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Comparison of somatic cell counts and cycle threshold values in bovine milk samples with varying numbers of pathogens detected by real-time PCR

Vergleich von somatischen Zellgehalten und ct-Werten in bovinen Milchproben mit unterschiedlicher Anzahl durch real-time PCR nachgewiesener Erreger

Susanne Spittel, Martina Hoedemaker

Summary

In this field study, the results obtained with the commercial PathoProof™ Mastitis PCR Assay were analysed under consideration of the number of detected pathogens, the somatic cell count (SCC) and the PCR cycle threshold (ct) value. On four Northern German dairy farms, 681 quarter milk samples from 173 cows with clinically normal udders and secretions and with varying SCC in the preceding Dairy Herd Improvement (DHI) sampling were collected and subjected to PCR. PCR resulted in 70.6 % positive samples. The positive samples contained one (56.3 %), two (34.3 %) or three or more (9.4 %) pathogens. For the most frequently found pathogens *Corynebacterium (C.) bovis,* coagulase-negative staphylococci (CNS), *Streptococcus (S.) uberis, Staphylococcus (S.) aureus* and *Trueperella (T.) pyogenes/Peptoniphilus (P.) indolicus,* samples were divided into monodetections, detections with another pathogen and detections with ≥2 further pathogens. Within those classes, means of the natural logarithm of the somatic cell count, mean ct-values and frequencies of samples >100 000 cells/ml were compared. For *S. uberis,* all parameters differed significantly between the pathogen classes, indicating that the two-species-limit from bacterial culture standards could be applied to PCR in order to differentiate between inflammation and contamination. For the other pathogens this could not be demonstrated.

Keywords: mastitis, dairy cow, *Streptococcus uberis*

Zusammenfassung

In dieser Feldstudie wurden Ergebnisse des kommerziellen PathoProof™ Mastitis PCR Tests unter Berücksichtigung der Anzahl nachgewiesener Erreger, des somatischen Zellgehaltes und des PCR-Schwellenwertes (ct-Wert) analysiert. In vier norddeutschen Milchviehherden wurden 681 Viertelanfangsgemelksproben von 173 Kühen mit klinisch gesunden Eutern und Sekreten, die unterschiedliche Zellgehalte in der letzten Milchleistungskontrolle hatten, gesammelt und mittels PCR untersucht. Die PCR erzielte 70.6 % positive Proben. Die positiven Proben enthielten einen (56.3 %), zwei (34.3 %) oder drei und mehr (9.4 %) Erreger. Für die am häufigsten nachgewiesenen Erreger *Corynebacterium (C.) bovis,* koagulase-negative Staphylokokken (KNS), *Streptococcus (S.) uberis, Staphylococcus (S.) aureus* und *Trueperella (T.) pyogenes/ Peptoniphilus (P.) indolicus,* wurden die Proben in Monoinfektionen, Mischinfektionen mit einem weiteren Erreger und Nachweise mit ≥2 weiteren Erregern eingeteilt. Innerhalb dieser Klassen wurden Mittelwerte des natürlichen Logarithmus des somatischen Zellgehaltes, Mittelwerte der ct-Werte und die Häufigkeit von Proben >100 000 Zellen/ml verglichen. Für *S. uberis* zeigten sich signifikante Unterschiede bei allen Parametern, die darauf hinweisen, dass die zwei-Erreger-Grenze aus Standards der klassischen bakteriellen Untersuchung auch für die PCR angewendet werden kann, um zwischen Entzündung und Kontamination zu unterscheiden. Für die anderen Erreger konnte dieser Nachweis nicht erbracht werden.

Schlüsselwörter: Mastitis, Milchkuh, *Streptococcus uberis*

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Introduction

Prevention, control and treatment of bovine mastitis require correct identification of mastitis pathogens. Bacterial culture is the current standard for isolating mastitis pathogens (National Mastitis Council, 1999). With the knowledge of genomic sequences of mastitis pathogens, highly sensitive and specific tests such as real-time PCR have been developed, which in future might complement or even replace culture-based methods. Both methods mainly differ in their approach: Bacteriology obtains viable cultures, whereas PCR identifies bacterial DNA. Molecular biology methods are usually more expensive than culture, but provide high sensitivity, high specificity and shorter analysis duration (Viguier et al., 2009). The present challenge with DNA-based tests is the lack of interpretation criteria for the exclusion of contaminants (Pyörälä, 2012).

