Evaluation of the Surveillance Program of *Streptococcus agalactiae* in Danish Dairy Herds

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ABSTRACT

The aim of this study was to evaluate the Danish surveillance program of *Streptococcus agalactiae* in dairy herds with respect to 1) fluctuation over time of the presence of *S. agalactiae* in bulk tank milk, 2) sensitivity and specificity of the bacteriological method used, and 3) contamination of bulk tank milk samples with milk from other herds. From June to September 1996, bulk tank milk was sampled from 100 Danish dairy herds seven times, with intervals of 2 wk. The samples were examined for the presence of *S. agalactiae* by four different methods: 1) by the method approved for the program, 2) after ultrasonic treatment of the milk before examination, 3) after freezing down the milk before examination, and 4) after selective preparation of the milk. Selected strains of *S. agalactiae* were examined by restriction fragment length polymorphism of the gene encoding rRNA to discriminate between the isolates.

Streptococcus agalactiae was found in eight of 96 herds in which *S. agalactiae* had never previously been found during the surveillance program. *Streptococcus agalactiae* was not found in all seven sampling rounds in any of the eight herds. Comparing the approved method with supplemental findings by the other methods, the estimated sensitivity was (95% confidence limits): 0.786 (0.628; 0.892) and the estimated specificity (95% confidence limits): 0.995 (0.985; 0.999). Using all four methods on the same sample could increase the sensitivity, but by comparing the methods individually, there was no significant difference between any of them ($P > 0.10$).

In milk samples from three herds, the ribotype of *S. agalactiae* was the same as in milk from herds sampled just before; therefore, it could not be ruled out that cross-contamination could occur.

Taking into account that *S. agalactiae* in bulk tank milk reflects the presence of *S. agalactiae* in at least one udder quarter, this investigation gives further reason to assume that *S. agalactiae* can be seen sporadically in several herds. A surveillance program based on annual bulk tank milk sample examinations will only detect a limited number of *S. agalactiae* infected herds. If the overall aim is to identify herds where the infection is established, annual bulk tank milk sample examinations combined with the information of number of colonies of *S. agalactiae* in the sample will be sufficient. (**Key words:** *Streptococcus agalactiae*, surveillance program, sensitivity, specificity)

Abbreviation key: BTMS = bulk tank milk sample, QMS = quarter milk sample.

INTRODUCTION

In 1950, it was estimated that approximately 30 to 40% of the 184,000 milk-producing herds in Denmark were infected with *Streptococcus agalactiae* (Danish Veterinary Service, 1981). Therefore, in 1954 a nationwide surveillance program, based on bacteriological examination of milk can and bulk tank milk samples (BTMS), was initiated, hand in hand with an eradication program. The sampling from 1964 to 1995 was carried out with changing intervals from quarterly to every second year. From 1995 the BTMS were examined every year.

The eradication strategy was based on identifying infected cows by quarter milk samples (QMS) and subsequently treating or culling these cows, in combination with improving milking procedures, milking equipment, and hygiene measures to control spread of the infection within the herd (Danish Veterinary Service, 1992).

The eradication program was compulsory until 1988. After this time it became voluntary, but still with prohibition to sell cows and pregnant heifers from herds declared to be infected.

Results from the surveillance program from 1992 to 2000, in which bulk tank milk was sampled from all herds with an interval of 8 to 12 mo, identified 0.4 to

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1.1% of all Danish dairy herds as newly infected during each examination (Danish Dairy Board, 2000).

Many studies have discussed the importance of the human and the bovine reservoirs of *S. agalactiae* in relation to bovine mastitis (Van den Heever and Giesecke, 1980; Jensen, 1982; Finch et al., 1984; Denning et al., 1989; and Jensen and Aarestrup, 1996). The discussion is particularly interesting when the prevalence of herds infected with *S. agalactiae* is low and when the focus is to find sources of the new introduction of *S. agalactiae* to dairy cows.

