* strains could provide information on pathogen-associated factors (Feil et al., 2003). *Spa* typing is a specific molecular method which classifies *S. aureus* strains by sequencing the X polymorphic hypervariable region of the staphylococcal protein A gene (*spa*) (Koreen et al., 2004). This typing method is well standardized and allows to compare *S. aureus* strains coming from different sources worldwide.

* Corresponding authors.
E-mail addresses: Francois.Malouin@USherbrooke.ca (F. Malouin), jean-philippe.roy@umontreal.ca (J.P. Roy).

https://doi.org/10.1016/j.vetmic.2018.11.024
Received 2 October 2018; Received in revised form 29 October 2018; Accepted 24 November 2018

Many bacteria including *S. aureus* have the ability to produce biofilm. Biofilm is an extracellular matrix that can reduce opsonisation by antibodies and phagocytosis. Furthermore, bacterial community in biofilm are less susceptible to antibiotics (Costerton et al., 1999; Jacques et al., 2010). Production of biofilm is tightly regulated by multiple regulatory gene systems. The quorum-sensing system Agr is one of the most influential regulatory systems that influences the formation of biofilm in *S. aureus* (Arciola et al., 2015). The activation of this system is proportional to the bacterial density and that of the autoinducing peptide (AIP) released in the environment by the bacteria (Novick, 2003). Low activation of the Agr system reduces the dispersion of the biofilm and thus promotes a more abundant production (Otto, 2013).

S. aureus have some virulence factors that help the establishment of an IMI (Sutra and Poutrel, 1994). They can be grouped in multiple categories like hydrolytic enzymes, hemolysins and leukocidins (cytolysins), and enterotoxins/superantigens. Enterotoxins like the staphylococcal enterotoxin G (*SEG*) and the toxic shock syndrome toxin (TSST-1) are superantigens that disturb the host immune response by causing a non-specific polyclonal activation of immune cells (Dinges et al., 2000). Leukocidin LukMF' is a two-component pore-forming cytolysin (Fromageau et al., 2010). Bovine neutrophils express a high quantity of CCR1 receptors at their surface, which are the targets of the LukMF' leukocidin and that make them vulnerable to lysis by this toxin (Vrieling et al., 2015).

Using *S. aureus* isolates from series of quarter milk samples, the objective of this study was to identify *S. aureus* genotypic and phenotypic characteristics modulating the duration of IMIs and manifestation of clinical or subclinical mastitis. To our knowledge, this study is the first of its kind because it compares series of quarter milk samples and associated isolates rather than single mastitis episodes and isolates, and also because it uses time survival analyses to determine bacterial factors associated with the duration of IMIs.

2. Material and methods

2.1. Bacterial isolates collection

Five hundred eighty four *S. aureus* isolates from quarter milk samples were characterized in the study. All isolates came from the Canadian Bovine Mastitis and Milk Quality Research Network Mastitis Pathogen Culture Collection (Faculté de Médecine vétérinaire, Université de Montréal, St-Hyacinthe, QC, Canada). As described in Reyher et al. (2011), the isolates were collected over a two-year period from 91 herds in the National Cohort of Dairy Farms from four different regions of Canada (Atlantic provinces, Quebec, Ontario, Western provinces). All *S. aureus* isolates coming from quarter samples with at least one colony of *S. aureus* from a 10-µL milk sample (100 cfu/ml) were kept in the mastitis pathogen culture collection. *S. aureus* isolates were Gram-positive cocci showing a positive catalase test, hemolysis on blood agar, and positive nuclease and coagulase tests (Reyher et al., 2011). All *S. aureus* isolates were archived with related data both at the farm and cow levels.

2.2. Types of quarter milk samples

Quarter milk samples were collected on several occasions during the two-year period: 1) during lactation, 2) prior to dry-off and after calving, and 3) when a clinical mastitis case was observed. More precisely, during lactation, quarter milk samples (termed lactation samples) were collected and consisted of 3 samplings, 3 weeks apart, during each of 3 periods of the year. Also, during one period of the year, 7 weekly lactation samples were taken. All this was done for 15 cows per herd (10 randomly selected and 5 that had most recently calved) with always the first lactation sample of the series taken on apparently healthy cows (no abnormal milk). Any quarter that presented clinical mastitis (clinical samples) during the sampling scheme (abnormal milk or a swollen quarter and/or sick cow) were recorded and samples were thereafter collected as a clinical case series. In such cases, quarter milk samples were repeated 2–3 weeks and again 4–5 weeks after diagnosis. In addition, within each herd, the first 15 cows to be dried off were sampled once between 4 to 2 weeks before dry-off and another time before dry-off (both termed dry cow samples), another sample was taken within 24 h of calving and one last sample was taken 1–2 weeks after calving (both termed fresh cow samples). Any quarter that presented clinical mastitis was thereafter treated as a clinical case. Quarter sample series with no clinical case were named subclinical series (subclinical samples). The somatic cell count (SCC) was available for most of the quarter samples. The SCC were measured by the Fossomatic method as described in Reyher et al. (2011).

Variable number of tandem repeats (VNTR) typing is described below and was used to infer the persistence of the same *S. aureus* strain in a quarter series of sequential isolates. This allowed to associate a specific strain to a clinical/subclinical status and a duration of infection. The duration of IMI (or the time for elimination of *S. aureus*) is defined further below. Complete characterization of *S. aureus* was performed on the first isolate of the quarter series.

2.3. Extraction of gDNA

The isolates were inoculated in 3 ml of brain heart infusion (BHI) (BD, Mississauga, ON, Canada) and incubated overnight. Genomic DNA was extracted using the kit Gen Elute kit according to manufacturer's recommandations (Sigma-Aldrich Canada, Oakville, ON, Canada) with few modifications. Lysostaphin incubation was increased from 30 min to 1h30 and proteinase K incubation time was increased from 10 min to 30 min.

2.4. Determination of the duration of infection by VNTR typing

The duration of the infection was determined by the time between samples having the first and the last isolates of the quarter milk series showing an identical VNTR profile. If their VNTR profiles differed, the other isolates of the series were also VNTR typed looking for sequential isolates showing the same VNTR profile thus redefining another series and duration of infection. The VNTR test was previously described by Sabat et al. (2003). The primers used were targeting the clumping factor genes A and B (*clfA* and *clfB*), staphylococcal protein A (*spa*), V8 serine protease (*ssp*) and Ser-Asp-rich fibronectin-binding protein (*srdCDE*) (Table 1). All VNTR PCR were performed using the GeneAmp PCR system 2700 (Life Technologies, Burlington, ON). The PCR reaction final volume was 25 µL and was composed of 1 × PCR buffer with $MgCl_2$ (New England BioLabs [NEB] Inc., Pickering, ON, Canada), 0.8 mM dNTP (Bioshop, Burlington, ON); 1.5 mM $MgCl_2$ (NEB); final primer concentration was 160 nM except for the *clfB* primers which were at 80 nM; final concentration of the Taq DNA polymerase (NEB) was 0.025 U/µl. The cycling settings were 5 min of denaturation at 94 °C; 20 cycles of amplification with 30 s at 90 °C, 30 s at 55 °C and 30 s at 72 °C; 5 min elongation at 72 °C. The amplicon was migrated on a 1.5% agarose gel, stained with bromide ethidium, visualized with a UV transmitter and photographed. As mentioned above, the duration of infection was determined by the time between samples having the first and the last isolates of the quarter milk series showing an identical VNTR profile, and only the first isolate of the quarter series with such a validated infection duration was then used for further characterization of the *S. aureus* strain (no duplication of isolates).