One of the first commercially available PCR-based tests, the PathoProof™ Mastitis PCR Assay, identifies eleven of the most common mastitis pathogens as well as the staphylococcal beta-lactamase resistance gene with an analytical sensitivity and specificity of 100 % (Koskinen et al., 2009). Additionally, the ct-value provides quantitative information on the genomic copies in the sample: The lower the ct-value, the higher the quantity of DNA in the sample. The PathoProof™ Mastitis PCR kit was compared with conventional culture in a first field study using milk samples from healthy as well as clinically and subclinically diseased udder quarters (Koskinen et al., 2010). Due to its high sensitivity, the PCR identified more positive samples and also more samples with two, three or more pathogens than bacterial culture. The authors pointed out that samples with more than two pathogens were considered contaminated in conventional bacteriology and that resampling should be recommended. They stressed the necessity for further studies to differentiate between relevant udder pathogens and contaminants in multiple species samples and emphasised the importance of additional information such as history of the cow, clinical symptoms and SCC (Koskinen et al., 2010).

The SCC is commonly used as a parameter for evaluating udder health. Intramammary infection, which leads to an inflammatory reaction with the recruitment of immune cells, influences SCC to a large extent (Harmon, 1994). Depending upon author, region and desired sensitivities and specificities, various cut-off values of the SCC, e. g. 100 000 cells/ml, 200 000 cells/ml or 500 000 cells/ml are used (Schukken et al., 2003). In German-speaking countries the current cut-off level is 100 000 cells/ml (DVG, 2002).

In this study it was hypothesised that PCR samples containing *S. uberis, S. aureus, C. bovis,* CNS or *T. pyogenes/P. indolicus* alone or in conjunction with one further pathogen (suspected infection samples) yield higher SCC and lower ct-values than samples with greater than or equal to two further pathogens (suspected contaminated samples).

Material and Methods

Dairy Herds

Four dairy farms in the region of Osnabrück (Lower Saxony, Germany) were selected for the collection of udder quarter milk samples from at least 40 cows per farm. Based on the current DHI records at the time of sampling, two farms had a bulk tank SCC below the German mandatory cell count limit of 400 000 cells/ml (Farm 1, Farm 4) and the other two farms had a higher somatic cell count (Farm 2, Farm 3) (see Tab. 1). Table 1 shows the geometric mean of the bulk tank SCC during the last twelve months before sampling, the average number of lactating cows during the previous twelve months and the herd milk yield per year. On all farms, cows were kept in free stalls with cubicles. All farmers milked in conventional milking parlours and performed post milking teat disinfection.

Animals

Subsequent to the final DHI sampling 40 cows per farm were selected for sampling according to their individual DHI SCC, in order to obtain an equal distribution among four SCC classes (>1 000 000 cells/ml, 400 000–999 000 cells/ml, 100 000–399 000 cells/ml and <100 000 cells/ml). The cows should be free from clinical mastitis. Farm 2 had a sufficient number of animals to fill all SCC classes. On the other farms, the percentage of cows with low SCC was slightly higher, or cows were sampled without a previous SCC result. In addition, attention was paid to sample cows at various stages of lactation and in different lactation numbers.

Milk samples

Sampling took place from July 2010-February 2011. Before milking, udder quarter samples were collected under aseptic conditions according to the guidelines of the German Veterinary Association (DVG, 2009). The sterile sampling tubes contained boric acid as preservative (Kabe Labortechnik GmbH, Nümbrecht, Germany), which is satisfactory for bacteriological analysis and somatic cell counting (FIL-IDF, 1981). Immediately after collection, the cooled milk samples were brought to the laboratory of the Weser-Ems DHI Association (Leer, Germany) for PCR analysis by means of PathoProof™ Mastitis Assay (Finnzymes-Thermo Fisher Scientific, Espoo, Finland) according to the manufacturer's guidelines. Somatic cell count was then determined by means of Fossomatic™ (Foss, Hillerod, Denmark). The number of animals sampled was 51, 41, 41