Human strains of *S. agalactiae* normally do not ferment lactose, but the ability to ferment lactose can be achieved after 8 to 10 passages in substrate containing lactose (Jensen, 1985). The same change must be assumed to take place in the mammary gland due to the content of lactose in milk. Records of the surveillance program in Denmark from 1992 to 1994, in which herds were sampled every 8 mo, showed on average 1.3 to 11.9% of the isolated strains to be lactose negative, which could imply a significant influence of the human reservoir, e.g., by continuously causing infections of cows in the herd. Furthermore, the chance of detecting *S. agalactiae* in BTMS and QMS is mainly determined by the number of colony-forming units (cfu) in the milk and the amount of milk cultured. The same records from 1992 to 1994 showed only 1 cfu in 12.7 to 20.2% of BTMS with *S. agalactiae* (Andersen and Huda, 1995).

This gave reason to believe that *S. agalactiae* could be found in more herds if the milk was sampled with shorter intervals, and if different methods of preparing the milk sample before culturing were used. Finally, during the surveillance program, it has been presumed that bulk tank milk with a very high number of *S. agalactiae* could cause subsequent samples to be contaminated.

Therefore, this study was initiated to evaluate the Danish surveillance program of *S. agalactiae* in dairy herds with respect to, 1) fluctuation over time of the presence of *S. agalactiae* in bulk tank milk, 2) sensitivity and specificity of the used bacteriological method, and 3) contamination of BTMS with milk from previously sampled herds.

MATERIALS AND METHODS

Sampling Scheme

The use of BTMS in this study was approved by a board of farmers' representatives, and the public authorities gave a written exemption from restrictions if *S. agalactiae* was found in the herds during the study. The owners were therefore not initially informed about the project to avoid deliberate influence on the results. The study should mirror the reality of the surveillance

program, and it was decided to use BTMS collected routinely during each delivery of milk. Furthermore, this way of sampling had some obvious logistic and economic benefits compared to manual sampling.

BTMS from 100 dairy herds were collected seven times with a 2 wk interval during the period June to September 1996. The dairy herds delivered milk to the same dairy plant and, based on results from the national surveillance program, were chosen in a region representative of the national herd level of new infections with *S. agalactiae*.

The herds were located on 10 milk collection routes. The contents of the bulk tanks were from 126 to 7997 L; the median was 1184 L (25% lower 816, 75% upper 1902).

BTMS were sampled with the same procedure as under the national surveillance program (Danish Veterinary Service, 1992). During weighing-in the milk from the bulk tank to the lorry, the first 30 L of milk was routinely flushed through the milk hose and pipes in order to avoid contamination by milk residues from former sampling. Hereafter, 90 ml of milk was successively taken out by means of "Spentrup automatic sampling system" (Mark IV, Spentrup Machine works, Spentrup, Denmark) and stored in plastic test tubes.

The test tubes were marked for later identification and immediately stored on ice. Within 24 h the samples were transported to Steins Laboratory A/S (Hjaltesvej 8, 7500 Holstebro, Denmark) for examination.

Laboratory Examinations

Bacteriology on bulk tank milk. Each BTMS was cultured using the commonly used ("approved") culture technique according to the Danish legislation (Danish Veterinary Service, 1992). For primary culture, 120 μ l of milk was mixed with 9 ml of a streptococci-selective media (5% bovine blood agar (Blood agar base CM55, Oxoid, Hampshire, England) with 1% wt/vol esculin supplied with neomycin sulphate (Pharmacia & Upjohn, Copenhagen, Denmark) 0.212 μ g/ml agar, polymyxin B sulphate (Sigma-Aldrich, Vallensbaek Strand, Denmark) 0.11 μ g/ml agar, and sodium fusidate (Løevens Chemical Factory, Ballerup, Denmark) 0.25 μ g/ml agar, and *Staphylococcus aureus* β -toxin), plated into a petri dish, and incubated for 18 to 24 h at 37°C. The next day the agar plates and the remaining milk from the BTMS were transported to Steins Laboratory A/S, Ladelundvej 85, 6650 Broerup, Denmark, for examination and further analysis.