2.5. Spa typing

The polymorphic X region of the *spa* gene was amplified using the primers spa-1113f and spa-1514 r (Table 1), as describe by Aires-de-Sousa et al. (2006). The PCR reaction was previously described in Veh

Table 1
Primer names and sequences, expected sizes of amplicons, and PCR conditions used to characterize *S. aureus* strains.

Primer name	Primer sequence[a]	Product size (bp)	PCR hybridization temperature	Reference
seg Fwd[b,c]	AATTATGTGAATGCTCAACCCGATC	642	50°C	Jarraud et al. (2002)
seg Rev[b,c]	AAACTTATATTGGAACAAAAGGTACTAGTTC			
tst Fwd[b,d]	TTCACTATTTGTAAAAGTGTCAGACCCACT	180	50°C	Jarraud et al. (2002)
tst Rev[b,d]	TACTAATGAATTTTTTTATCGTAAGCCCTT			
femA Fwd[b,d]	ACAGCTAAAGAGTTTGGTGCCTIIIIIGATAGCATGC	729	50°C	Jarraud et al. (2002)
femA Rev[b,d]	TTCATCAAAGTTGATATACGCTAAAGGTIIIIICACACGGTC			
lukM Fwd[b,c]	TGGATGTTACCTATGCAACCTAC	780	55 °C	Veh et al. (2015)
lukM Rev[b,c]	GTTCGTTTCCATATAATGAATCACTAC			
spa-113f[e]	TAAAGACGATCCTTCGGTGAGC	100– 442	63°C	Aires-de-Sousa et al. (2006)
spa-1514 r[e]	CAGCAGTAGTGCCGTTTGCTT			

[a] Fwd = forward; Rev = reverse.
[b] Used for the detection of virulence genes.
[c] Used in simplex.
[d] Used in multiplex PCR.
[e] Used for spa typing.

et al. (2015). The presence of the amplicon was confirmed on a 1% agarose gel and then sequenced at the *Plateforme d'Analyses Biomoléculaires de l'Université Laval* (Quebec, QC, Canada). The sequences were analyzed with the software Bionumerics v5.10 (Austin, TX, USA). The *spa* plug-in was used to compare the sequence obtained to the *spa* database server (http://www.spaserver.ridom.de/). One specific *spa* type was assigned to each strain.

2.6. Virulence gene PCR

The presence of the virulence genes *seg, tst* and *lukM* was detected by PCR. Detection of the gene *femA* was used as a positive control. *FemA* encoded for the biosynthesis of a cell-wall protein that is universally carried by *S. aureus* (Mehrotra et al., 2000). All primer sequences are presented in Table 1. One PCR multiplex (*tst* and *femA* genes) and two additional simplex PCR (*seg* and *lukM* genes) were performed. The final volume for each PCR reaction was 25 µl and consisted of 1X PCR buffer (NEB), 0.4 mM of dNTP (Bioshop), 80 nM of each primer for simplex PCR and 40 nM of each primer for the multiplex PCR; 0.025 U/ µL Taq DNA polymerase (NEB). All the PCR reactions were performed on the GeneAmp PCR system 2700 (Life Technologies). The cycling conditions were 4 min à 95 °C for initial denaturation, 35 cycles of 30 s at 95 °C followed by 30 s at primer specific temperature (Table 1) and 1 min at 68 °C. A final extension of the product was performed for 10 min at 68 °C. The gDNA of the *S. aureus* strain N315 was used has a positive control for the presence of *seg* and *tst* genes. *S. aureus* strain RF122 was used has a positive control for the *lukM* gene.

2.7. Biofilm production in vitro

S. aureus strains were grown on BHI agar supplemented with 0.25% glucose and were incubated overnight at 35°C. For each strain, a 0.5 McFarland standard suspension was prepared in BHI supplemented with 0.25% glucose. One hundred µl of each suspension were inoculated in a flat bottom cellular culture treated 96-well plates in quadruplicate. The plates were then incubated at 35°C for 24 h. After incubation, plates were washed three times with 200 µL of PBS, then air dried and stained with a 0.01% (w/v) crystal violet (Sigma-Aldrich Canada, Oakville, ON, Canada) solution for 30 min. The plates were washed with distilled water twice and the biofilm was dissolved in 100% ethanol. The absorbance was measured at 540 nm by spectrometry (Bio-Tek Instruments, Winooski, VT). Each strain's biofilm production was normalized to the biofilm production of strain Sa2539 (value of 1.00), which produces a stable and moderate amount of biofilm. For each strain, biofilm quantification was performed three independent times. The means of the biofilm production from the strains were used for the subsequent statistical analysis.

2.8. Statistical analysis

2.8.1. Case-control study with clinical cases against series without any clinical case

The case group contained all the quarter series (with isolates of the same VNTR profile) showing at least one clinical case during the sampling period. The strains from these series were compared to a control group, which contained all series showing no manifestation of clinical mastitis in the series, although a *S. aureus* strain was detected in the series samples (subclinical mastitis). The distribution of the *spa* type between strains causing clinical and subclinical mastitis was compared using the Pearson chi-square test (x^2). The distribution of virulence genes in the strains of the case and control groups was also compared using x^2 test. The production of biofilm was compared using Wilcoxon signed rank test.

2.8.2. Kaplan-Meier (time survival) analysis

The samples used for this statistical test were all the samples for which the duration of infection was validated by VNTR typing. The time of elimination of the *S. aureus* strain responsible for a quarter series was defined as the time from the first positive sample to the last positive sample followed by at least two negative samples. If the elimination was not confirmed, right-censoring was applied at the last observation available. In this case, the data were considered to be type 2 interval censored (Huang and Wellner, 1997). Survival analysis with non-parametric likelihood estimators of the survivor distribution was used to estimate the time of elimination (Gentleman and Geyer, 1994). The time of survival analysis compared sample types, *S. aureus spa* types, possession or not of virulence genes, and biofilm production. Weighted log-rank tests were applied on the variables (Sun, 1996).

