Evaluation of an O antigen Enzyme-Linked Immunosorbent Assay for Screening of Milk Samples for *Salmonella dublin* Infection in Dairy Herds

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ABSTRACT

Levels of antibodies to the O antigens (O:1,9,12) of *Salmonella dublin* were tested in 1355 serum, 1143 cow milk and 160 bulk milk samples from dairy herds using an enzyme-linked immunosorbent assay (ELISA). In order to define the background reaction, milk samples from all lactating cows and serum samples from 9 animals were collected in each of 20 salmonellosis-free herds located on the island of Bornholm, where cattle salmonellosis has not been reported. Similar samples were collected from all stalled animals in 10 herds with recent (<6 months) outbreaks of salmonellosis located in Jutland, where salmonella infection is enzootic. Using herd history of salmonellosis, herd location and clinical status of the herds as criteria, the optimal cutoff in the milk ELISA was determined as being at least 5% of the samples having optical density >0.5, resulting in herd sensitivity of 1.0 and herd specificity of 0.95. While none of the sera in the herds from Bornholm was ELISA positive, 2 herds had a few reactors in the milk ELISA. Using the same cutoff, all but 1 bulk milk sample from 150 herds on Bornholm was ELISA-negative, and all 10 salmonellosis-positive herds from Jutland were ELISA-positive. A significant correlation was found between ELISA reactions in milk and in serum of cows (34% and 32% respectively, r_s = 0.69, $P < 0.001$). The results indicate the possibility of applying cow and bulk milk samples to screening and, eventually, regular certification of herds.

RÉSUMÉ

Les niveaux d'anticorps dirigés contre l'antigène O de *Salmonella dublin* (O:1, 9, 12) à partir de 1355 échantillons de sérum, 1143 échantillons de lait provenant de vaches et 160 échantillons de lait provenant de réservoirs de troupeaux laitiers ont été déterminés à l'aide d'une épreuve immuno-enzymatique (ELISA). À partir de 20 troupeaux déclarés exempt de salmonellose sur l'île de Bornholm, un lieu où la salmonellose bovine n'est pas rapportée, les niveaux de base des réactions ont été établis dans des échantillons de lait de toutes les vaches en lactation et du sérum de 9 animaux de chacun des troupeaux. Une série d'échantillons similaires ont été prélevés dans 10 troupeaux ayant eu un épisode récent (moins de 6 mois) de salmonellose, sur l'île de Jutland où la salmonellose est enzootique. À l'aide de critères tels que les antécédents du troupeau en rapport avec la salmonellose, la localisation du troupeau et l'état clinique du troupeau, une valeur limite optimale pour le test ELISA sur les échantillons de lait a été établie comme étant celle où au moins 5 % des échantilllons ont une valeur de densité optique >0.5, donnant une sensibilité de troupeau de 1.0 et une spécificité de troupeau de 0.95. Aucun des échantillons de sérum en provenance des animaux de l'île de Bornholm n'était positif au test ELISA, et deux troupeaux avaient quelques réacteurs au test ELISA effectué avec le lait. En utilisant la même valeur seuil, tous les échantillons de réservoir de lait sauf un provenant de 150 troupeaux sur l'île de Bornholm étaient négatifs, et les 10 troupeaux positifs de l'île de Jutland étaient positifs. Une corrélation significative existait entre les résultats de l'ELISA à partir du lait et du sérum des animaux (r^2 = 0.69, $P < 0.001$). Il semble donc possible d'utiliser des échantillons de lait, du réservoir ou de chaque animal, comme spécimen pour effectuer des épreuves de tamisage et éventuellement de certification pour la salmonellose. (*Traduit par Dr Serge Messier*)

INTRODUCTION

Salmonella dublin, a host-adapted serotype causing widespread infections in cattle herds (1), is the major salmonella serotype isolated in Danish dairy herds (2). Infections with *S. dublin* are not only economically important in the dairy industry, but pose a serious human health hazard, as salmonellosis is one of the most prevalent zoonotic diseases (3). The bacterium has a high propensity for persisting in latent carriers and to survive for long periods in the animal and its environment (4,5). Although *S. dublin* predominantly causes disease in calves, cows are reported to constitute the majority of latent carriers (6). Systemic salmonella infections give rise to production of specific serum antibodies which can be detected in milk samples (7). Salmonellae may also locally colonize the mammary gland of cows and be shed in the milk (8–11), which can give rise to significant production of specific immunoglobulins in the

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udder (12). Testing milk for antibodies to the strongly immunogenic O antigen factors (13,14), may therefore facilitate the identification of salmonella-infected herds. Milk samples are easier and more economical to collect than blood samples, and application of milk samples in an enzyme-linked immunosorbent assay (ELISA) for detection of carrier cows has already been reported (7). However, to our knowledge, there is no report on a herd-based application of milk samples for the detection of antibodies to salmonella. The purpose of the present study was, therefore, to evaluate the ability of an antibody-specific, O antigen based ELISA to correctly identify *S. dublin* herd infection by screening milk samples. A similar approach based on serum samples has been earlier found possible (15). Furthermore, the serum and milk ELISA results were compared, and correlation of results of cow milk with bulk milk titer was investigated.