Colonies showing β -hemolytic activity were counted (1, 2, 3, 4, 5, 5 to 10, 11 to 20, and >21) on each plate. One β -hemolytic *S. agalactiae* suspected colony per plate was selected and cultured on 5% bovine blood

agar containing esculin 1% (wt/vol) for 24 h at 37°C. Following this, isolates being positive in CAMP-test on bovine blood agar (Barrow and Feltham, 1993) and positive in a Lancefield group B latex agglutination test (Streptex, Murex Biotech Ltd., Kent, England) were identified as *S. agalactiae*. Finally, all *S. agalactiae* strains were tested for their ability to ferment broth-based lactose (Barrow and Feltham, 1993).

The remaining milk from each BTMS was split into three aliquots of which each was subjected to one of the following three culture techniques:

- 1) Preculture ultrasonic treatment of milk followed by culture using the standard culture technique: The milk was treated with an ultrasonic unit Elma Transonic T 700/H (Elma-Hans Schmidbauer, Singen, Germany), 35 kHz for 20 min, according to the manufacturer's instructions, and plated as described above for the standard method.

- 2) Preculture freezing of milk followed by culture using the standard culture technique: The milk was stored at -20°C for 24 h, thawed at room temperature, and plated after the standard protocol.

- 3) Preculture incubation of milk added a selective inhibitory substance followed by culture using the standard culture technique: A 10-ml sample was added to 1.1 µg of polymyxin B sulphate (Sigma-Aldrich) and incubated at 37°C for 4 h prior to standard culture.

All *S. agalactiae* strains were stored at -80°C in 50% glycerol and kept within the strain collection of the laboratory.

Testing for Cross-Contamination

The milk collection routes, the order of the samples taken from each sampling round, as well as the order of samples and examinations at the laboratories, were recorded.

Cross-contamination could occur as a result of milk remnants in the Spentrup sampling equipment from a previously sampled herd infected with *S. agalactiae* or during handling at the laboratories.

All recordings were scrutinized together with the results from the bacteriological examination and 29 subsamples with *S. agalactiae* were selected from the 15 herds. These strains of *S. agalactiae*, which could be suspected for cross-contamination, were sent to The Danish Veterinary Institute, Bülowsvej 27, 1790 Copenhagen V, Denmark, and examined by restriction fragment length polymorphism of the gene encoding rRNA to discriminate between the different isolates as described by Jensen and Aarestrup (1996).

Statistics

Sensitivity and specificity at the level of BTMS. The commonly used and "approved" laboratory exami-

nation was evaluated by calculating the sensitivity and specificity of the method (Table 1), and classification of the results was performed on the level of subsamples.

BTMS in which *S. agalactiae* was found by only the approved laboratory examination method were defined as "unconfirmed positive." Samples in which *S. agalactiae* was found by any but the approved laboratory examination method were defined as "false negative BTMS". The sensitivity was calculated as: Number of positive in the approved method and at least in one other method/Total number of positive by the approved and - or positive in at least one of the other methods ($a/(a+c)$ in Table 1). The specificity was calculated as Negative by all four methods/Total number of unconfirmed by the approved method, and negative in all four methods ($d/(b+d)$ in Table 1). The calculations were approximations due to lack of a proper golden standard.

Sensitivity and specificity at herd level. A herd was classified positive, if *S. agalactiae* was found by one of the four diagnostic methods during the sampling period (Table 1). Herds in which *S. agalactiae* was isolated by only the approved method were defined as "unconfirmed positive herds." Herds in which *S. agalactiae* was isolated by any of the three other methods in at least one or more examination rounds and not by the approved method were defined as "false negative herds." Sensitivity and specificity was calculated as described at the level of BTMS.