Parametric regression proportional hazard models for interval censored data with Weibull baseline distribution were fit separately for sample type, *spa* type, biofilm production, virulence genes, and also within a model considering all of them.

All statistics were realized with R version 3.3.1 (R Core Team, 2015), and add-on packages, notably packages interval (Fay and Shaw, 2010) and *icenReg* (Anderson-Bergman, 2016).

3. Results

3.1. Description of the S. aureus isolates

In total, 353 distinct quarter series with at least one isolation of *S. aureus* were compared. From these 353 quarter series, there was 119 series composed of a single positive isolate of *S. aureus*, which was thereafter eliminated from the quarter based on two following samples testing negative for *S. aureus*. The duration of infection associated to

those 119 isolates was the short time extending from the first *S. aureus*-positive sample to the next sampling, which was negative. Besides those 119 single-isolate series, 234 quarter series could have up to 18 isolates (collected from 18 quarter milk samples). For those multiple-isolate series, 234 pairs of isolates were formed with the first and the last isolates of the series to validate the persistence of the same strain in each series using VNTR typing. This validation thus determined the duration of infection for strains from multiple-isolate series (see below).

3.2. Validation of persistent strains from quarter series by VNTR typing

As mentioned above, VNTR typing was performed to determine the duration of the *S. aureus* IMIs for multiple-isolate series. The infection was considered to be persistent if the VNTR profiles for the first and last quarter series isolates were the same. If the first and last isolates presented different profiles, the whole series of isolates was VNTR typed to validate the exact duration of the infection by a specific strain. In total, from the 234 pairs of isolates studied, 195 pairs showed a persistent strain based on VNTR typing (195 quarter series having a *S. aureus* strain with a validated duration), meaning that 39 pairs were composed of isolates with different VNTR profiles (possible reinfection with a different strain or a co-infection). The characteristics of the first isolate of the pairs with a validated duration (195 persistent strains) were compared to the characteristics of the 119 short-duration strains from the single-isolate series and were all included in the Kaplan-Meier (time survival) analysis.

3.3. Spa typing

The compilation of all *spa* types found for each of the first strain of the 353 studied series is reported in Table 2. There were 22 different *spa* types found among these strains and 57 strains were non typable (no PCR amplification). A novel *spa* type with 14 repeats was discovered in the series (t16275). The main *spa* types ($n \geq 10$), were t529 (n = 115), t267 (n = 67), t359 (n = 36), t605 (n = 18), t2445 (n = 11) and t13401 (n = 10). The distribution of the *spa* types based on the sample type (lactation-subclinical, lactation-clinical, dry cow, fresh cow) did not differed when considering all *spa* types (Pearson test, $p > 0.1$), but differed if comparing the 6 main *spa* types and a group formed of the non typable strains together with all minor *spa* types ($p < 0.05$).

3.4. Virulence genes

The distribution of the virulence genes for each of the first strain of the 353 series is presented in Table 2. The non typable strains (n = 57) were not characterized for a total of 296 strains tested for *seg* and *tst* and 294 strains tested for *lukM* (two additional strains also not tested). Among all the *S. aureus* strains tested, 31.8% were positive for *seg* (n = 94). The presence of virulence genes varied with specific *spa* types. The *seg* positive strains were mainly found in *spa* type t529 (82/94, 87.2%). The second and third most populated *spa* types, t267 and t359, only included 2.1% and 6.4% of the *seg* positive strains, respectively.

For the *tst* gene, only 11 strains were positive, which is too few to find a strong statistical significance.

The gene *lukM* gene was present at a higher rate than *seg* with 78.6% of all tested strains being positive (231/294 strains). The three main *spa* types (t529, t267 and t359) included 44.6%, 25.5% and 13.9% of the *lukM* positive strains found in the study, respectively.

3.5. Biofilm production

The ability to produce biofilm *in vitro* was investigated by the crystal

Table 2
Spa type, virulence genes and biofilm production of the first *S. aureus* strain of each quarter series, which were also classified by sample type (subclinical, clinical, dry-cow, fresh-cow).

Spa type	n	Type of *S. aureus* strain[a] (n = 353)[b]				Virulence gene			Biofilm production[c]
		Subclinical (n = 137)	Clinical (n = 82)	Dry cow (n = 79)	Fresh cow (n = 55)	*seg*	*lukM*	*tst*	
t529	115	38	32	25	20	82	103	9	1.02
t267	67	27	15	12	13	2	59	1	1.10
NT[d]	57	27	8	15	7	NA[e]	NA[e]	NA[e]	NA[e]
t359	36	13	14	1	8	6	32	0	1.09
t605	18	8	1	9	0	0	1	1	2.18
t2445	11	3	4	4	0	0	11	0	0.59
t13401	10	5	1	2	2	0	2	0	3.59
t3380	9	3	2	3	1	0	8	0	1.11
t521	7	1	4	1	1	1	6	0	1.21
t1965	5	3	1	1	0	1	5	0	0.70
t1166	3	2	0	0	1	0	0	0	0.91
t11215	2	2	0	0	0	0	0	0	4.13
t1190	2	1	0	1	0	0	2	0	0.83
t1236	2	1	0	0	1	0	1	0	1.32
t015	1	0	0	0	1	1	0	0	1.26
t021	1	0	0	1	0	1	0	0	4.11
t10610	1	0	0	1	0	0	0	0	2.33
t12186	1	0	0	1	0	0	1	0	5.22
t16275	1	1	0	0	0	0	0	0	4.31
t127	1	0	0	1	0	0	0	0	3.68
t177	1	0	0	1	0	0	0	0	2.58
t224	1	1	0	0	0	0	NA[e]	0	NA[e]
t9129	1	1	0	0	0	0	NA[e]	0	NA[e]

[a] Based on sample type (lactation-subclinical, lactation-clinical, dry cow, fresh cow).
[b] There were 353 quarter series studied and the first *S. aureus* strain of each series was *spa* typed. The non typable strains (n = 57) were not further characterized for a total of 296 strains tested for *seg*, *tst* and biofilm production and 294 strains tested for *lukM*.
[c] Data presented are the median values for all the strains of the indicated *spa* type. Each strain's biofilm production was normalized to the biofilm production of strain Sa2539 (value of 1.00), which produces a stable and average amount of biofilm.
[d] NT, non typable (no amplification of *spa* by PCR).
[e] NA, not available.