TABLE 1: *Characterisation of the four study herds regarding somatic cell counts (SCC), number of lactating cows and milk yield.*

	bulk tank SCC in current dairy herd improvement sampling (in cells/ml)	geometrical mean of bulk tank SCC in previous 12 months (in cells/ml)	average number of lactating cows in previous 12 months	herd milk yield in 2010 (in kg)
Farm 1	142 000	275 000	72	9 566
Farm 2	534 000	335 000	132	9 214
Farm 3	511 000	320 000	58	10 340
Farm 4	151 000	202 000	39	11 904

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and 40 on Farm 1–4, respectively. Ten cows had one atrophic udder quarter (Farm 1: n=3, Farm 2: n=3, Farm 3: n=2, Farm 4: n=2). One udder quarter sample broke. Therefore, a total of 681 udder quarters from 173 cows were analysed.

Statistical Analysis

The data were processed in Microsoft® Excel 2002 (Microsoft Corporation, Redmond, USA) and subjected to descriptive analysis using SAS® (Version 9.1.3) (SAS Institute Inc., Cary, USA). DATA steps calculated the natural logarithm of the quarter milk sample SCC (in 1000 cells/ml) (lnSCC) and classified samples according to their SCC in classes of ≤100 000 cells/ml and >100 000 cells/ml. Then, five different datasets were generated selecting samples PCR positive for *S. uberis, S. aureus, T. pyogenes/P. indolicus,* CNS or *C. bovis.* Within those pathogen datasets, samples were classified according to the number of all pathogens detected in the sample in classes of one pathogen (monodetection), two pathogens (mixed detection) and ≥3 pathogens. Means of lnSCC and ct-value were calculated, values expressed as mean ± standard error of the mean (SEM) and compared by the Kruskal Wallis test (PROC NPAR1WAY). In case of significant differences paired Wilcoxon post-hoc tests were performed to a Bonferroni corrected level of significance. Frequencies of samples ≤100 000 cells/ml and >100 000 cells/ml were compared using Fisher's exact test (PROC FREQ). The significance level was set at $p<0.05$ (Sachs, 2004).

Results

The proportion of PCR positive samples was 70.6 % (n=481). Among the positive samples the most frequently found pathogens were *C. bovis* (67.6 %), CNS (48.9 %), *S. aureus* (13.5 %), *T. pyogenes/P. indolicus* (10.2 %) and *S. uberis* (10.0 %). Less frequently detected pathogens were: *Streptococcus (S.) dysgalactiae* (n=10), *Escherichia (E.) coli* (n=6), *Enterococcus* spp. (n=4), *Klebsiella* spp. (n=2) and *Streptococcus (S.) agalactiae* (n=1). In the positive samples, 56.3 % (n=271), 34.3 % (n=165), 7.9 % (n=38), 1.0 % (n=5) and 0.4 % (n=2) contained one, two, three, four and five pathogens, respectively.

Table 2 shows the distribution of monodetections and mixed detections with another pathogen or with ≥2 further pathogens for different bacterial species. While *S. uberis* and *T. pyogenes/P. indolicus* were more common in the mixed samples with one or several further pathogens, CNS, *C. bovis* and *S. aureus* were mostly detected as monoculture or with only one further pathogen. With increasing amounts of pathogen findings per sample, the inflammatory response, i. e. the number of samples above 100 000 cells/ml, and/or the mean somatic cell counts decreased for *S. uberis,* increased for *S. aureus,* varied for CNS and did not show any differences for *T. pyogenes/P. indolicus* and *C. bovis* (Tab. 2). For *S. uberis,* the significantly different ($p<0.05$) mean lnSCC corresponded to 1 436 000 cells/ml (lnSCC: 7.27) for monodetections, 773 000 cells/ml (lnSCC: 6.65) for mixed detections with one further pathogen and to 146 000 cells/ml (lnSCC: 4.99) with several further pathogens. In 95 % of the mixed detections the second pathogen was CNS or *C. bovis.* In the ≥2 further pathogens samples those minor pathogens accounted for 85 % and 75 %, respectively (Tab. 3).