RESULTS AND DISCUSSION

Cross-Contamination of BTMS, and Bulk Tank Milk Subsamples

Ten different subtypes of *S. agalactiae* were found in subsamples from 15 herds. In three herds there was a marked coincidence between the same ribotype and a higher number of colony-forming units in the BTMS from herds just previously sampled in the same examination round. BTMS from these three herds were categorized as possibly cross-contaminated, and the number of herds suspected to be really infected was reduced to 12.

Ribotyping is considered to have a higher discrimination index than biochemical subtyping, and has been used earlier for the discrimination of strains of *S. agalactiae* (Denning et al., 1989; Jensen and Aarestrup, 1996). Together with comparison of the number of cfu/120 µl from the suspected contamination source herds (Table 2), it could be assumed that *S. agalactiae* from herds with a high number of *S. agalactiae* in bulk tank milk can be transferred to samples from following herds. This, in spite of flushing the sampling equipment with 30 L of milk before sampling, is initiated. The number of colony-forming units in the contaminated

Table 1. Classification of the diagnostic results for the approved laboratory examination compared to the combined information from ultra wave, freezing down and enrichment.

Method	True positive samples or herds	True negative samples or herds	Total
Positive approved method	a-Positive by approved method and at least in one other method	b-Positive by approved method but negative in the other three methods (unconfirmed positive)	Total positive by approved method
Negative approved method	c-Negative by approved method but positive in one of the other methods (false negative)	d-Negative by all four methods	Total negative by approved method
Total	(a + c)-Total number of positive by the approved method and/or positive in at least one of the other methods	(b + d)-Total number of unconfirmed by the approved method and negative in all four methods	Total number of samples or herds

sample seems to be low, and by using only the approved method, only one of the four cross-contaminated samples would have been detected positive (Table 2).

When connecting the hose to the outlet of a full bulk tank, air and about 2 L of milk remnants from the previous bulk tank was seen bubbling into the milk. If *S. agalactiae* were present in the remnants, it would be introduced to the bulk tank milk. In herds with smaller tanks, a suction pipe was used without being cleaned between herds. During the sampling, milk was seen splashing over the test tube, which in itself could cause contamination of samples, test tubes, and so on. BTMS with *S. agalactiae* were found on six of the 10 routes covering 60 herds. On the remaining four routes covering 40 herds, *S. agalactiae* was not found. The role of the lorry in a possible transmission of *S. agalactiae* from herd to herd is relevant to consider.

Findings at the Level of BTMS

Seven hundred examinations of the BTMS by each of the four methods were performed during the study. The findings of *S. agalactiae* in BTMS are shown in Table 3. Three BTMS were positive in the subsample examined by the approved method but negative in the

corresponding subsamples examined by the other three diagnostic methods.

These unconfirmed positive BTMS were from three different herds. The number of cfu/120 μ l found was one, two and four, respectively.

A total of nine subsamples were negative by the approved method but positive in at least one of the other three methods (Table 3). The number of cfu/120 l counted in these samples were ten or higher in two cases and below five in the remaining seven cases.

Studies made by Villanueva et al. (1991) on QMS, have shown that freezing of the milk samples before culturing can increase the number of cfu/120 ml of *S. agalactiae* by 2.5 after splitting up the chain. Schukken et al. (1999) could not establish any similar increase. Murdough et al. (1996) demonstrated that the viability of *S. agalactiae* in QMS was not influenced by freezing. Ultrasound treatment was used in this study based on the same idea, but bulk tank milk is mechanically affected by both milking, pumping of the milk to the milk lorry, and in particular by the sampling procedure. This may cause the streptococci chains to be split up, even before special laboratory preparation. We found no significant increase in sensitivity by one method compared with other methods.

Table 2. Colony forming units per 120 μ l in suspected cross-contaminated samples and in bulk tank milk samples from source herds.