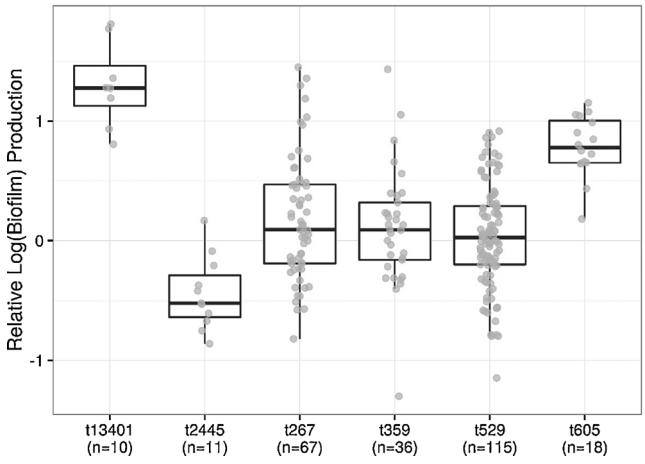

Fig. 1. Boxplots of the biofilm production *in vitro* for *S. aureus* strains of the major *spa* types (n ≥ 10). Each dot represents one strain. The horizontal bar represents the median of biofilm production for each *spa* type. The boxes represent the interquartile interval. Biofilm production was different among the six main *spa* types ($p < 0.001$). More specifically, *spa* type t13401 produces more biofilm than all the other main *spa* types ($p < 0.001$), excepted for t605.

violet staining method. A total of 296 out of the first strains of the 353 series were compared (the non-typable strains were excluded). The median of biofilm production for each *spa* type is shown in Table 2. The distribution of biofilm production for the six main *spa* types is presented in Fig. 1. Biofilm production was different among the six main *spa* types ($p < 0.001$). More specifically, *spa* type t13401 produces more biofilm than all the other main *spa* types ($p < 0.001$), excepted for t605. The *spa* type t605 produces more biofilm than t529, t267, t359, and t2445 ($p < 0.001$). The *spa* type t2445 also produces less biofilm than t267 ($p < 0.001$), t359 ($p < 0.01$), and t529 ($p = 0.01$) (Fig. 1).

Biofilm production was also compared according to the presence or absence of virulence genes. The *seg* positive strains produce less biofilm than the *seg* negative strains ($p < 0.01$). The *tst* and *lukM* positive strains produce less biofilm than the *tst* and *lukM* negative strains, respectively ($p < 0.001$). It is noteworthy that the high biofilm producer *spa* types t605 and t13401 mostly do not possess the *lukM* gene (Table 2).

3.6. Strain characteristics and association with a clinical episode within a series

All quarter milk series with at least one case of clinical mastitis (106 series, case group) were compared to all quarter milk sample series without any case of clinical mastitis (247 series, control group). The descriptive statistics showed a different *spa* type distribution between the subclinical and clinical series ($p < 0.05$) (Table 3). The presence of virulence genes among strains of those two groups was also compared. A significant difference was found with the presence of the *lukM* gene. The series with at least one clinical mastitis episode included 94% of *lukM* positive strains compared to the subclinical mastitis series with 80% of *lukM* positive strains ($p < 0.01$) (Table 3).

In the logistic regression model for this case-control study, the only factor that was significantly different was the biofilm production *in vitro* ($p = 0.05$). The conclusion was that for each log of biofilm production, there was a 97% increased chance to have clinical mastitis.

3.7. Time survival analysis

Time survival analysis according to the presence/absence of virulence genes showed no significant difference when each virulence gene was considered alone (Fig. 2A and B). Note that no time survival analysis was performed with the *tst* gene as a variable since there were too few positive strains.

Table 3
Descriptive comparison of *S. aureus* strains associated with quarter sample series with subclinical and clinical cases.

Strain characteristic		Series with only subclinical cases (n = 247) n (%)	Series with at least one clinical case (n = 106) n (%)	p value[1]
spa type	t529	74 (30.0)	41 (38.7)	P < 0.05
	t267	46 (18.6)	21 (19.8)	
	t359	22 (9.0)	14(13.2)	
	t605	17 (6.8)	1 (0.9)	
	t13401	7 (2.8)	3 (2.8)	
	t2445	5 (2.0)	6 (5.7)	
	Others and NT[2]	76 (30.8)	20 (18.9)	
Superantigen gene	*seg*	86 (33.6)	29 (35.4)	p > 0.1
	tst	12 (5.2)	4 (4.9)	p > 0.1
Presence of *lukM*		161 (80.0)	75 (94.0)	P < 0.01

[1] Pearson test.
[2] Include strains with minor *spa* types (n < 10) as well as non typable (NT) strains (those with no PCR amplification for *spa*).

For the time survival analysis according to biofilm production, strains were divided between the strong biofilm producers, which produce more biofilm than the median of the overall dataset, and the weak biofilm producers, which produce less biofilm than the median of the overall dataset. The time to elimination of those two groups (high and low biofilm producers) was then investigated. No difference could be observed for the time to elimination with only the biofilm as the main factor (Fig. 2C).

Time survival analysis, according to the sample type and *spa* type, also did not show any statistical difference between these categories (Fig. 3A and 3B).

The survival analysis was then followed by a logistic regression, which included all variables (type of samples, *spa* type, biofilm production, presence of virulence genes). In this multivariate model of the logistic regression, time to elimination was shorter with the strains of the lactation-subclinical series when compared to the lactation-clinical series ($p < 0.05$), with the strains in the clinical samples having an increase of 48% in the possibility that the bacteria persist longer than the strains from the subclinical samples. Strains from the *spa* types t359 ($p < 0.05$) and t529 ($p < 0.05$) were 4.47 and 3.50 times more likely to be eliminated from the quarters than strains from *spa* type t13401, respectively.

3.8. Somatic cell counts

The somatic cell count (SCC) is routinely used to evaluate udder health and to detect possible IMIs. The SCC was available for most of the quarter samples in this study. Lactation-subclinical samples were as expected associated with a lower log SCC (5.5) compared to lactation-clinical (8.1), dry-cow (6.5) and fresh-cow (8.2) samples ($p < 0.001$). Interestingly, persistent strains (> 100 days within a quarter) were associated with samples having higher log SCC compared to samples associated with strains that were eliminated (≤100 days within a quarter) with 7.4 log *vs* 5.5 log cells/ml of milk, respectively ($p < 0.001$). Note that these values are the specific quarter milk sample SCC from the first sample of the series (*i.e.*, of the quarter sample from which the first isolate of the series came from).