TABLE I. Within-run variations of OD values for control samples in the *S. dublin* milk ELISA[a]

Milk ELISA	Control samples	Plates (*n*)	Mean OD (*x*)	Standard deviation	Coefficient of variation (%)
IgG	Positive	30	1.25	0.18	14
	Weak positive	30	0.37	0.05	15
	Negative	30	0.04	0.008	18
IgA	Positive	5	1.59	0.18	11
	Weak positive	5	0.34	0.04	12
	Negative	5	0.03	0.006	23

[a]The mean of duplicate measurements of the control samples were used in the calculations

TABLE II. Herd specificity and herd sensitivity of *S. dublin* milk ELISA as a function of cut-off level

Salmonellosis status of herds	Cutoff (OD)	ELISA[a] positive herds (*n*)	Sensitivity[b]	Specificity[c]	DPR[d]
Salmonellosis-positive ($n = 10$)[e]	0.3	10	1.0	—	0.9
	0.5	10	1.0	—	0.95
	0.7	9	0.9	—	0.9
Salmonellosis-free ($n = 20$)[f]	0.3	2	—	0.90	
	0.5	1	—	0.95	
	0.7	0	—	1.0	

[a]Herds with at least 5% ELISA positive cows were defined as positive
[b]The proportion of salmonellosis-positive herds detected as ELISA positive above the cutoff OD indicated
[c]The proportion of salmonellosis-negative herds detected as ELISA negative below the cutoff OD indicated
[d]Differential positive rate calculates Sensitivity − (1 − Specificity)
[e]Herds from the *S. dublin* enzootic area of Jutland with clinic outbreak verified bacteriologically within the previous 6 mo
[f]Herds with no herd history of salmonellosis from the Island of Bornholm where there has been no reports of cattle salmonellosis

MATERIALS AND METHODS

STUDY POPULATION

Due to the low diagnostic sensitivity of salmonella culturing in subclinically infected herds (1,4), 2 cattle populations with different prevalences of salmonellosis were included in the study; herds from an island where the disease does not occur, and herds from areas with an endemic occurrence. These 2 populations were comparable in breed, housing and feeding regimen. The salmonellosis-free herds were not investigated bacteriologically for the presence of salmonellae.

SALMONELLOSIS-FREE HERDS (1–20)

Blood and milk samples were collected from 20 closed herds located on the island of Bornholm, where no cattle salmonellosis has been reported. In each herd, blood samples were collected from 9 animals, including 3 calves and two 1st, 2nd, and 3rd lactating cows, respectively. In addition, a pool of quarter milk samples was collected on 1 occasion from all lactating cows ($n = 582$) within 3 mo after collecting the serum samples ($n = 180$). The average number of cows per herd was 29 (range 13–50) and the average number of animals was 50. Within 3 mo after obtaining cow milk and serum samples, a bulk milk sample was collected on a single occasion from all milk-producing herds ($n = 150$), regardless of disease status, on the island of Bornholm.

SALMONELLOSIS-POSITIVE HERDS (21–30)

Blood samples were obtained from all stalled calves and cows (range 67–216), and milk samples were collected from all the lactating cows in 10 herds with recent (<6 mo) clinical outbreaks of *S. dublin* infection from areas of Jutland. The average number of animals per herd was 120. Bulk milk samples were also obtained on the same occasion. In these herds, several animals showed clinical signs of salmonellosis at the time of outbreak, clinical cases of salmonellosis were present in the herd during the time of sampling, and *S. dublin* was isolated from feces or organs at postmortem of at least one of the diseased animals by the serotyping section of the Danish Veterinary Laboratory. All the aforementioned conditions were present in all 10 herds. The time of outbreak in each herd was registered as the day the first clinical case was reported.

In 8 of these herds (21–28), cow milk and bulk milk samples were collected within 3 mo after obtaining the blood samples. These herds were used for testing the correlation between serum and milk ELISA on a herd basis when samples were not taken simultaneously, as may often be the case. In herds 29 and 30, the milk and blood samples were collected simultaneously in all 180 cows in order to compare the ELISA results for milk and serum ELISA in individual animals.

CULTURE METHOD

Fecal or organ samples collected at postmortem were incubated in selective enrichment medium of selenite broth, plated onto brilliant green agar, and serotyped as described (15).

SERA AND MILK

Sera were prepared from blood samples and kept at −20°C until use.