Possible cross-contaminated bulk tank milk sample						Bulk tank milk sample from herd sampled just before suspected cross-contaminated herd					
Laboratory method - cfu/120 μ l						Laboratory method - cfu/120 μ l					
Herd	Round	Appr.	Ultraw.	Enrich.	Freez.	Herd	Round	Appr.	Ultraw.	Enrich.	Freez.
A	1	0	10	5	4	D	1	10	5	2	5
A	6	0	0	0	1	D	6	10 – 20	10 – 20	10 – 20	10 – 20
B	3	0	1	0	0	E	3	>50	>50	20 – 30	20 – 30
C	4	1	1	0	1	E	4	20 – 30	20	8	5

Table 3. The test results from the 700 samples with each four applied diagnostic methods (cross-contaminated samples included).

	Approved	Ultrawave	Freezing	Enrichment	Positive in all four methods	Positive in at least one method
Positive in this method and at least one other method	33	34	28	32		
Only positive in this method	3	1	0	4		
Total number of positive by the method	36	35	28	36	22	45

Improving the sensitivity of a surveillance program when a small number of bacteria are excreted normally requires improvement of the method or repeated examinations (Martin et al., 1987; Noordhuizen, 1997). The results from the surveillance program from 1992 to 2000 showed an annual new infection rate from 0.4 to 1.6% of all Danish dairy herds (Danish Dairy Board, 2000). The high percentage of herds pointed out as infected during the project must be attributed to both the repeated examinations and the use of supplementary methods, but the repeated examinations could also reflect the *dynamics* of the infection in the herds more than just a matter of diagnostic sensitivity.

Calculation of the sensitivity of testing for *S. agalactiae* is quite difficult because of the lack of a golden standard. The golden standard used for evaluating the approved method was defined and based on the test results from the other three applied methods. Based on this approximation, the sensitivity of the approved method was quite low, 0.786 at sample level and 0.800 at herd level (Table 4).

Findings at Herd Level

During the study period, a total of 15 herds were classified positive for *S. agalactiae* (Table 4). Four of these herds were known as infected with *S. agalactiae* at the beginning of the project and three were excluded due to suspected cross-contamination. Of the remaining

eight herds *S. agalactiae* was found in the bulk tank milk in one of the seven sampling rounds in five herds, in two of the seven sampling rounds in one herd, in three of the seven sampling rounds in one herd, and in four of the seven sampling rounds in one herd (Figure 1).

Two herds were found positive in one BTMS, respectively, with only one and two cfu/120 μ l. In three herds, *S. agalactiae* was not detected by the approved method. The four herds known as infected at the beginning of the project have been infected in 1 (two herds), 5 and 7 yr, respectively. Generally, no distinct pattern with regard to the number of cfu/120 μ l in the sample were identified, but the known infected herds (herds 1, 2, and 3 in Figure 1) tended to have consistently more cfu/120 μ l than the other herds. Therefore, it is reasonable to assume that persistently infected herds have several cows excreting *S. agalactiae* and that the bovine reservoir is well established.

Based on the results, it is most likely that infected cows periodically shed *S. agalactiae*, and/or are continuously newly infected and self-cured or cured by treatment. Cows infected with human strains, were demonstrated to have a higher self-cure rate than cows infected with bovine strains (Jensen, 1982). The dynamics of the infection could be attributed to a continuous infection by the human strains or a reservoir outside the udder.

Table 4. Classification of the diagnostic results for the approved laboratory examination compared to the combined information from ultrawave, freezing down, and enrichment. *Bulk tank milk sample evaluation* (cross-contaminated samples included) (n = 700). *Herd evaluation* (cross-contaminated samples excluded) (n = 100).

Method	True positive methods/herds	True negative methods/herds	Total methods/herds
Positive in the approved method	33/12	3 ¹ /2 ³	36/14
Negative in the approved method	9 ² /3 ⁴	655/83	664/86
Total	42/15	658/85	700/100

¹Unconfirmed positive samples.