4. Discussion

Currently, the only answer dairy producers receive from the bacteriology performed on milk samples is the species of bacteria that is found. This information does not provide the dairy producer and the

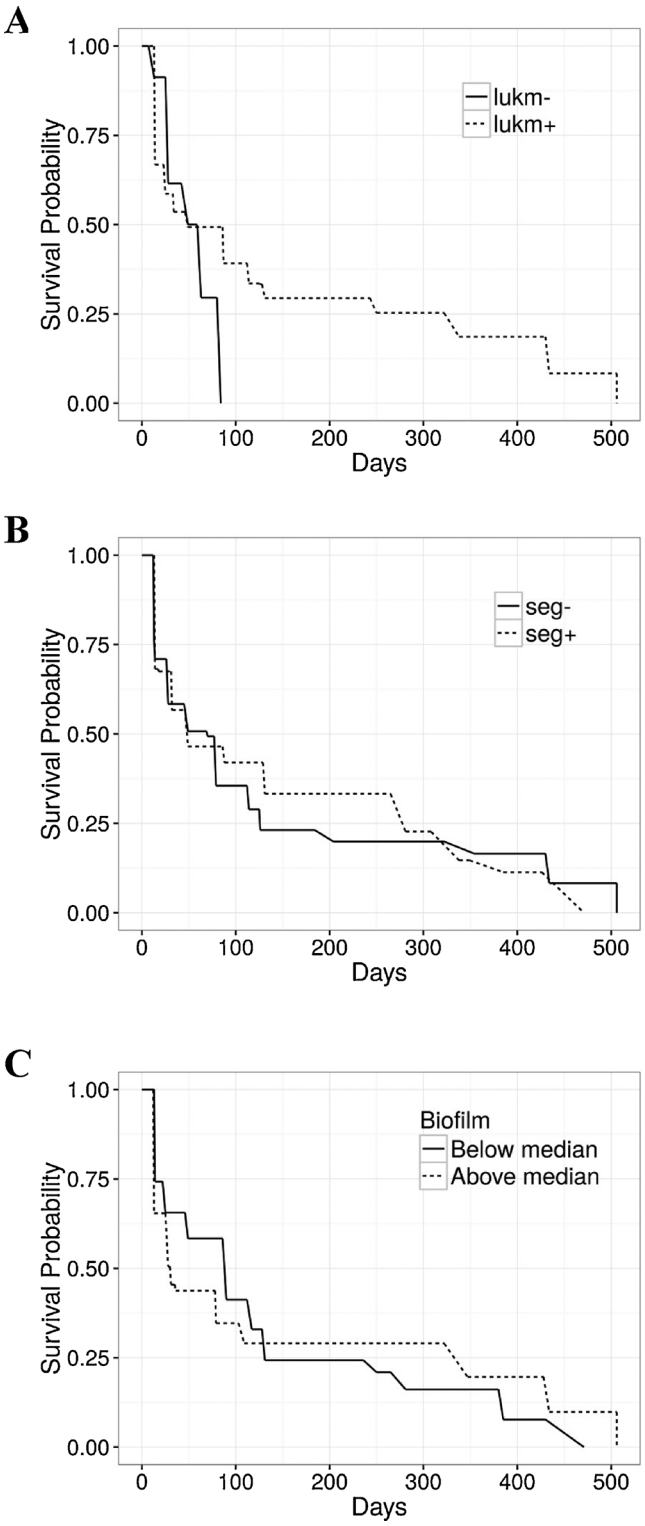

Fig. 2. Kaplan-Meier plots of the survival probability and duration of IMIs (days) for (A) the presence or absence of the *lukM gene*, (B) the presence or absence of *seg gene*, and (C) the *S. aureus* strains which produce more biofilm than the overall median and the strains that produce less biofilm than the median.

accompanying veterinarian with guidance for their decision to treat or not the *S. aureus* infection or to cull the infected animal. The objective of this study was to identify genotypic and phenotypic characteristics of *S. aureus* strains that could be associated to clinical or subclinical mastitis or that could be used to predict the duration of *S. aureus* IMIs.

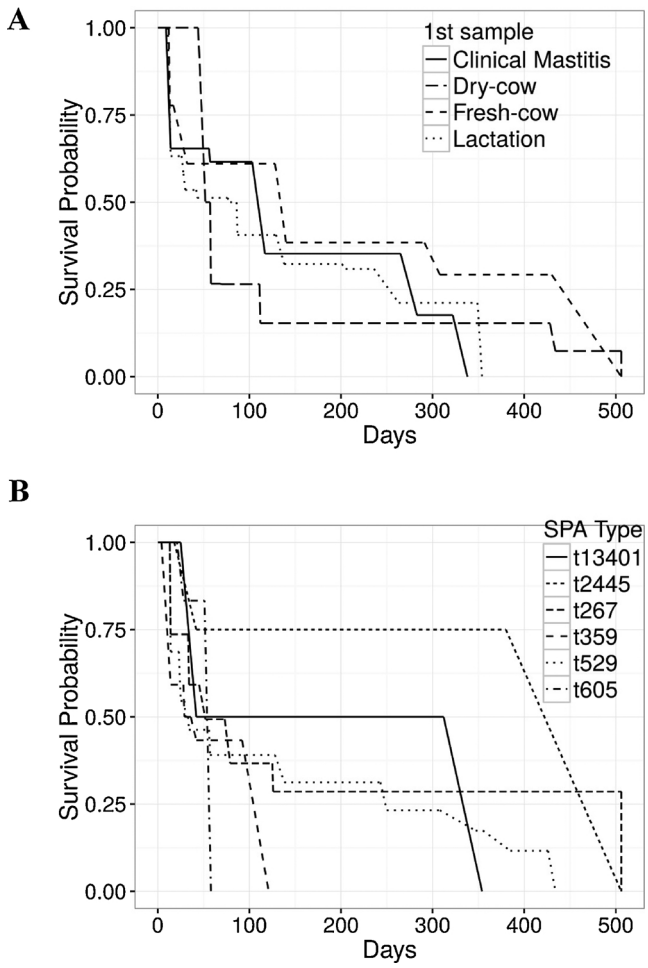

Fig. 3. Kaplan-Meier plots of the survival probability and duration of *S. aureus* IMIs (days) for (A) the types of samples (lactation-subclinical, lactation-clinical, dry-cow, and fresh-cow samples), and (B) the main *spa* types.

Such a knowledge would certainly help case management decisions.

The *spa* typing method is frequently used to characterize *S. aureus* isolates. Studies compared this technique to other typing methods like pulse field gel electrophoresis (PFGE) and found that *spa* typing was as good as such a technique to discriminate *S. aureus* strains of different clonal origins (Rodriguez et al., 2015). The *spa* types found in the present study were similar to those found in other studies conducted on Canadian bovine IMI strains (Said et al., 2010; Veh et al., 2015). The three main *spa* types encountered in this work (t529, t267 and t359) were also recovered from other IMI cases around the world (Bar-Gal et al., 2015; Cremonesi et al., 2015). The present work associated some genotypic and phenotypic features to specific *spa* types, like the high percentage of *seg* positive strains among t529. The ability to produce biofilms *in vitro* was also different between *spa* types. We however noticed several non typable strains in this study. An alternative typing method could have been the use of a 14-loci VNTR typing approach (Pourcel et al., 2009), which could have perhaps segregate some of the non typable strains. In addition, PCR amplification of the 16S–23S rRNA intergenic spacer was used successfully to identify *S. aureus* strains that are highly contagious (*e.g.*, genotype B) as detected in some countries in Europe (Cosandey et al., 2016).