TABLE III. ELISA results for all cows in ten dairy herds with history of outbreak of salmonellosis from the *S. dublin* enzootic area of Jutland

Herd number	Sample[b] interval (days)	Calves[a]		Cows				Correlation[e] both tests		Bulk milk ELISA OD
		n	Serum[c] positive *n* (%)	*n*	Serum[c] positive *n* (%)	Milk[d] positive *n* (%)	Positive both tests *n* (%)	r_s	*P*	
21	85	85	53 (62)	49	19 (39)	18 (37)	14 (29)	0.62	<0.001	0.81
22	159	59	23 (39)	50	13 (26)	14 (28)	6 (12)	−0.05	NS	0.90
23	14	53	26 (49)	43	17 (40)	12 (28)	9 (20)	0.72	<0.001	1.50
24	42	33	12 (36)	34	26 (77)	27 (79)	25 (74)	0.86	<0.001	3.63
25	145	44	11 (25)	25	3 (12)	3 (12)	3 (12)	0.58	<0.005	0.60
26	70	91	41 (45)	52	10 (19)	12 (23)	6 (11)	0.50	<0.001	1.90
27	23	64	42 (65)	73	28 (38)	20 (27)	19 (26)	0.90	<0.001	1.40
28	90	26	1 (4)	55	11 (20)	12 (22)	8 (15)	0.49	<0.001	0.56
29[f]	145	64	8 (12)	59	19 (32)	29 (49)	18 (31)	0.85	<0.001	1.75
30[f]	92	95	21 (22)	121	32 (26)	45 (37)	28 (23)	0.82	<0.001	3.20
TOTAL		614	238 (39)	561	178 (32)	192 (34)	136 (24)[g]	0.69	<0.001	

[a]All stalled calves, heifers and bulls. Dry cows were not included in either categories
[b]Time of blood sampling after observing the clinical cases reported by the veterinarian
[c]ELISA OD of >0.3
[d]ELISA OD of >0.5
[e]Spearman value (r_s) and the significance level (*P*) for correlation of results between the two ELISAs in each animal on a herd basis
[f]Milk and serum samples were taken on the same occasion
[g]Kappa = 0.60, $P < 0.001$ for correlation of results between the two ELISAs calculated on the basis of total number of animals

Cow milk samples were pooled from udder, and bulk milk samples were collected in connection with a weekly quality control analysis. The samples were transported at 4°C, and on arrival, the milk samples were centrifuged at 2000 × *g* at 4°C. The fat layer was removed and the defatted samples were kept at −20°C without addition of any preservative.

PREPARATION OF ANTIGEN

The *S. dublin* strain (Div 800,83) was originally isolated at the Danish Veterinary Laboratory, Denmark, from a cow with clinical salmonellosis. The lipopolysaccharide (LPS) fraction was prepared using the phenol extraction method as described earlier (15).

SERUM ELISA

Serum samples were tested in an indirect ELISA as described previously (15). Briefly, microwell plates were coated with LPS, test sera were added in a dilution of 1:800 and the bound antibodies were detected using a horseradish peroxidase (HRP)-labelled rabbit antiserum to bovine immunoglobulin. A test setup was considered valid if the negative control serum had an optical density (OD) of 0.02 to 0.09, and the positive control serum had an OD of 2.5 to 3.5 (15).

MILK ELISA

The test condition was similar to the serum ELISA. Microwell plates (PolySorp, Cat. # 475094, Nunc, Denmark) were coated with 100 µL of a solution of *S. dublin* LPS (85 ng/well) in carbonate buffer and incubated overnight at 4°C. The plates were blocked by adding 200 µL of phosphate-buffered saline (PBS) containing 0.5 M NaCl, 0.1% Tween-20, and 4% polyvinylpyrrolidone-40T (PVP, Sigma Chemical Co., St. Louis, Missouri). Cow or bulk samples were added undiluted (100 µL in duplicate) and incubated at room temperature for 30 min on a plate shaker (1200 RPM). Positive, weak-positive, and negative control samples were also added undiluted in duplicate to the wells in column 11 in every plate. The positive reference samples were from cows with recent clinical salmonellosis, and the negative control milk was a bulk sample from a salmonellosis-free, healthy herd with no previous vaccination against the organism. The control samples were not from animals in the study herds. Column 12 was used as a blank with no samples. The plates were incubated for 1 h at room temperature and washed 5 times in the high salt PBS buffer. The secondary antibody was HRP-labelled, affinity-purified, goat antiserum to bovine IgG (Cat # 14-12-02, Kirkegaard & Perry Lab, Maryland) diluted 1:1000 in the PBS buffer containing 1% (W/V) skimmed milk powder, which was tested and found negative for antibodies to *S. dublin*. After addition of 100 µL of the labelled antibody, the plates were incubated for 1 h at room temperature and washed as before. Finally, the substrate (H_2O_2) and indicator (1,2-OrthoPhenyl-Diamine, OPD) were added, and after 15 min, the color development was stopped. A test setup was considered valid with a blank OD of <0.02, a negative control OD of 0.02 to 0.09, a weak positive OD of 0.3 to 0.4, and a positive control OD of 1.1 to 1.5 (Table I).