²False negative samples. Estimated sensitivity (95% confidence limits): 0.786 (0.628 ; 0.892). Estimated specificity (95% confidence limits): 0.995 (0.985 ; 0.999).

³Unconfirmed positive herds.

⁴False negative herds. Estimated herd sensitivity (95% confidence limits): 0.800 (0.514 ; 0.947). Estimated herd specificity (95% confidence limits): 0.976 (0.910 ; 0.996).

Figure 1. Dairy herds in which *Streptococcus agalactiae* were isolated in the bulk tank milk during seven examination rounds with 2-wk intervals. Numbers in columns refers to herd number. 100 herds examined in each round.

In herds 10 and 11 (Figure 1), lactose negative isolates of *S. agalactiae* were found. Samples from these two herds were only found positive for *S. agalactiae* by one examination round. In herd 4 both isolates fermenting lactose and isolates not fermenting lactose were found, indicating both established infection in the udder and newly introduced infection by a human source. During the surveillance program from 1992 to 1994, with nationwide bulk tank milk examinations every eighth month, 6.7% (1.3 to 11.9%) of the isolated strains of *S. agalactiae* were not fermenting lactose (Andersen and Huda, 1995). It must be reasonable to assume that these strains have origin in the human reservoir.

Streptococcus agalactiae does not multiply within the milking system and bulk tank at temperatures below 27°C (Gonzalez, 1986). Therefore, if no contamination from human sources has occurred, cows might have been infected with *S. agalactiae* from the human reservoir within the past 2 wk (Jensen, 1985). Self-cure rates for infections caused by strains not fermenting lactose are, based on results of experimental infections, assumed to be high, and the difference between the bovine and human strains with respect to numbers of bacteria excreted per milliliter of milk are significantly lower by human strains (Jensen, 1982). This could be of substantial influence on the dynamics of the infection.

In only one herd of the eight herds categorized as newly infected, *S. agalactiae* was detected in all bulk tank milk examination rounds. The farmers were not

informed of the project and had no reason to act against the infection.

To run an effective eradication program such as the Danish national *S. agalactiae* program, it is essential to classify the status of the herds correctly. This study shows that a limited number of herds are incorrectly classified (false negative). These herds pose a risk for horizontal spread of the bacteria to noninfected herds by either animal movements or fomite transmission. Extrapolating from this study approximately 1% of all the samples in the Danish National *S. agalactiae* Program are false negative.

In Denmark the herds are submitted to restrictions when *S. agalactiae* are found in QMS or in bulk tank milk on two subsequent BTMS, as a consequence, farmers must be interested in a high specificity of the method. In relation to the surveillance program, the estimated specificity at herd level must be considered as acceptable, 0.995 (0.985; 0.999).

Each examination round can be considered as one result by the annual national surveillance program. Therefore, it is reasonable to believe that further examinations with 2-wk intervals will cause detection of even more herds infected with *S. agalactiae*. Furthermore, the infection will be permanently established in some herds and only transient in others.

CONCLUSION

In Denmark one can expect to find approximately 1% of herds newly infected with *S. agalactiae* by each

examination of bulk tank milk with 2-wk intervals. *Streptococcus agalactiae* can only be detected once in many of these herds, either because the infection does not establish in the herd, because the number of bacteria shed to the bulk tank milk is low, or because the shedding is fluctuating. Using several methods for examination on the same bulk tank milk sample will increase the number of findings of *S. agalactiae* in bulk tank milk. However, in order to identify all herds that are newly infected with *S. agalactiae* or all herds where a latent or persistent infection is flaring up, it is necessary to examine bulk tank milk from all herds with a frequency beyond what is realistic.

Cross-contamination of the bulk tank milk sample can occur during sampling due to milk residues from formerly sampled and infected herds in the sampling equipment. If the overall aim is to identify herds where the infection is established, annual bulk tank milk sample examinations combined with the information of number of colonies of *S. agalactiae* in the sample will be sufficient.

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