Biofilm production gives the ability to bacteria to resist against a multitude of hazards. It gives protection against antibiotics that have difficulty to reach the inner layer of the biofilm (Davies, 2003). The inner layer is also populated with bacteria with a slower metabolism that are less susceptible to antibiotics (Huang et al., 1995). The efficacy of the host immune system is also severely impaired by the presence of

biofilms (Domenech et al., 2013; Hernandez-Jimenez et al., 2013). In this study, the virulence gene *seg* was strongly associated with lower biofilm production. The same observation was made with the other virulence genes *tst* and *lukM*. Overall, the presence of these virulence genes is associated with a lower production of biofilm. On the other hand, we found in the logistic regression of the case-control study that isolates with a higher production of biofilm were more inclined to cause symptoms (clinical mastitis). The strains causing clinical mastitis had 1.48 more chance to persist (longer time to elimination from the quarter) than strains from the lactation series causing only subclinical mastitis. Whether or not biofilm increases the chance to provoke clinical symptoms by promoting persistency of *S. aureus* needs to be investigated further.

The leukocidin MF' is a leukocidin mostly present in bovine-associated strains of *S. aureus* (Hata et al., 2008; Haveri et al., 2007). Vrieling et al. (2015), demonstrated that the pore-forming toxin leukocidin MF' specifically targets the CCR1 receptor, which is abundant on the surface of bovine neutrophils, to promote efficient killing. When comparing *S. aureus* strains from clinical and subclinical mastitis cases, we found that the isolates from clinical samples have a higher percentage of *lukM* positive strains (94% vs 80%). Although this difference appears to be small, this finding is reinforced by the recent study of Hoekstra et al. (2018), which showed that isolates producing high levels of lukMF' were cultured significantly more frequently from clinical than subclinical mastitis cases. The presence of *lukM* alone does not provoke inflammation (Fromageau et al., 2011), but the presence of the leukocidin may help in the establishment of the infection. The *spa* types that more frequently do not have the *lukM* gene produce more biofilm. This may indicate that there is more than one efficient strategy for establishing an IMI, such as low biofilm producers may need the leukocidin to protect themselves from the immune system of the host, whereas high biofilm producers may succeed without the presence of such a virulence factor.

Several studies attempted to identify "the" critical bacterial factor leading to a clinical or subclinical manifestation of bovine IMIs (Le Marechal et al., 2011; Ote et al., 2011; Wolf et al., 2011). The present study utilized a broader approach by using quarter sample isolate series taken from fresh cows, through lactation and up to the dry period. Hence, the characteristics of strains from quarter milk series with at least one clinical mastitis case were compared to strains from the quarter milk series without any case of clinical mastitis. Also, for the first time, a wide range of *S. aureus* strains and sample types was used in a time survival analysis in an attempt to associate the strains' characteristics to the duration of IMIs. However, the longitudinal study previously performed to investigate bovine IMIs among Canadian dairy farms (Reyher et al., 2011), and from which we retrieved the isolates and associated data, was not specifically designed to completely support our time survival analysis. For instance, we needed to reconstruct each quarter milk series in a chronological order to use them in a time survival analysis. This means that the time gaps between the isolates varied among the series that were compared and unexpected events may have happened during those inconstant time gaps. For example, between samples, the infection could have been cured and a new infection could have occurred with the same strain. The duration of infection or time to elimination may be falsely increased in those cases. Even with such restrictions, the time survival analysis gave us interesting insights into strains that are more prone to persist (longer time to elimination from the quarter) than others.

In a previous study done by our team, the presence of the gene coding for enterotoxin G (*seg*) in *S. aureus* strains causing subclinical mastitis were correlated to a lesser chance to persist by 66.0% during the lactation period (Veh et al., 2015). In that study, persistence was strickly evaluated over the lactation period (subclinical cases only) or over the dry-off period, which were generally shorter periods than those investigated in the present study. In the present study, the duration of infection could be set as a numerical value (numbers of days infected), included strains from both subclinical and clinical cases as well as from dry-cow and fresh-cow type samples and was observed over a longer period of time. By observing over a longer period of infection and by including clinical cases, the mere presence of the *seg* gene did not reduce the overall persistence of the *S. aureus* strains during lactation.

Time survival analyses performed according to the presence or absence of specific virulence genes, overall biofilm production or *spa* types showed no significant difference when these characteristics were considered by themselves. In fact, differences were only observed when the logistic regression combined all the variables in one statistical test. This indicates that to determine the duration of infection by a particular *S. aureus* strain, multiple variables must be considered. In the multivariable logistic regression model, strains from clinical-lactation type samples were more persistent than subclinical-lactation strains. Some studies showed that a higher SCC increases the chance of persistence of the infection (Pinzon-Sanchez and Ruegg, 2011). We also found this tendency, because persistent strains (>100 days within a quarter) were associated to samples having higher log SCC compared to strains that were eliminated (≤ 100 days). Another factor or variable that could have been examined is antibiotic resistance that could of course contribute to persistence when antibiotic treatment is involved. Indeed, in a recent study (Ster et al., 2017), we compared isolates that persisted after three different types of extended therapies and showed that a reduced antibiotic susceptibility may have a role in persistence. However, because this previous study showed that persistence could occur for most of the isolates despite antibiotic susceptibility, we decided not to determine the susceptibility profile of the 353 isolates used in the present study.

Interestingly, the logistic regression model showed that the *spa* types t529 and t359 were predicted to have more chance of being eliminated than the *spa* type t13401. Those two *spa* types possess more virulence genes and produce less biofilm than t13401. Other factors not quantified in this study may also influence the persistence of strains, but for these *spa* types a difference in survival could be found. These observations indicate that *spa* type could be suited to identify persistent strains but more work is needed to obtain more precise prognostic guidelines. For this purpose, a prospective study where cows would be sampled at the start of the first lactation, at regular intervals, and for more than one lactation would allow a better follow-up and thus a more accurate survival analysis.

In this study, *S. aureus* phenotypic and genotypic characteristics were evaluated to predict the duration of IMI. Duration of infection according to the *spa* types should be further investigated as a prognostic tool to help udder health management decision-making. However, the bovine host genetic and immune status, and the influence of the environment and of management practices are also factors that could help prediction. Barkema et al (2006) showed that cow and quarter level factors such as parity and the position of the quarter (front vs rear) can affect the outcome of *S. aureus* IMI. Management practices such as the duration of antibiotic treatment can also have an impact on the cure rate. Host factors and management practices were out of the scope of this study but will have to be considered as determining factors in future studies.