MILK IgA

The test setup and control samples was the same as the milk ELISA, with the exception of the secondary antibody which was HRP-labelled, affinity-purified, sheep antiserum to bovine IgA (Cat # A10-121P, Bethyl Lab, Texas).

POTENCY OF MILK ELISA

The titer of IgG antibodies to the LPS of *S. dublin* in milk samples from 5 high-reactant cows in herd 30 was measured in the milk ELISA. The test setup was the same as the milk ELISA but the samples were serially diluted in the negative control milk.

DETERMINATION OF CUTOFF

The cutoff value for the ELISA was evaluated and determined on a herd basis and not on the basis of individual animals in the herds. The herd sensitivity was defined as the ability of the ELISA to correctly identify the known diseased herds (ELISA-

positive herds/total diseased herds), and herd specificity was defined as the ability of the ELISA to correctly identify the salmonellosis-free herds (ELISA-negative herds/total nondiseased herds (16)). The cutoff value was defined as the level of antibody activity which determined negative or positive reactor status for a given herd. A herd was regarded milk ELISA positive if at least 5% of cows had OD values above the selected cutoff; i.e. herds with greater than 95% of the cows having OD values below the cutoff were negative within the 95% confidence limit.

In the calculation of the cutoff OD, an interval of 0.2 was used (Table II), since the OD interval of 0.1 was within the limit of test variation (Table I). The differential positive rate (DPR), which expresses the difference between the true positive ratio and the false positive ratio at various cutoff values, was used to obtain the cutoff value associated with the highest sensitivity and specificity (17).

STATISTICAL ANALYSIS

The correlation between the milk and serum ELISAs was evaluated using the Spearman rank correlation coefficient (r_s), and the level of significance (P) was calculated for a two-tailed test (18). The kappa test was also used for estimating the agreement between milk and serum ELISA positive test results in samples from the same animals in 10 herds (16). The Spearman test has the advantage of assigning a rank order to each OD value showing correlation in the intensity of reaction between milk and serum samples, whereas the kappa test treats the results as either positive or negative.

