Serodiagnosis of *Salmonella dublin* Infection in Danish Dairy Herds Using O-Antigen Based Enzyme-linked Immunosorbent Assay

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ABSTRACT

Usefulness of two enzyme-linked immunosorbent assays (ELISA) for screening of dairy herds for antibodies to lipopolysaccharide (LPS) of *Salmonella dublin* (O:1,9,12) was investigated. Sera (3097) were collected from 40 dairy herds located in three areas of Denmark with different prevalence of salmonellosis: ten salmonellosis-free herds from the island of Samsø where there is no history of salmonellosis, ten salmonellosis-free herds from the island of Sealand where outbreaks are infrequent, and 20 salmonella infected herds from Jutland where salmonellosis is enzootic. The samples were analyzed for antibodies to *S. dublin* LPS using an indirect (O:9,12) and a blocking (O:9) ELISA. Using herd history of salmonellosis, herd location and clinical state of the herds as reference, the herd sensitivity and herd specificity of the tests were 100% and 100% in the indirect ELISA and 95% and 100% in the blocking ELISA, respectively. A significant correlation was found between the two tests ($r_s = 0.46$, $p < 0.001$). However, the indirect ELISA detected more seropositive animals than the blocking ELISA (17% vs. 7%, respectively). In calves from Sealand, level of background reaction was significantly lower ($p < 0.001$) compared to the heifers and the cows. The percentages of seropositive calves in both tests were higher ($p < 0.01$) in comparison to cows (19 vs. 8 in indirect ELISA, and 14 vs. 6 in blocking ELISA, respectively). Results of the study indicated that it is possible to apply LPS ELISA in serological screening for salmonellosis.

RÉSUMÉ

Deux épreuves ELISA (enzyme-linked immunosorbent assay) ont été évaluées pour la détection, dans des troupeaux de bovins laitiers, d'anticorps contre le lipopolysaccharide (LPS) de *Salmonella dublin* (O:1,9,12). Un total de 3097 sérums provenant de 40 troupeaux de bovins laitiers situés dans trois régions du Danemark ont servi à l'étude. Des 40 troupeaux, dix provenaient de l'île de Samsø et dont tous les animaux étaient reconnus exempts de salmonellose; dix autres troupeaux, exempts de salmnellose, provenaient de l'île de Sealand, sur laquelle les épisodes de salmonellose sont rares; enfin 20 troupeaux affectés de salmonellose provenaient de Jutland, région dans laquelle la salmonellose est enzootique. La recherche d'anticorps anti-*S. dublin* a été effectuée au moyen d'un ELISA indirect (O:9,12) et d'un ELISA par compétition (O:9). En se basant sur l'histoire des troupeaux en ce qui concerne la salmonellose, sur leur localisation géographique et sur leur statut sanitaire, la sensibilité et la spécificité des tests, au niveau du troupeau, ont été établies à respectivement 100 % et 100 % pour l'ELISA indirect et à 95 % et 100 % pour l'ELISA par compétition. Une corrélation significative a été trouvée entre les deux tests ($r_s = 0,46$, $p < 0,001$). Toutefois, l'ELISA indirect a détecté plus d'animaux séropositifs que l'ELISA par compétition (17 % vs 7 %). Avec les sérums des veaux provenant du Sealand, les réactions de bruits de fond étaient significativement plus faibles ($p < 0,001$) que celles avec les sérums des taures et des vaches. Avec les deux tests, les pourcentages de veaux séropositifs étaient respectivement plus élevés ($p < 0,01$) que ceux obtenus chez les vaches (19 vs 8 avec l'ELISA indirect et 14 vs 6 avec l'ELISA par compétition). Les résultats de cette étude indiquent qu'il est possible d'identifier, avec un ELISA utilisant le LPS comme antigène, les animaux infectés par *S. dublin*. (*Traduit par Dr Robert Higgins*)

INTRODUCTION

Salmonellosis is an important disease in cattle, and infections caused by *Salmonella dublin* in particular, are common all over the world (1). In the period from 1980 to 1992, more than 70% of salmonella outbreaks in cattle in Denmark were caused by serotype *dublin* (2). *Salmonella dublin* belongs to the group of zoonotic salmonella serotypes which is associated with human food poisoning (3). Cattle infected with *S. dublin* may become carriers and apparently healthy cows may excrete the organism intermittently (4). This makes identification of herds with enzootic *S. dublin* infection difficult. Furthermore, the existing bacteriological tests are time consuming and costly (5). There is therefore an increasing interest in applying of enzyme immunoassays for diagnosis.