Table 3 shows the composition of mixed detections. The most frequently involved pathogens were *C. bovis* and CNS, followed by *T. pyogenes/P. indolicus* and *S. uberis.*

Whilst *C. bovis,* CNS, *T. pyogenes/P. indolicus* and *S. uberis* were found abundantly in cows from all four farms, *S. aureus* was not detected on Farm 2. Fifty-seven out of 65 PCR detections originated from 24 cows on Farm 4 with a reported history of *S. aureus* problems. The SCC in those samples ranged from 2000–2 823 000 cells/ml. Four samples came from four cows on Farm 1 (SCC 330 000–1 284 000 cells/ml) and a further four samples from four cows on Farm 3 (SCC 289 000–4 540 000 cells/ml).

TABLE 2: *Polymerase-chain reaction positive samples for various mastitis pathogens grouped by number of pathogen species present in the sample; number and percentage of samples with a somatic cell count (SCC) > 100 000 cells/ml, mean ± standard error of the mean (SEM) of natural logarithm of somatic cell count (lnSCC; SCC in 1000 cells/ml) and cycle threshold (ct) value.*

	number of further pathogens detected		*Streptococcus uberis* (n=48)	*Staphylococcus aureus* (n=65)	*Trueperella pyogenes/ Peptoniphilus indolicus* (n=49)	coagulase-negative staphylococci (n=235)	*Corynebacterium bovis* (n=325)
total	0	n	8	11	5	97	147
	1	n	20	46	15	102	140
	≥ 2	n	20	8	29	36	38
SCC >100 000 cells/ml	0	n	8	3	1	32	71
		%	100.0*	27.3	20.0	33.0[a]	48.3
	1	n	18	18	5	53	71
		%	90.0*	39.1	33.3	52.0[b]	50.7
	≥ 2	n	12	5	6	14	17
		%	60.0*	62.5	20.7	38.9[ab]	44.7
lnSCC	0	mean ± SEM	7.27[a] ± 0.19	3.39* ± 0.58	3.87 ± 1.14	4.03[a] ± 0.17	4.62 ± 0.10
	1	mean ± SEM	6.65[a] ± 0.33	4.63* ± 0.22	4.19 ± 0.49	4.85[b] ± 0.14	4.93 ± 0.13
	≥ 2	mean ± SEM	4.99[b] ± 0.39	5.35* ± 0.55	3.82 ± 0.25	4.23[ab] ± 0.26	4.53 ± 0.24
ct value	0	mean ± SEM	29.31* ± 1.18	32.26 ± 1.19	33.52 ± 0.88	33.84 ± 0.24	31.68 ± 0.19
	1	mean ± SEM	29.01* ± 0.83	32.45 ± 0.53	35.25 ± 0.47	33.57 ± 0.24	31.59 ± 0.20
	≥ 2	mean ± SEM	32.50* ± 0.93	29.69 ± 1.27	34.95 ± 0.38	33.07 ± 0.40	32.35 ± 0.39

[a,b] Within columns: Means and frequencies with different superscripts differ significantly ($p<0.05$). * Within columns: Global test obtained significant differences ($p<0.05$), but post-hoc test with Bonferroni correction did not.

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TABLE 3: *Composition of mixed detections of selected pathogens with Streptococcus (S.) uberis (UBE), Staphylococcus (S.) aureus (AUR), Trueperella (T.) pyogenes/Peptoniphilus (P.) indolicus (PYO), coagulase-negative staphylococci (CNS), Corynebacterium (C.) bovis (BOV), Enterococcus spp. (ENT), Escherichia coli (ECO), Klebsiella spp. (KLE), Streptococcus dysgalactiae (DYS), Streptococcus agalactiae (AGA).*