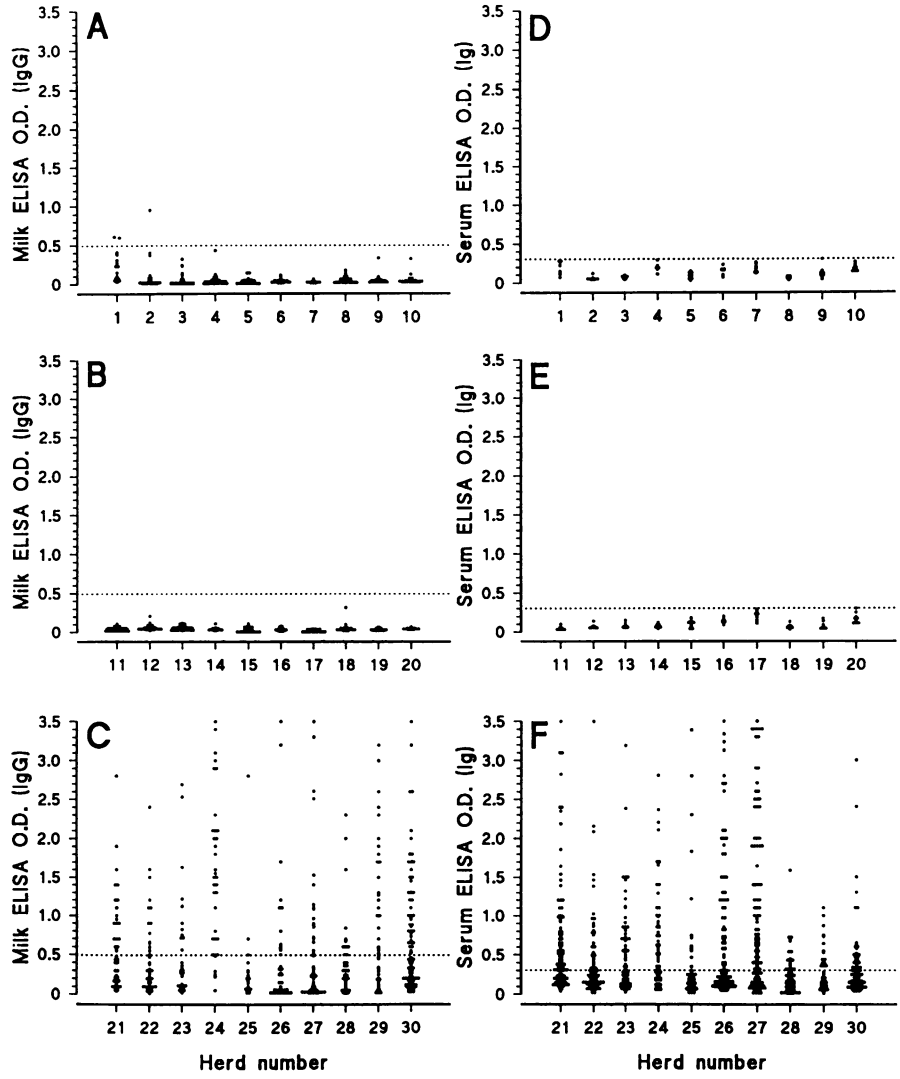

Fig. 1. Results of indirect ELISAs for detection of antibodies to LPS (O:1,9,12) of *S. dublin* in 30 cattle herds with following clinical status: A and B, cows in salmonellosis-negative herds (1–20) from Bornholm (island with no occurence of cattle salmonellosis) tested in milk ELISA; C, cows in salmonellosis-positive herds (21–30) from Jutland (area with endemic *S. dublin* infection) tested in milk ELISA; D and E, serum samples from 9 cows and calves in salmonellosis negative-herds (1–20) from Bornholm tested in serum ELISA; F, cows and calves in salmonellosis-positive herds from Jutland tested in serum ELISA. Each point represents the mean of 2 measurements in 1 animal, and the dotted lines show the optimal cutoff level of OD 0.3 in serum ELISA and OD of 0.5 in milk ELISA.

RESULTS

TEST PRECISION

In the milk ELISA, the within-run variation was assessed by determining the coefficient of variation (CV) for the control samples in 30 valid plates for IgG-ELISA and 5 valid plates for IgA-ELISA. The mean and standard deviations ($x \pm$ SD) and CV for the positive, weak-positive, and negative control samples are shown in Table I. Test samples with OD values close to the cutoff level (0.4 to 0.6), and duplicate results with CV > 15 were retested and the confirmed results are reported here.

MILK ELISA

A total of 1143 cow milk samples was tested. The number of salmonellosis-free herds being detected as positive in the milk ELISA decreased by increasing the OD value from 0.3 to 0.7 (Table II). The highest DPR was obtained at an OD of 0.5. At this cutoff level, there was one ELISA positive herd (no. 1, 11% with OD > 0.5) among the salmonellosis-free herds on Bornholm, and no ELISA negative herds among the salmonellosis-positive herds in Jutland (herd specificity 0.95 and herd sensitivity 1.0, Table II). In the salmonellosis-positive herds, the proportion of the milk ELISA positive cows ranged from 12% to 79%, with an average of 34% (Table III).

BULK MILK

As cow milk OD would create the basis of background reaction of the bulk milk OD, the cutoff OD of 0.5 for the cow milk samples was also used for the bulk samples. A total of 160 bulk samples was tested. At the selected cutoff OD, there was only one supposed false positive herd (no. 2) from Bornholm with OD of 2.1 (Fig. 2), while all 10 salmonellosis positive