The immune response to the O-antigen of salmonella lipopolysaccharide (LPS) is thought to be of major importance in the host defence against salmonella (6), and cattle apparently respond promptly to the LPS antigen of salmonella (7,8). There are several reports on application of serological methods, such as enzyme-linked immunosorbent assay (ELISA), to detect antibodies to

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TABLE I. Information on dairy herds tested in *S. dublin* ELISAs

Herd location	Herd no.	Salmonella prevalence	Salmonellosis history of the herds	Number of animals tested	n
Samsø	1–10	No	No	All	497
Sealand	11–20	Low	No	30/herd	300
Jutland	21–40	Endemic	Yes	All	2300

S. dublin LPS in cattle (9,10). However, most of these ELISAs were designed to identify individual carriers within herds. To our knowledge there is no published work on the evaluation of LPS ELISA in screening large series of cattle herds.

Salmonella dublin belongs to the serogroup D_1 with the O:1,9,12 antigenic factors (11). However, an indirect ELISA may also detect response to O:1,O:9 or O:12 factors in other salmonella serogroups. This problem of cross-reactivity can partially be reduced by using a blocking ELISA based on monoclonal antibodies (MAb) against the O:9 factor (present only in D serogroups D_1, D_2 and D_3). Besides, the usefulness of ELISAs based on MAb in screening of dairy herds needs to be documented.

The purpose of the present study was to evaluate, on a herd basis, the application of an indirect ELISA and a MAb based (O:9) blocking ELISA. These tests were intended for screening and certification of herds regarding presence or absence of antibodies to salmonellae. Furthermore, sensitivity and specificity of these two tests were compared for diagnosis of herd infection.

MATERIALS AND METHODS

STUDY POPULATION

We used different populations for testing the ELISAs, because of the low diagnostic sensitivity of salmonella culture in subclinically infected herds. Few carrier animals in subclinically infected herds shed salmonella intermittently, and then only in low numbers. Such culture negative herds may well house several nonshedding carriers, which may be seropositive by ELISAs. In order to circumvent this obstacle we chose three areas of Denmark with varying prevalence of *S. dublin* infection: Jutland, Samsø and Sealand (Table I). *Salmonella dublin* is endemic in areas of Jutland, whereas the small island of Samsø has a limited number of herds, free of salmonellosis, and a relatively restricted cattle trade with Jutland. The island of Sealand with low prevalence of salmonellosis was included as an example of a larger island, compared to Samsø, with some cattle trade with Jutland. The clinical status of the herds, including isolating salmonella, and geographical prevalence of salmonellosis were used as the main criteria for defining the populations (Table I).

CULTURE METHOD

Approximately 1 g feces was incubated overnight at 42°C in 25 mL selenite broth (Oxoid CM395, UK). Ten microliters of the selenite broth was plated onto brilliant green agar plate (Oxoid CM329) and incubated overnight at 37°C. One salmonella suspect colony was plated to obtain pure culture for serotyping (11). Postmortem tissue (approximately 10 g) was homogenized in Stomacher, transferred to 90 mL peptone water and incubated overnight at 37°C. One milliliter of this culture mixture was added to 9 mL selenite broth and incubated over night at 42°C. The subsequent procedure was similar to culturing of feces.

SERA

Sera were obtained once from animals in 40 dairy herds. In some herds the age of the sampled animals was known. For data analysis, animals in these herds were divided into three age groups: calves, up to 6 months old; heifers, 6–12 months old; and cows, more than 12 months old.

Blood samples were collected from all animals in each of ten herds from Samsø (herds no. 1–10). The age of these animals was not available.

Ten other herds (no. 11–20) were from the island of Sealand with approx. one outbreak of cattle salmonellosis per 1000 herds per year. In each herd, blood samples were collected from ten calves, ten heifers and ten cows.

The third group was herds with recent outbreaks of salmonellosis from areas of Jutland where *S. dublin* is enzootic (herd no. 21–40). These herds had clinical signs of salmonellosis and *S. dublin* was isolated from feces or tissue sample at necropsy. Blood samples from all animals in these herds were taken within one year after the clinical outbreak. Sera were kept at −20°C until use. The age of individual animals was known in eight herds in this group.