Acknowledgements

This research was supported by a contribution from the Dairy Research Cluster initiative [Dairy Farmers of Canada (Ottawa, ON, Canada), Agriculture and Agri-Food Canada (Ottawa, ON, Canada), and the Canadian Dairy Commission (Ottawa, ON, Canada)] through the Canadian Bovine Mastitis and Milk Quality Research Network (St-Hyacinthe, QC, Canada). This project was also partially funded by the Regroupement de recherche pour un lait de qualité optimale (Op + lait; St-Hyacinthe, QC, Canada) supported by the Fonds de Recherche du Québec-Nature et Technologies. S.P.-J. and M.C. obtained studentships from Op + lait, G.M. from the Natural Science and Engineering

Research Council (NSERC) of Canada, D.B.B. and E.D. from the NSERC CREATE program in milk quality. Finally, we thank Eric Frost and Sophie Michaud for access to the Bionumerics software and databases.

References

Anderson-Bergman, 2016. C, and the Eigen team for Eigen library. *icenReg*: Regression Models for Interval Censored Data. R Package Version 1.3.2.

Aires-de-Sousa, M., Boye, K., de Lencastre, H., Deplano, A., Enright, M.C., Etienne, J., Friedrich, A., Harmsen, D., Holmes, A., Huijsdens, X.W., Kearns, A.M., Mellmann, A., Meugnier, H., Rasheed, J.K., Spalburg, E., Strommenger, B., Struelens, M.J., Tenover, F.C., Thomas, J., Vogel, U., Westh, H., Xu, J., Witte, W., 2006. High interlaboratory reproducibility of DNA sequence-based typing of bacteria in a multicenter study. J. Clin. Microbiol. 44, 619–621.

Arciola, C.R., Campoccia, D., Ravaioli, S., Montanaro, L., 2015. Polysaccharide intercellular adhesin in biofilm: structural and regulatory aspects. Front. Cell. Infect. Microbiol. 5, 7.

Bar-Gal, G.K., Blum, S.E., Hadas, L., Ehricht, R., Monecke, S., Leitner, G., 2015. Host-specificity of *Staphylococcus aureus* causing intramammary infections in dairy animals assessed by genotyping and virulence genes. Vet. Microbiol. 176, 143–154.

Barkema, H.W., Schukken, Y.H., Zadoks, R.N., 2006. Invited review: the role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. J. Dairy Sci. 89, 1877–1895.

Cosandey, A., Boss, R., Luini, M., Artursson, K., Bardiau, M., Breitenwieser, F., Hehenberger, E., Lam, T., Mansfeld, M., Michel, A., Mosslacher, G., Naskova, J., Nelson, S., Podpecan, O., Raemy, A., Ryan, E., Salat, O., Zangerl, P., Steiner, A., Graber, H.U., 2016. *Staphylococcus aureus* genotype B and other genotypes isolated from cow milk in European countries. J. Dairy Sci. 99, 529–540.

Costerton, J.W., Stewart, P.S., Greenberg, E.P., 1999. Bacterial biofilms: a common cause of persistent infections. Science 284, 1318–1322.

Cremonesi, P., Pozzi, F., Raschetti, M., Bignoli, G., Capra, E., Graber, H.U., Vezzoli, F., Piccinini, R., Bertasi, B., Biffani, S., Castiglioni, B., Luini, M., 2015. Genomic characteristics of *Staphylococcus aureus* strains associated with high within-herd prevalence of intramammary infections in dairy cows. J. Dairy Sci. 98, 6828–6838.

Davies, D., 2003. Understanding biofilm resistance to antibacterial agents. Nat. Rev. Drug Discov. 2, 114–122.

Dinges, M.M., Orwin, P.M., Schlievert, P.M., 2000. Exotoxins of Staphylococcus aureus. Clin. Microbiol. Rev. 13, 16–34 table of contents.

Domenech, M., Ramos-Sevillano, E., Garcia, E., Moscoso, M., Yuste, J., 2013. Biofilm formation avoids complement immunity and phagocytosis of *Streptococcus pneumoniae*. Infect. Immun. 81, 2606–2615.

Fay, M.P., Shaw, P.A., 2010. Exact and asymptotic weighted logrank tests for interval censored data: the interval R package. J. Stat. Softw. 36.

Feil, E.J., Cooper, J.E., Grundmann, H., Robinson, D.A., Enright, M.C., Berendt, T., Peacock, S.J., Smith, J.M., Murphy, M., Spratt, B.G., Moore, C.E., Day, N.P., 2003. How clonal is *Staphylococcus aureus*? J. Bacteriol. 185, 3307–3316.

Fromageau, A., Cunha, P., Gilbert, F.B., Rainard, P., 2011. Purified *Staphylococcus aureus* leukotoxin LukM/F' does not trigger inflammation in the bovine mammary gland. Microb. Pathog. 51, 396–401.

Fromageau, A., Gilbert, F.B., Prevost, G., Rainard, P., 2010. Binding of the *Staphylococcus aureus* leucotoxin LukM to its leucocyte targets. Microb. Pathog. 49, 354–362.

Gentleman, R., Geyer, C.J., 1994. Maximum likelihood for interval censored data: consistency and computation. Biometrika 81, 618–623.

Halasa, T., Huijps, K., Osteras, O., Hogeveen, H., 2007. Economic effects of bovine mastitis and mastitis management: a review. Vet. Q. 29, 18–31.

Hata, E., Katsuda, K., Kobayashi, H., Nishimori, K., Uchida, I., Higashide, M., Ishikawa, E., Sasaki, T., Eguchi, M., 2008. Bacteriological characteristics of *Staphylococcus aureus* isolates from humans and bulk milk. J. Dairy Sci. 91, 564–569.

Haveri, M., Roslof, A., Rantala, L., Pyorala, S., 2007. Virulence genes of bovine *Staphylococcus aureus* from persistent and nonpersistent intramammary infections with different clinical characteristics. J. Appl. Microbiol. 103, 993–1000.

Hernandez-Jimenez, E., Del Campo, R., Toledano, V., Vallejo-Cremades, M.T., Munoz, A., Largo, C., Arnalich, F., Garcia-Rio, F., Cubillos-Zapata, C., Lopez-Collazo, E., 2013. Biofilm vs. Planktonic bacterial mode of growth: which do human macrophages prefer? Biochem. Biophys. Res. Commun. 441, 947–952.

Hoekstra, J., Rutten, V., Sommeling, L., van Werven, T., Spaninks, M., Duim, B., Benedictus, L., Koop, G., 2018. High Production of LukMF' in *Staphylococcus aureus* field strains is associated with clinical bovine mastitis. Toxins (Basel) 10.

Huang, C.T., Yu, F.P., McFeters, G.A., Stewart, P.S., 1995. Nonuniform spatial patterns of respiratory activity within biofilms during disinfection. Appl. Environ. Microbiol. 61, 2252–2256.