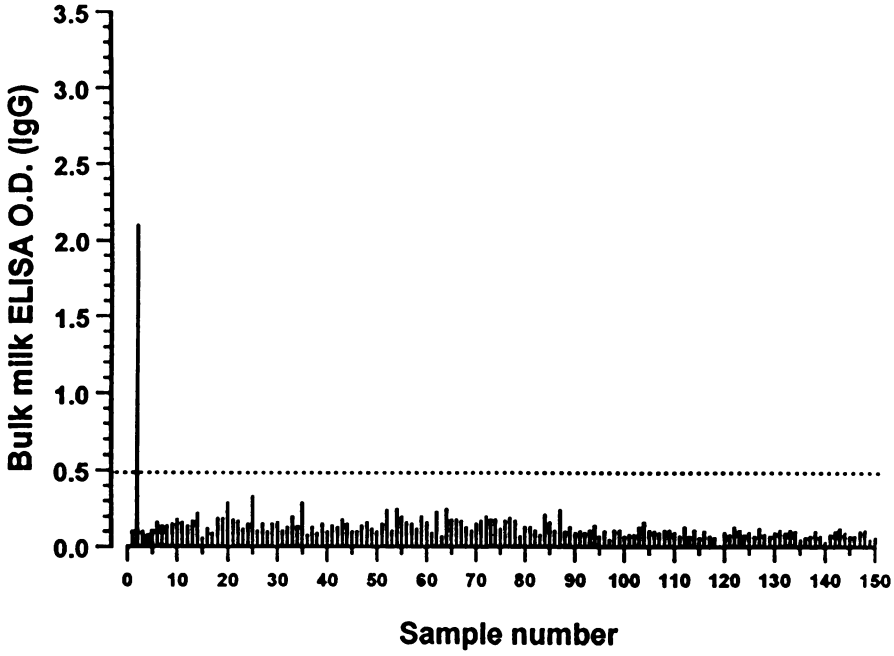

Fig. 2. Results of milk ELISA for detection of antibodies to LPS (O:1,9,12) of *S. dublin* in bulk milk samples of all 150 dairy herds from the island of Bornholm with no prevalence of cattle salmonellosis. Samples 1–20 correspond to herds 1–20 in Fig. 1. The dotted lines show the optimal cutoff level of 0.5 in the milk ELISA, and each histogram represents the mean of 2 measurements for each sample.

herds from Jutland had OD values above the cutoff level (Table III). The 9 tested sera from the supposed false positive herd on Bornholm were ELISA-negative. However, follow-up studies is this herd revealed the presence of a few milk- and serum-positive cows (data not shown), still without any clinical signs of salmonellosis. Comparison of herd size and bulk milk titer in all 10 salmonellosis-positive herds showed no significant correlation (data not shown).

COMPARISON BETWEEN MILK AND SERUM REACTIONS

The ELISA reactions in each herd for tests based on serum or milk are shown in Fig. 1. All 10 herds with outbreaks of salmonellosis were positive in both serum and milk ELISA (Fig. 1C and 1F). None of the salmonellosis-negative herds was positive in the serum ELISA (Fig. 1), in which at least 1 sample should have an OD of >0.3 in order for a herd to be considered ELISA-positive (15). Figure 1C includes the results of serum ELISA for both cows and calves, whereas Figure 1F includes only the results of milk ELISA in the lactating cows. Comparison of the overall results in all 10 herds pooled together showed a highly significant ($P > 0.001$) correlation ($r_s = 0.69$, kappa = 0.60) between the ELISA reactions of serum and milk samples in the individual cows in all except herd 22. In general, the numbers of cows positive in the milk and serum tests were comparable (34% and 32%, respectively (Table III)). The correlations between the milk and serum results for the 180 cows in herds 29 and 30, in which serum and milk samples were taken on the same occasion, were high ($r_s > 0.8$, Table III and Fig. 3). A similar high level of correlation was also found in herds 24 and 27, in which milk and serum samples were taken on different occasions (r_s of 0.86 and 0.90, respectively, Table III). In Figures 3A and 3B, the position of most correlation points above the 45° diagonal illustrates the higher potential of the milk ELISA to detect the specific antibodies in comparison with the serum ELISA. Only 1 cow in herd 29, and 5 cows in herd 30, had OD values above the cutoff in their serum samples, but below the cutoff in their milk samples.

MILK IgA

This test was included in the study in order to elucidate its potential for detection of IgA antibodies to *S. dublin* in milk, and to compare it with the milk IgG ELISA. In a preliminary study, milk samples from all cows in 2 salmonellosis-free herds (1 and 2) from Bornholm were tested, and all had OD <0.5 (data not shown), which was used to define the background reaction. Choosing the OD of 0.5 as a provisional cutoff, 9 cows in herd 30, and none in herd 29, were milk IgA ELISA positive and milk IgG ELISA negative (Fig. 3C and 3D). Thus, the IgG ELISA detected substantially more seropositive animals than IgA ELISA.

POTENCY OF MILK ELISA

This part of the study was designed to simulate the bulk milk situation and assess the potency of milk ELISA in detecting a minimum number of ELISA positive cows in a bulk sample. Milk samples were tested from 5 cows in herd 30, with similar high OD values of 3.2 to 3.9 (Fig. 4). While OD values for some of the samples dropped rapidly following dilution in the negative control milk, other samples continued to be ELISA positive at much higher dilution levels (dilution range 16–256, Fig. 4), thus indicating that the ELISA OD of bulk milk will be affected by both the number of milk ELISA positive cows and the antibody titer of individual samples.

There was some correlation, though not significant, between the percentage of milk ELISA positive cows and the OD of bulk samples in herds 21 to 30 ($r_s = 0.53$, $P < 0.1$). Herds with a similar percentage of milk positive cows had large variations in the OD of the bulk samples, thus indicating that the antibody titer in individual milk samples is of great importance. Consistent herewith, the mean herd OD of milk ELISA showed a significant correlation with the herd bulk milk OD ($r_s = 0.70$, $P < 0.05$, data not shown).