PREPARATION OF ANTIGENS

The *S. dublin* strain (Div 800,83) was originally isolated from a cow with clinical salmonellosis and kept in the collection at the National Veterinary Laboratory, Denmark. The LPS antigen was prepared using the phenol extraction method (12). The antigen used for screening of hybridoma cell lines producing MAb was the LPS fraction from *S. berta* (O:9,12) prepared by a slightly modified method including an extra step of cold acetone extraction (13). The LPS fraction was treated with proteinase-K (Sigma, Missouri) as described (14). The purity of the LPS was tested in polyacrylamide gel-electrophoresis (SDS-PAGE) using silver staining.

PRODUCTION OF MONOCLONAL ANTIBODY

Balb/c mice were immunized i.p. four times two weeks apart with 100 μL of a heat-killed and saline washed whole cells (2×10^8 cells/mL) of *S. berta* in Freund's incomplete adjuvant. The mice were boosted three days prior to fusion with the same amount of antigen in saline. The fusion procedure and production of MAb were as described (15).

Monoclonal antibodies were screened by two ELISAs; one test using supernatant from sonicated (3×15 sec, 9 kilocycles) *S. berta* cells and another test using the LPS fraction. A MAb designated 3.75 was selected for use in the blocking ELISA. The hybridoma cell-lines were produced during another study at our laboratory concerning serodiagnosis of *S. berta* in chickens.

DOT BLOT ASSAY

The specificity of the MAbs was determined by dot blot assay (16) using 60 salmonella isolates of the following nine serotypes: O:4, O:7, O:8, O:9, O:3,10, O:11, O:1,3,19, O:13, O:16 (11). One hundred microliters (2×10^7 cells/mL) of each isolate was applied to a nitrocellulose sheet using a 96-well blotting equipment (Schleicher & Schuell, Germany). The wells were sucked dry and the nitrocellulose removed and blocked by phosphate-buffered saline (PBS) containing 2% Tween-20 (PBS-T). The culture supernatants were diluted 1:10 and incubated for 1 h with the nitrocellulose sheet. Following three washings in PBS-T 0.1% the membrane was incubated 1 h with horse-radish peroxidase (HRP)-labelled rabbit antiserum to mouse immunoglobulins (Ig) diluted 1:2000 (P260, Dako, Denmark). Tetramethylbenzidine was used as indicator (17). Clones producing MAbs reacting with serogroup D_1 salmonellae and not serogroup B and C were selected and isotyped using a commercial kit (Zymed, California).

INDIRECT ELISA

By using checker board titration, the appropriate concentration of *S. dublin* LPS for coating 96-well, flat-bottomed PolySorp microwell plates (Cat.# 475094, Nunc, Denmark) was found to be 340 ng/mL and the serum dilution 1:800. Plates were coated with 100 µL of a solution of LPS (34 ng/well) in coating buffer (0.1 M sodium carbonate buffer, pH 9.6) and incubated overnight at 5°C. The plates were blocked with 200 µL of PBS containing 0.5 M NaCl, 0.05% (V/V) Tween-20 (PBSS-T), and 1% (W/V) polyvinylpyrrolidone-40T (PVP, Sigma, PBSS-T-PVP) for 1 h and then washed five times in PBSS-T. Test sera were diluted 1:800 in PBSS-T-PVP and added in duplicate to the plates which were then incubated for 1 h at room temperature and then washed as before. A positive and negative control serum was also diluted 1:800 and added in quadruplicate to eight wells in column 11. The HRP-labelled rabbit antiserum to bovine Ig (P159, Dako) was diluted 1:4000 in PBSS-T-PVP and 100 µL was added to each well. The plates were then incubated for 1 h at room temperature and washed as before. Finally, 100 µL of the substrate and indicator was added (8 mg 1,2-orthophenyldiamine dihydrochloride, 12 mL 0.1 M citrate, pH 5 and 5 µL H_2O_2). After 10 min the color development was stopped by adding 100 µL of 0.5 M H_2SO_4. The optical density (OD) was read at 490 nm with 650 nm as reference using a Dynatech plate reader (Molecular Devices, U.S.A.). A test set-up was considered valid if the negative control serum had an OD of 0.02–0.09, and the positive control serum had an OD of 2.5–3.5.