Huang, J., Wellner, J.A., 1997. Interval censored survival data: a review of recent progress. In: In: Lin, D.Y., Fleming, T.R. (Eds.), Proceedings of the First Seatle Symposium in Biostatistics Lecture Notes in Statistics, vol. 123. Springer, New York, NY, pp. 123–169.

Jacques, M., Aragon, V., Tremblay, Y.D., 2010. Biofilm formation in bacterial pathogens of veterinary importance. Anim. Health Res. Rev. 11, 97–121.

Jarraud, S., Mougel, C., Thioulouse, J., Lina, G., Meugnier, H., Forey, F., Nesme, X., Etienne, J., Vandenesch, F., 2002. Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. Infect. Immun. 70, 631–641.

Koreen, L., Ramaswamy, S.V., Graviss, E.A., Naidich, S., Musser, J.M., Kreiswirth, B.N., 2004. Spa typing method for discriminating among *Staphylococcus aureus* isolates: implications for use of a single marker to detect genetic micro- and macrovariation. J. Clin. Microbiol. 42, 792–799.

Le Marechal, C., Seyffert, N., Jardin, J., Hernandez, D., Jan, G., Rault, L., Azevedo, V., Francois, P., Schrenzel, J., van de Guchte, M., Even, S., Berkova, N., Thiery, R., Fitzgerald, J.R., Vautor, E., Le Loir, Y., 2011. Molecular basis of virulence in *Staphylococcus aureus* mastitis. PLoS One 6, e27354.

Mehrotra, M., Wang, G., Johnson, W.M., 2000. Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance. J. Clin. Microbiol. 38, 1032–1035.

Melchior, M.B., Vaarkamp, H., Fink-Gremmels, J., 2006. Biofilms: a role in recurrent mastitis infections? Vet. J. 171, 398–407.

Novick, R.P., 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol. Microbiol. 48, 1429–1449.

Ote, I., Taminiau, B., Duprez, J.N., Dizier, I., Mainil, J.G., 2011. Genotypic characterization by polymerase chain reaction of *Staphylococcus aureus* isolates associated with bovine mastitis. Vet. Microbiol. 153, 285–292.

Otto, M., 2013. Staphylococcal infections: mechanisms of biofilm maturation and detachment as critical determinants of pathogenicity. Annu. Rev. Med. 64, 175–188.

Pinzon-Sanchez, C., Ruegg, P.L., 2011. Risk factors associated with short-term post-treatment outcomes of clinical mastitis. J. Dairy Sci. 94, 3397–3410.

Pourcel, C., Hormigos, K., Onteniente, L., Sakwinska, O., Deurenberg, R.H., Vergnaud, G., 2009. Improved multiple-locus variable-number tandem-repeat assay for *Staphylococcus aureus* genotyping, providing a highly informative technique together with strong phylogenetic value. J. Clin. Microbiol. 47, 3121–3128.

R Core Team, 2015. R: a Language and Environment for Statistical Computing. R Foundation for statistical Computing, Vienna, Austria.

Reyher, K.K., Dufour, S., Barkema, H.W., Des Coteaux, L., Devries, T.J., Dohoo, I.R., Keefe, G.P., Roy, J.P., Scholl, D.T., 2011. The National Cohort of Dairy Farms–a data collection platform for mastitis research in Canada. J. Dairy Sci. 94, 1616–1626.

Rodriguez, M., Hogan, P.G., Satola, S.W., Crispell, E., Wylie, T., Gao, H., Sodergren, E., Weinstock, G.M., Burnham, C.A., Fritz, S.A., 2015. Discriminatory indices of typing methods for epidemiologic analysis of contemporary *Staphylococcus aureus* strains. Medicine (Baltimore) 94, e1534.

Sabat, A., Krzyszton-Russjan, J., Strzalka, W., Filipek, R., Kosowska, K., Hryniewicz, W., Travis, J., Potempa, J., 2003. New method for typing *Staphylococcus aureus* strains: multiple-locus variable-number tandem repeat analysis of polymorphism and genetic relationships of clinical isolates. J. Clin. Microbiol. 41, 1801–1804.

Said, K.B., Ismail, J., Campbell, J., Mulvey, M.R., Bourgault, A.M., Messier, S., Zhao, X., 2010. Regional profiling for determination of genotype diversity of mastitis-specific *Staphylococcus aureus* lineage in Canada by use of clumping factor A, pulsed-field gel electrophoresis, and spa typing. J. Clin. Microbiol. 48, 375–386.

Ster, C., Lebeau, V., Leclerc, J., Fugere, A., Veh, K.A., Roy, J.P., Malouin, F., 2017. In vitro antibiotic susceptibility and biofilm production of *Staphylococcus aureus* isolates recovered from bovine intramammary infections that persisted or not following extended therapies with cephapirin, pirlimycin or ceftiofur. Vet. Res. 48, 56.

Sun, J., 1996. A non-parametric test for interval-censored failure time data with applicaiton to AIDS studies. Stat. Med. 15, 1387–1395.

Sutra, L., Poutrel, B., 1994. Virulence factors involved in the pathogenesis of bovine intramammary infections due to Staphylococcus aureus. J. Med. Microbiol. 40, 79–89.

Veh, K.A., Klein, R.C., Ster, C., Keefe, G., Lacasse, P., Scholl, D., Roy, J.P., Haine, D., Dufour, S., Talbot, B.G., Ribon, A.O., Malouin, F., 2015. Genotypic and phenotypic characterization of *Staphylococcus aureus* causing persistent and nonpersistent subclinical bovine intramammary infections during lactation or the dry period. J. Dairy Sci. 98, 155–168.

Vrieling, M., Koymans, K.J., Heesterbeek, D.A., Aerts, P.C., Rutten, V.P., de Haas, C.J., van Kessel, K.P., Koets, A.P., Nijland, R., van Strijp, J.A., 2015. Bovine *Staphylococcus aureus* secretes the leukocidin LukMF' to kill migrating neutrophils through CCR1. MBio 6, e00335.

Wolf, C., Kusch, H., Monecke, S., Albrecht, D., Holtfreter, S., von Eiff, C., Petzl, W., Rainard, P., Broker, B.M., Engelmann, S., 2011. Genomic and proteomic characterization of *Staphylococcus aureus* mastitis isolates of bovine origin. Proteomics 11, 2491–2502.

Zadoks, R.N., van Leeuwen, W.B., Kreft, D., Fox, L.K., Barkema, H.W., Schukken, Y.H., van Belkum, A., 2002. Comparison of *Staphylococcus aureus* isolates from bovine and human skin, milking equipment, and bovine milk by phage typing, pulsed-field gel electrophoresis, and binary typing. J. Clin. Microbiol. 40, 3894–3902.