Supplementary file

Risk factors of *S. aureus* intramammary infection in pre-calved dairy heifers under grazing conditions and molecular characterization of isolates from heifers and cows

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Detailed Materials & methods

Herd Information

Prevalence of *S. aureus* IMI at herd level was determined by taking composite milk samples of every lactating cow in each herd. Thereafter, dairy farms were visited at a three-month interval to obtain mammary secretion samples from pregnant dairy heifers, within 20 of expected calving day. Samples from the total number of pre-calved heifers within 20 days of expected calving day available at each visit were taken. In most cases, the total number of heifers that calved during the year was sampled. In those dairy farms where this was not possible due to the visit schedule established, at least 70% of the pre-calving heifers were sampled.

A questionnaire was developed to obtain information about the management practices carried out in each dairy farm that could be associated with the presence of *S. aureus* IMI in heifers at pre-calving. The purpose and importance of the survey was previously explained to the farmers, emphasizing that responses would be confidential, since the interest was not the experience or practices applied by any particular farmer but the frequency of events at the population level. A copy of the questionnaire is available below.

Sampling

Heifers were immobilized in a chute, secretion samples were taken aseptically and teats were dipped in a 0.5% iodophor solution after sample collection. Both lactating cows and heifer samples were collected by trained personnel participating in this study.

Bacteriological examination

Samples were mixed thoroughly and 10 µl were streaked onto half plate (for cow milk composite samples) or quarter plate (for heifer quarter secretion samples) of 5% calf blood agar using a sterile loop. Plates were incubated at 37°C for 24 h and for further 24 h in case no growth was observed.

A culture with more than two different bacterial species was regarded as contaminated and not considered for analysis. However, the presence of at least one colony of *S. aureus* on blood agar was considered as a positive identification even if another colony type was detected. Detection limit for *S. aureus* was 100 cfu/ml. Bacteria were stored at -80°C in trypticase soy broth (TSB) (Britania, Buenos Aires, Argentina) added with 15% glycerol until processed.

Molecular characterization of isolates

Selection criteria for herds were high *S. aureus* prevalence in lactating cows, different *S. aureus* prevalence in pre-calved heifers between herds, and different geographical location (online Supplementary Table 1).

Pulse field gel electrophoresis (PFGE): Similarity between PFGE types was evaluated with BioNumerics version 6.6 (Applied Maths, Belgium) using Dice coefficient. Dendrograms were generated using Unweighted Pair Group Method with Arithmetic Mean (UPGMA), (optimization value = 1.0, position tolerance = 2.0%).

Virulence genes amplification: PCR was performed in duplicate in a total volume of 20µl containing: 1x PCR buffer, 2mM $MgCl_2$, 0.2 mM dNTPs (Productos Bio-Lógicos, Buenos Aires, Argentina), 1U/µl Taq DNA polymerase (Fermentas, Thermo Fisher Scientific), 0.3 µM of each sense and antisense primer and 50 ng of genomic DNA. Amplification was carried out on Ivema T21 thermal cycler (Ivema, Argentina) using a program as follows: an initial 5-min denaturation step at 94°C, followed by 30 cycles of 30 s of denaturation at 94°C, 30 s of annealing at aT, and 1 min of extension at 72°C; with a final extension step

65 at 72°C for 5 min. Positive controls and blank reactions were included. PCR products were
66 analyzed by electrophoresis on GelRed (Biotium, USA)-stained 1% or 1.5% agarose gels
67 (Biodynamics, B.A., Argentina).
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HEIFER SURVEY

1. CALVING SYSTEM: a. Continuous b. Seasonal

Indicate calving season and month of highest expected calving number

2. Time calf spent with dam.

3. Time between calving and first milking.

4. Time of colostrum milking.

5. Calves rearing system: 2. Individually tethered b. hutches c. Collective

d. Other (specify)

6. Colostrum usage: a. directly to calves b. Freezing c. Other (specify)

7. Feeding of heifer calves: a. Milk b. Pasteurized milk c. Milk replacer

d. Other (specify).

8. Feeding times per day, volume administered and temperature.

9. Other feeds during rearing time (besides milk and milk replacer).

a. What feed?

b. When did administration of other feeds begin?

c. Weaning time

10. Time grazing started

11. Post-weaning

a. In the same dairy farm? YES NO

b. Do heifer calves have contact with animals from other dairy farms? YES NO

Observations: Provide details of breeding and rearing cycles if necessary.

12. Dams vaccination schedule.

13. Heifer calves vaccination schedule.

14. Heifer calves parasiticide administration schedule.

15. Do you apply fly control in the rearing facility?

16. There is an established control program for: a) Mastitis b) brucellosis

c) Tuberculosis.

17. Age (months) and weight at first insemination.

18. Type of insemination: a. natural b. artificial

19. Are there any records of clinical mastitis in heifers? YES NO

20. Percentage of clinical mastitis in heifers in first third of lactation (if recorded).

21. Origin of cow replacements. Own dairy farm external dairy farm

22. If external, please specify origin, age and time when they get in contact with calves of own dairy farm.

23. Number of heifers per year.

105 Table S1. Characteristics of dairy herds selected for molecular epidemiology studies.

	S. aureus heifers IMI prevalence	*S. aureus* lactating cows IMI prevalence	Province	Nº heifers isolates evaluated	Nº lactating cows isolates evaluated	Total Nº isolates evaluated
Herd 1 (H1):	32.3%	24.8%	Santa Fe	14	19	33
Herd 2 (H2):	3.5%	20%	Buenos Aires	5	18	23
Total Nº isolates evaluated				19	37	56

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Table S2. PCR primers and conditions for identification of *Staphylococcus aureus* genes.