S. Berta 1,9,12	S. Bovismorbificans 6,8
S. Dublin 1,9,12[Vi]	S. Istanbul 8
S. Enteritidis 1,9,12	S. Amsterdam 3,10[15][15,34]
S. Typhi 9,12[Vi]	S. Senftenberg 1,3,19
S. Agona 1,4,[5],12	S. Aberdeen 11
S. Typhimurium 1,4,[5],12	S. Worthington 1,13,23
S. Rissen 6,7,14	S. Adeoyo 16

Fig. 1. Reaction pattern of MAb 3.75 in dot blot assay against 14 salmonella serotypes from nine serogroups. Presence of a solid circle indicates a positive reaction.

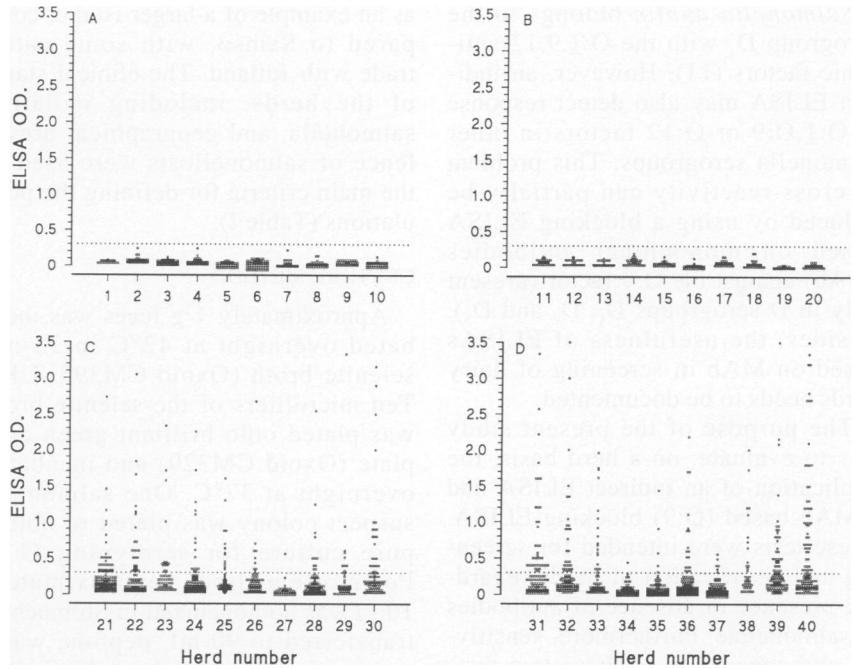

Fig. 2. Results of indirect ELISA for detection of antibodies to LPS antigen of *S. dublin* (O:9,12) in 40 cattle herds with the following clinical status : A, negative herds from Samsø (no history of salmonellosis); B, negative herds from Sealand (rare outbreak of salmonellosis); C and D, positive herds from Jutland (endemic salmonella infection). Each point represents mean of two measurements in one animal, and each group of points represents all samples in that herd. Dotted line shows the optimal cut-off level of OD 0.3.

BLOCKING ELISA

PolySorp plates were coated with 100 µL (34 ng) per well of the same LPS solution as the one used in the indirect ELISA and incubated overnight at 5°C. The plates were blocked with PBS with total NaCl content of 0.15 M, 0.05% Tween-20 (PBS-T), and 1% PVP (PBS-T-PVP) for 1 h and then washed five times in PBS-T. Test sera (100 µL) were diluted 1:10 in PBS-T-PVP (2%) and added in duplicate to each well. At the same time, a positive and a negative control serum in four dilutions of 1:10, 1:20, 1:40 and 1:80 was added to eight wells of column 12 on each plate (8 control sera on each plate). No serum samples were added to column 11 which contained the antigen. The plates were then incubated for

TABLE II. Herd specificity and herd sensitivity of *S. dublin* ELISAs as a function of cut-off level[a]

	Indirect ELISA				Blocking ELISA			
Clinical status of the herds	Cut-off (OD)	ELISA positive herds(n)	SENS[d] %	SPEC[d] %	Cut-off (%)	ELISA positive herds(n)	SENS[d] %	SPEC[d] %
Salmonellosis-positive:	0.1	20	100	—	30	20	100	—
	0.2	20	100	—	40	19	95	—
(n = 20)[b]	0.3	20	100	—	50	19	95	—
Salmonellosis-negative:	0.1	14	—	30	30	3	—	85
	0.2	4	—	80	40	1	—	95
(n = 20)[c]	0.3	0	—	100	50	0	—	100