pathogen	number/species of further pathogens		UBE	AUR	PYO	CNS	BOV	ENT	ECO	KLE	DYS	AGA
S. uberis	1	n	(20)	1	0	12	7	0	0	0	0	0
		%	(100.0)	5.0		60.0	35.0					
	≥ 2	n	(20)	3	7	17	15	0	1	0	4	0
		%	(100.0)	15.0	35.0	85.0	75.0		5.0		20.0	
S. aureus	1	n	1	(46)	2	1	42	0	0	0	0	0
		%	2.2	(100.0)	4.3	2.2	91.3					
	≥ 2	n	3	(8)	4	0	7	1	0	0	0	1
		%	37.5	(100.0)	50.0		87.5	12.5				12.5
T. pyogenes/ P. indolicus	1	n	0	2	(15)	7	5	0	0	0	1	0
		%		13.3	(100.0)	46.7	33.3				6.7	
	≥ 2	n	7	4	(29)	24	24	0	1	1	3	0
		%	24.1	13.8	(100.0)	82.8	82.8		3.4	3.4	10.3	
coagulase-negative staphylococci	1	n	12	1	7	(102)	81	0	0	0	1	0
		%	11.8	1.0	6.9	(100.0)	79.4				1.0	
	≥ 2	n	17	0	24	(36)	30	1	3	0	6	0
		%	47.2		66.7	(100.0)	83.3	2.8	8.3		16.7	
C. bovis	1	n	7	42	5	81	(140)	2	2	1	0	0
		%	5.0	30.0	3.6	57.9	(100.0)	1.4	1.4	0.7		
	≥ 2	n	15	7	24	30	(38)	1	3	1	4	0
		%	39.5	18.4	63.2	78.9	(100.0)	2.6	7.9	2.6	10.5	

Discussion

In this study, 45 out of 481 PCR positive samples (9.4 %) contained more than two detected pathogens. In bacterial culture, differentiation of contamination and infection is a major concern when interpreting sample results. According to the National Mastitis Council (1999) samples containing three or more dissimilar colony types are regarded as contaminated and resampling should be advised. The German guidelines (DVG, 2009) consider the specific organism, the number of colonies and the somatic cell count for the interpretation of bacterial growth in milk samples. CNS and coliforms, for example, are not regarded as etiologically important when SCC is below 100 000 cells/ml and bacterial counts are low whereas findings of *S. aureus* would be significant (DVG, 2009). The aim of this study was to evaluate the suitability of the two-species-limit for the differentiation of infection and contamination in mastitis PCR results. The hypothesised indicators for infection were high lnSCC, high percentages of samples >100 000 cells/ml and low ct-values (high numbers of genomic copies in the sample). The opposite was expected for contamination.

As this study was designed as a field study, PCR was performed on a single sample with the risk of false-positive bacterial findings of contaminating environmental pathogens as well as of false-negative bacterial results of contagious pathogens with low level shedding from an infected udder. Furthermore, other factors than infection status, e. g. lactation number and lactation status, may influence SCC. Their effect on SCC in quarters without infections was absent or minor depending upon investigation (Sheldrake et al., 1983; Laevens et al., 1997) and therefore not taken into account in this study.

For *S. uberis* significant differences in the percentage of samples above the threshold of 100 000 cells/ml, the somatic cell counts and the ct-values between monodetections, mixed detections with another pathogen (the two suspected infection groups) and detections with ≥2 further pathogens (the suspected contamination group) could be demonstrated. The difference in somatic cell counts was the only measure to be confirmed by post-hoc tests to a Bonferroni corrected level of significance, indicating that samples with ≥2 further pathogens showed a clearly weaker or nearly absent inflammatory reaction compared to the other groups. In conjunction with the greater number of detected species it could be hypothesised that those samples with a mean lnSCC corresponding to 146 000 cells/ml were contaminated with environmental *S. uberis.* However, the lower SCC might also indicate early infection. For *S. uberis* which can be found on many extramammary sites on the cow and in the cow's environment, it is crucial to differentiate between infection and contamination. In bacteriology, significance of streptococci detections requires at least 1000 colony-forming units per millilitre and SCCs above 100 000 cells/ml (DVG, 2009). Therefore, culture standards for discrimination of *S. uberis* infection and contamination seem to be applicable for mastitis PCR, too. As the cows in this study were clinically normal, the authors classified the discovered *S. uberis* infections (mono- and mixed detections with another pathogen) as subclinical and/or chronic infections.