DISCUSSION

The total immunoglobulin concentration in cow serum is about 30 times higher than in the milk (19), but the isotype compositions are different, thus making an immediate comparison of the ELISA results from these 2 types of samples difficult. By diluting the serum samples 800 times in the serum ELISA (15), it was partially compensated for this difference in concentrations. Nevertheless, the milk ELISA, because of the application of undiluted samples, would theoretically still detect a 25-fold lower

concentration of specific antibodies than the serum ELISA. This assumption was supported by the fact that the average herd OD was substantially higher in the milk ELISA (data not shown), and more cows were positive in the milk ELISA compared to the serum ELISA (Table III). This higher potency and subsequently higher background may also, in part, explain the lower specificity of the milk ELISA (0.95) compared to the serum ELISA (1.0). The unspecific background reaction, related to the selected cutoff level, may be due to infections with other salmonella serotypes from serogroup D (O:9,12), or B (O:4,12), sharing some of the O antigen factors with *S. dublin* (15,20,21).

The high sensitivity of the milk ELISA, at the expense of its lower specificity, seems to be useful for surveillance screenings, as this may increase the chance of tracing the infection. Positive reactions could subsequently be verified by the serum ELISA or by culturing. The functionality of the selected cutoff of the milk ELISA was supported by the finding that all herds with salmonellosis had bulk milk OD readings above 0.5. The results of cow milk samples demonstrated the relevance of applying this cutoff in herds with a recent (<6 mo) outbreak of salmonellosis. However, herds with older outbreaks may well have fewer seroreactors and lower antibody titer; i.e. the cutoff OD of 0.5 in the milk ELISA is only provisional and needs to be further adjusted depending on the purpose of screening, which in some instances may include identification of older persistent infections.

Similar considerations apply to the results of bulk samples. The lack of a significant correlation between the percentage of milk positive cows and the bulk milk OD values, together with the data on variation in the potency of cow milk samples (Fig. 4), indicate that the number of ELISA-positive cows is not the only factor affecting the bulk milk reaction. The differences in cow milk volume contributing to the herd bulk milk may also affect the bulk milk titer. Higher potency of some cow milk samples may well be due to the increased penetration of udder by serum proteins as a result of mastitis, and the subsequent epithelial damage (22).

IgG is the major immunoglobulin class in the mammary secretions of

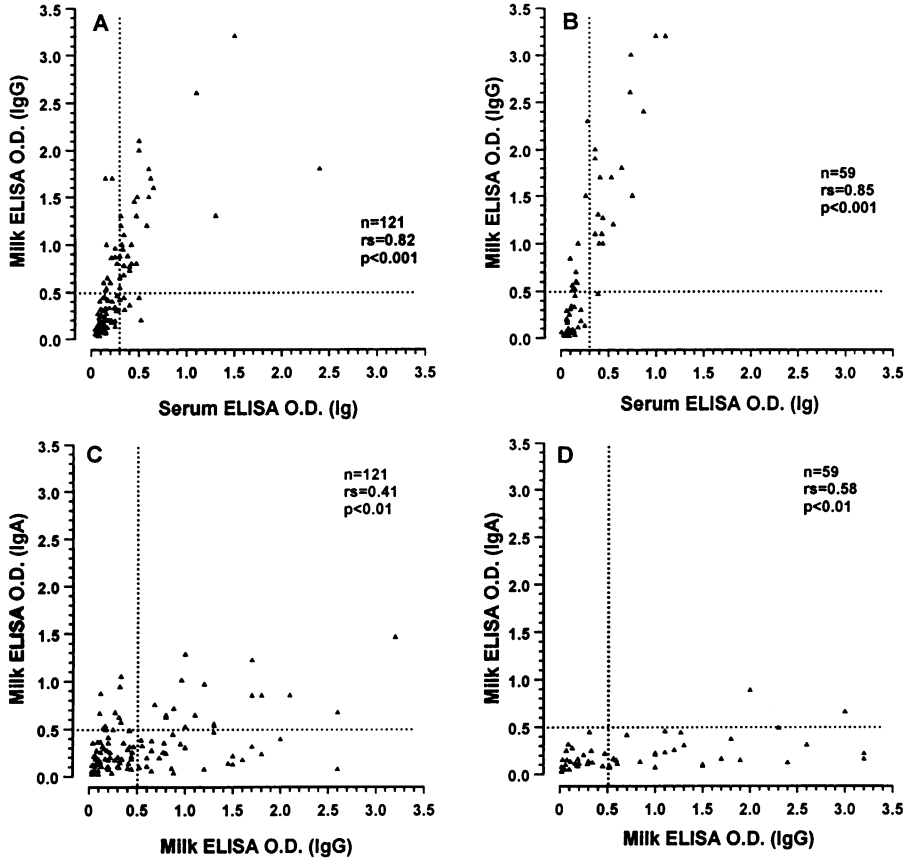

Fig. 3. Comparison of ELISA results for detection of antibodies to LPS (O:1,9,12) of *S. dublin* in serum and milk samples taken on the same occasion from all lactating cows (*n*) in 2 dairy herds within 3 mo after clinical outbreak of salmonellosis: A, serum vs. milk samples in herd 30; B, serum vs. milk samples in herd 29; C, milk IgG titer vs. milk IgA titer in herd 30; D, milk IgG titer vs. milk IgA titer in herd 29. The dotted lines show the optimal cutoff level of OD 0.3 in the serum ELISA and OD of 0.5 in milk ELISA. Spearman rank correlation coefficient (r_s) and the level of significance (*P*) are indicated.