[a] Herds with at least one seropositive animal were defined as ELISA positive
[b] *S. dublin* isolated within the last 12 months from at least one animal
[c] No known history of salmonellosis
[d] SENS = herd sensitivity, SPEC = herd specificity

TABLE III. ELISA results in dairy herds from the *S. dublin* enzootic area of Jutland

Herd number	Animals n	Indirect[a] ELISA n (%)	Blocking[b] ELISA n (%)	Positive in both tests n (%)	Correlation[c] r_s	p
21	232	31 (13)	11 (5)	9 (3)	0.04	NS
22	105	30 (29)	13 (12)	13 (12)	0.73	<0.001
23	60	16 (27)	5 (8)	5 (8)	0.52	<0.001
24	141	16 (11)	10 (7)	9 (6)	0.64	<0.001
25	175	14 (8)	5 (3)	5 (3)	0.36	<0.001
26	76	9 (12)	6 (8)	4 (5)	0.60	<0.001
27	55	1 (2)	1 (2)	0 (0)	0.48	<0.001
28	133	11 (8)	7 (5)	4 (3)	0.58	<0.001
29	57	11 (19)	1 (2)	1 (2)	0.54	<0.001
30	105	23 (22)	31 (30)	16 (15)	0.71	<0.001
31	120	37 (32)	7 (6)	7 (6)	0.59	<0.001
32	119	31 (26)	19 (16)	13 (11)	0.66	<0.001
33	74	18 (24)	1 (1)	1 (1)	0.66	<0.001
34	124	1 (1)	0 (0)	0 (0)	0.40	<0.001
35	124	5 (4)	1 (1)	1 (1)	0.02	NS
36	183	19 (10)	16 (9)	11 (6)	0.46	<0.001
37	110	7 (6)	9 (8)	2 (2)	0.68	<0.001
38	38	13 (34)	5 (13)	5 (13)	0.89	<0.001
39	124	41 (33)	5 (4)	4 (3)	0.49	<0.001
40	145	54 (37)	12 (8)	11 (8)	0.73	<0.001
Total	2300	389 (17)	165 (7)	121 (5)[d]	0.46	<0.001

[a] Number of seropositive animals with OD of >0.3
[b] Number of seropositive animals with blocking >50%
[c] Spearman value (r_s) and the significance level (p) for correlation of results between two ELISAs in each animal on a herd basis
[d] Kappa = 0.37, $p < 0.001$ for correlation of results between two ELISAs calculated on the basis of total number of animals

1.5 h at room temperature and washed as before. A 1:4000 dilution of MAb 3.75 in PBS-T (100 µL) was added to each well and the plate was incubated for 1 h at room temperature. Rabbit antiserum to mouse Ig labelled with HRP (P260, Dako) was added (100 µL) in a dilution of 1:4000 in PBS-T-PVP (0.5%) for the detection of bound MAb. Finally, 100 µL of the substrate and indicator was added as described in the indirect ELISA. After 10 min the color development was stopped and the OD was read at 490 nm with 650 nm as reference. The results were calculated as the percentage of test OD related to the average OD of the eight wells in column 11, containing the antigen and MAb but not sera. This estimated the ability of each serum in blocking the binding of MAb to LPS antigen. Only test setups with the following conditions were considered valid: The four increasing dilutions of the negative control serum with a blocking ability of 10 to 0%, and the four increasing dilutions of the positive control serum with a blocking ability of 100 to 60%.

DETERMINATION OF CUT-OFF VALUES

Cut-off values for seropositivity were determined on a herd basis and not for individual animals in the herds. A herd was considered infected by *S. dublin* if several animals had shown clinical signs of salmonellosis during the previous 12 months, if salmonellosis was a persistent problem in the herd, and if *S. dublin* could be isolated from feces or postmortem organs of at least one of the diseased animals. Herds were considered salmonella negative if they originated from the islands of Samsø or Sealand and had no history of salmonellosis.

A herd was regarded as ELISA positive if at least one animal in the herd was seropositive at the selected cut-off levels. Herd sensitivity was defined as the proportion of infected herds that tested positive in ELISA. Herd specificity was the proportion of noninfected herds that tested negative in ELISA. The cut-off value was set to achieve the best possible herd specificity and herd sensitivity.