Primer	Sequence (5′–3′)	Amplified fragment	Annealing temperature (aT, °C)	Amplicon size (bp)	Reference
Cap5k	F- GTCAAAGATTATGTGATGCTACTGAG R- ACTTCGAATATAAACTTGAATCAATGTTATACAG	*capsular type 5*	55	361	Verdier et al., 2007
Cap8k	F- GCCTTATGTTAGGTGATAAACC R- GGAAAAACACTATCATAGCAGG	*capsular type 8*	55	173	Verdier et al., 2007
α-tox	F- GCAGATTCTGATATTAAT R- ATTTGTCATTTCTTCTTT	*α-toxin*	50	879	Designed (Primer Blast Software)
β-tox	F- AAAGGAGTGATAATGATG R- CTATTTACTATAGGCTT	*β-toxin*	50	1008	Camussone et al., 2014
ClfA	F- GAAAATAGTGTTACGCAATCT R- CTCTGGAATTGGTTCAATTTC	*Clumping factor A*	51	1554	Camussone et al., 2014
ClfB	F-TGCAAGTGCAGATTCCGAAAAAAAC R-CCGTCGGTTGAGGTGTTTCATTTG	*Clumping factor B*	62	194	Klein et al., 2012.
FnBPA	F-CGACACAACCTCAAGACAATAGCGG R-CGTGGCTTACTTTCTGATGCCGTTC	*Fibronectin binding protein A*	62	133	Pereyra et al., 2016
FnBPB	F-ACGCTCAAGGCGACGGCAAAG R-ACCTTCTGCATGACCTTCTGCACCT	*Fibronectin binding protein b*	62	197	Pereyra et al., 2016
icaA	F-CTTGCTGGCGCAGTCAATAC R-CCAACATCCAACACATGGCA	*Intercellular adhesion A*	*55*	178	Pereyra et al., 2016
icaD	F-CGCTATATCGTGTGTCTTTTGGA R-TCGCGAAAATGCCCATAGTT	*Intercellular adhesion D*	*55*	*164*	Pereyra et al., 2016

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Klein RC, Fabres-Klein MH, Brito MA, Fietto LG, Ribon Ade O. 2012. *Staphylococcus aureus* of bovine origin: genetic diversity, prevalence and the expression of adhesin-encoding genes. Veterinary Microbiology 160, 183–188.

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Table S3. Lactating herd prevalence, prepartum heifer prevalence and prepartum heifer quarter prevalence for each herd included in the study

Herd	*S. aureus* heifers IMI prevalence	*S. aureus* heifers IMI quarter prevalence	*S. aureus* lactating cows IMI prevalence
1	32.3	11.29	24.8
2	3.5	1.04	20.0
3	15.3	5.56	28.8
4	40.5	14.19	31.3
5	0.0	0.00	12.9
6	14.5	5.25	40.0
7	14.0	5.26	46.0
8	34.4	10.94	7.0
9	6.1	2.87	8.0
10	21.6	6.08	3.6
11	2.7	0.68	8.6
12	12.0	3.52	20.3
13	8.1	2.03	0.0
14	2.8	0.70	10.7
15	12.9	5.00	6.3
16	10.7	2.68	6.9
17	14.0	5.00	14.9

Table S4. Distribution of explanatory variables selected (*P*<0.15) by univariate analysis (generalized linear model) for potential association with the prevalence of *S. aureus* in pre-calved heifers.

Variable	Level	Number of heifers sampled	Number of heifers positive to *S. aureus* (%)	OR (95%CI)	*P*-Value
S. aureus prevalence in cows	<0.15	788	68 (8.6)	1.72 (1.24-2.39)	**0.001**
	>0.15	686	96 (14.0)		
Time the calf stayed with their dam after birth	<24 h	509	40 (7.85)	1.73 (1.19-2.51)	**0.001**
	>24 h	965	124 (12.84)		
Time between birth and first milking	<24 h	1047	109 (10.41)		0.172
	>24 h	427	55 (12.88)		
Calves rearing system	Stake system	1293	134 (10.36)	1.72 (1.11-2.64)	**0.014**
	Collective system	181	30 (16.57)		
Calves reared in the farm	No	181	30 (16.57)	1.82 (1.18-2.81)	**0.007**
	Yes	1262	124 (9.82)		
Type of calf feeding	Milk replacer or pasteurized milk	288	27 (9.37)		0.392
	Milk from hospital herd	834	92 (11.03)		
	Milk	352	45 (12.78)		
Time calves were fed milk	30 to 45 days	103	13 (12.62)		0.584
	60 days	1122	108 (9.62)		

	80 days	37	3 (8.10)		
Post-weaning heifer calves reared in the farm	No	318	33 (10.37)		0.847
	Yes	1125	121 (10.75)		
Contact with other animals	No	1135	116 (10.22)		0.287
	Yes	308	38 (12.33)		
Fly control	No	1295	129 (9.96)		
	Yes	148	25 (16.89)	1.84 (1.15-2.93)	**0.011**
Weight at first service	450 Kg	142	17 (11.97)		0.216
	280 Kg	303	24 (7.92)		
	320-370 Kg	998	113 (11.32)		
Clinical mastitis percentage at first third of lactation in heifers	NS	1047	112 (10.69)		0.663
	10-14%	248	29 (11.69)		
	2-4%	148	13 (8.78)		
Origin of replacements	External	151	17 (11.25)		0.805
	From the own dairy farm	1292	137 (10.60)		

Bold numbers represent significant association