ruminants, and the subclass IgG1 is selectively transported from blood into colostrum and milk (19). Although IgA is quantitatively a minor isotype in ruminant milk, local antigenic stimulation of the mammary gland initiates considerable IgA production (12,23). Assuming a high degree of isotype-specificity for the labelled antibodies applied in the present study, the very high number of IgG positive cows, compared to the number of IgA positive cows, would seem to indicate the presence of systemic infection in the 2 herds tested and not mammary gland infection (22–26). As the milk ELISA was intended for screening of herds possibly housing carrier animals, the choice of IgG isotype as the detector antibody seems then to be appropriate.

In our previous study (15), herds from the island of Samsø were included as a salmonellosis-free material. However, due to availability of milk samples from the island of Bornholm at the time of this study, this island was included here. The island of Bornholm, besides being free of salmonellosis, is geographically more isolated than the island of Samsø and has a greater economic impact in Danish animal meat production. The defined salmonellosis-free area of Bornholm is intended as a national, field-based experiment for integrated control of important zoonotic infections. It was, therefore, encouraging to observe that all but one bulk sample on Bornholm were ELISA negative. The supposed false positive result could be due to a low-grade infection with some of the food-borne serotypes, giving rise to cross-reacting antibodies (21). However, the possibility also remains that this herd was in fact *S. dublin* infected, in which case the specificity of the milk ELISA will be even higher than calculated.

It must be noted that the study included herd populations with extremes in levels of herd infection. Although this is a proper springboard

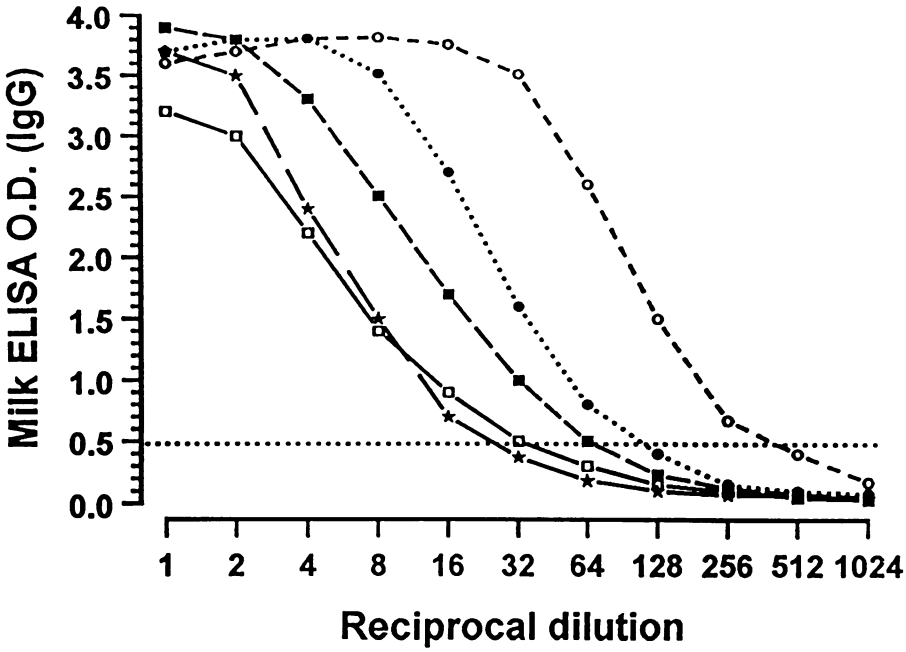

Fig. 4. The titration of milk ELISA for detection of antibodies to LPS (O:1,9,12) of *S. dublin* in samples from 5 cows with high OD values from herd 30.

for evaluation of the test, the real test will be on subclinically infected herds.

In conclusion, the results indicate the possibility of screening herds using cow milk instead of serum samples. Bulk samples seem to be useful in identifying salmonella-positive and negative herds, and for the prediction of prevalence of infected herds, as reported for other cattle diseases (27,28). However, no single factor, such as the proportion of seropositive cows, the mean OD values of cow milk samples, nor the age of outbreak could be used alone to predict the OD reaction. Finally, application of the O antigen ELISAs for screening subclinically infected herds needs to be further investigated.

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