STATISTICAL ANALYSIS

Age-related ELISA results were analyzed by the Kruskal-Wallis one way analysis of variance based on Wilcoxon scores. Correlation between the ELISAs was evaluated by Spearman rank correlation coefficient, and level of significance was calculated for a two-tailed test. The kappa test was used on the correlation

of total number of seropositive animals as well (18).

RESULTS

MONOCLONAL ANTIBODY

In the screening of the culture supernatants the MAb (IgG_1) assigned 3.75 reacted with the crude and LPS antigens, and in the dot blot assay showed a reaction pattern indicating specificity towards the O:9 epitope of the serogroup D_1 (Fig. 1).

ANTIGEN

The SDS-PAGE of the LPS gave a typical S-type LPS ladder pattern in which the spacing was indicative of a LPS composed of an O-chain with a large repeating oligosaccharide unit (14). Enzymatic treatment of the LPS fraction with Proteinase-K did not reduce binding of the positive control serum to the antigen in the indirect ELISA, indicating lack of protein in the LPS fraction. Presence of proteins in the LPS fraction may cause some cross-reactivity with other bacterial antigens. This lack of protein contamination was further verified in the SDS-PAGE.

TEST PERFORMANCE

In the indirect ELISA, the daily variation in the OD of the negative control serum was much smaller than the positive control serum. The mean ± standard deviation and coefficient of variation (CV) of the control sera from 30 valid plates from 30 different days were 3.24 ± 0.33(10%) for the positive and 0.05 ± 0.02(41%) for the negative. Sera with OD close to the cut-off level at the first run were tested two more times. From these sera only those being above the cut-off for a second time were considered as positive. Similar results were obtained when the ELISA was used in another laboratory in Denmark.

Daily variations in the blocking ELISA had less effect on the test sera since all values were related to the maximum binding of MAb on the same day. The mean ± SD (CV) of the blocking percent of control sera diluted 1:10 from 30 valid plates were 97 ± 1.2(1%) and 4 ± 3.7(97%).

CUT-OFF LEVEL

As seen in Table II, the number of salmonellosis-free herds testing positive in the indirect ELISA decreased by increasing the OD value from 0.1 to 0.3. Choosing an OD of 0.3 as cut-off value, gave no false negative or false positive herds in the indirect ELISA (herd specificity and herd sensitivity 100%).

Determining an optimal cut-off level for the blocking ELISA was more difficult, since no cut-off value resulted in complete separation of positive and negative herds. A 50% blocking level of the MAb 3.75 by test serum was set as cut-off in order to obtain the best herd specificity (100%) for serogroup D_1 and at the same time obtain the highest possible herd sensitivity (95%, Table II).

ELISA RESULTS

A total of 3097 sera from cows, heifers and calves in 40 dairy herds were tested. Information on the age of animals was available for all herds from Sealand and eight herds from Jutland (Table I).

The ELISA results are illustrated in Fig. 2 and Fig. 3. For the sake of clarity, only results from ten herds were presented in each sub-figure. Figure 2 shows OD values obtained with the indirect ELISA. In Fig. 3, the numbers on the Y-axis show to which percent each sample was able to block the binding of MAb 3.75 in the blocking ELISA.

In the infected herds, there was a wide variation in the level of antibodies to LPS among animals. Also, percentage of seropositive animals varied substantially among the herds (Table III). Herds no. 27 and no. 34 had only one seropositive animal with ELISA values slightly above the cut-off levels.

In all but herd no. 30 more animals were seropositive in the indirect ELISA than in the blocking ELISA (17% vs. 7%, respectively). In the indirect ELISA, 75% of the infected herds had more than ten seropositive animals, whereas only 30% of these herds had more than ten seropositive