What appeared to be clear for *S. uberis,* could not be proven for the other mastitis pathogens in this study. For *S. aureus,* even an inverse tendency was obtained with the lnSCC. Whilst eight PCR positive samples for *S. aureus* on Farms 1 and 3 were connected with increased SCC suggesting an intramammary infection, the remaining 57 PCR results on Farm 4 complicated the interpretation of the *S. aureus* findings. As this farm had a history of *S. aureus* problems, the positive results could have been due either to latent *S. aureus* infections or to a contamination from the teat canal or the teat skin during sampling. In a Finnish study involving two farms with *S. aureus* problems, Haveri

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et al. (2008) found a higher susceptibility for intramammary infection when *S. aureus* was present around the teat orifice. Yet, it cannot be ruled out that extramammary sites such as the teat skin might be contaminated by intramammary infections (Haveri et al., 2008). Additionally, differences in genotype are discussed as having or not having an influence on somatic cell count and epidemiology (Zadoks et al., 2000; Middleton et al., 2002). In a recent study on the detection of *S. aureus* intramammary infection by PCR and culture, Cederlöf et al. (2012) claimed that neither culture nor PCR was a perfect test and that changes in ct-value cutoffs changed the underlying disease definition from a truly/heavily infected cow at lower PCR ct-values to a *S. aureus* positive cow at higher ct-value cutoffs. Cederlöf et al. (2012) also recommended interpreting ct-values with other information e. g. SCC.

In this study, mean lnSCC were lowest and mean ct-values were highest in all groups of samples with *T. pyogenes/P. indolicus.* The mean lnSCC values (in 1000 cells/ml) corresponded to 48 000 cells/ml (lnSCC 3.87), 66 000 cells/ml (lnSCC 4.19) and 46 000 cells/ml (lnSCC 3.82), which was inferior to the inflammatory limit of 100 000 cells/ml. The low bacterial load and the missing inflammatory reaction might suggest either contamination or a reservoir of *T. pyogenes/P. indolicus* close to the teat canal. In literature, there are hints that *T. pyogenes* and *P. indolicus* are natural cohabitants of healthy cattle (Sørensen, 1976). In case of teat injuries or drying off of otherwise infected udder quarters, the presence of *T. pyogenes* close to the teat canal might be a risk factor for ascending udder infections.

The minor pathogen *C. bovis* accounted for two thirds of the positive samples with PCR. For the three groups, mean lnSCC (in 1000 cells/ml) corresponded to 101 000 cells/ml (lnSCC 4.62), 139 000 cells/ml (lnSCC 4.93) and 93 000 cells/ml (lnSCC 4.53), respectively, indicating almost no inflammatory reaction. None of the tests revealed significant differences within the groups. Huxley et al. (2002) rated a high frequency of *C. bovis* as indicator for insufficient post-milking teat disinfection. A Portuguese study, comparing the results of bacterial culture from samples taken either with the conventional sampling method or via a teat cannula, showed that with the conventional method washouts of *C. bovis,* which colonises the teat canal without causing intramammary infection, are possible (Bexiga et al., 2011).

In this study, half of the PCR positive samples contained CNS which are considered as part of the physiological teat flora and opportunistic coloniser of the teat canal and the mammary gland (Huxley et al., 2002). The higher proportion of samples above 100 000 cells/ml and the higher lnSCC in the CNS plus another pathogen group might be due to an inflammatory reaction caused by *S. uberis* representing the second pathogen in 11.8 % of the mixed samples. In another study with a CNS udder quarter prevalence of 26 %, the authors did not consider the most frequently isolated CNS as important pathogens for bovine mastitis. Especially findings of *Staphylococcus haemolyticus* were rather associated with colonisation or contamination during sampling (Pate et al., 2012). On the other hand, Supré et al. (2011) found a species-specific effect of CNS on SCC: Depending on the CNS species, infected quarters had a SCC comparable to non-infected *(S. cohnii)* or to *S. aureus* infected quarters *(S. chromogenes, S. simulans, S. xylosus).* The positive CNS results with the PathoProof™ real-time PCR test were neither able to be assigned to a (CNS) species nor was a possible coexistence of various CNS species discovered.

The use of a two-species-limit according to bacterial culture standards showed differences in the inflammatory reaction (SCC) and bacterial loads (ct-value) for *S. uberis* positive samples suggesting an inflammation on the one hand and contamination on the other hand. However, for *S. aureus, T. pyogenes/P. indolicus,* CNS and *C. bovis* positive samples this could not be demonstrated. Whereas the importance of CNS, *C. bovis* and *T. pyogenes/P. indolicus* positive signals with PCR remains unclear but limited in a practical context, the many positive *S. aureus* signals represent a new challenge for mastitis control programmes involving grouping of animals.

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