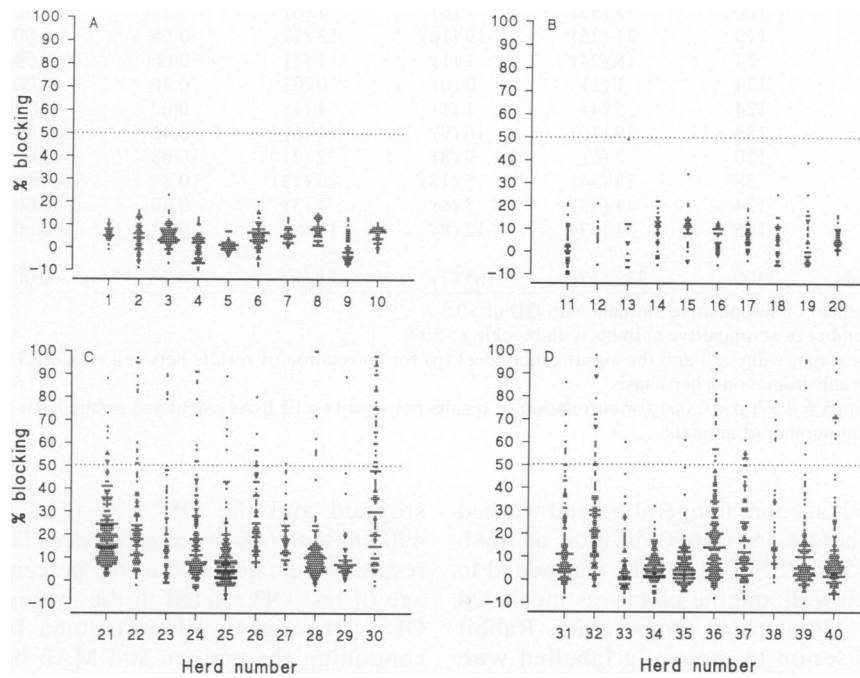

Fig. 3. Results of blocking ELISA for detection of antibodies to LPS antigen of *S. dublin* (O:9) in 40 cattle herds with the following clinical status: A, negative herds from Samsø (no history of salmonellosis); B, negative herds from Sealand (rare outbreak of salmonellosis); C and D, positive herds from Jutland (endemic salmonella infection). Each point represents mean of two measurements in one animal, and each group of points represents all samples in that herd. Dotted line shows the optimal cut-off level of 50%.

TABLE IV. Age-related *S. dublin* ELISA results in different dairy herds

Location	n	Mean ± SD		ELISA positive (%)	
		Indirect (OD)	Blocking (%)	Indirect	Blocking
Sealand[a]					
Calves	96	0.04±0.03[c]	2.0±6.8[c]	0	0
Heifers	98	0.06±0.04	6.4±7.9	0	0
Cows	98	0.06±0.04	8.2±8.8	0	0
Jutland[b]					
Calves	170	0.22±0.39	20.0±24.7	33 (19)[d]	23 (14)[e]
Heifers	116	0.16±0.32	15.4±18.9	10 (9)	9 (8)
Cows	587	0.13±0.27	15.4±18.5	45 (8)	36 (6)

[a] Island with rare outbreak of cattle salmonellosis (10 herds with 30 cattle were tested)
[b] Part of Denmark with endemic cattle salmonellosis (eight herds with all cattle included were tested)
[c] $p < 0.001$ in Kruskal-Wallis one way analysis of variance. No significant difference among age groups where not given
[d] Significantly higher numbers of seropositive calves than heifers and cows tested in the same ELISA (Chi-squared test, $p < 0.001$)
[e] Significantly higher numbers of seropositive calves than heifers and cows tested in the same ELISA (Chi-squared test, $p < 0.01$)

animals in the blocking ELISA. Comparing individual ELISA results from both tests, there was a significant ($p < 0.001$) correlation between the tests (Table III). The degree of test correlation in the herds from Samsø was $r_s = 0.39$ ($p < 0.001$) and the herds from Sealand $r_s = 0.42$ ($p < 0.001$). However, results of the two ELISAs from herds no. 21 and no. 35 from Jutland did not correlate significantly.

The ELISA results in the three age categories are shown in Table IV. Calves in the healthy herds from Sealand had significantly ($p < 0.001$) lower levels of ELISA values compared to heifers and cows. Conversely, in salmonella infected herds calves tended to have higher levels of antibodies to LPS in comparison to heifers and cows, although the difference was not statistically significant. In both tests, the percentage of seropositive calves from Jutland was significantly higher than heifers and cows (Table IV).

DISCUSSION

There are four major findings in the present study. First, it was possible to select cut-off levels for seropositivity that were in agreement with the salmonella clinical status in the herds. Second, calves in salmonellosis-free herds had lower levels of background reaction compared to the older age categories, i.e. heifers and cows. Third, there was a significantly positive correlation between the indirect ELISA and the blocking ELISA, but the indirect ELISA, in general, detected more seropositive animals than the blocking ELISA. Finally, the percentage of seropositive animals varied substantially among the infected herds.

Salmonella dublin infection in cattle under both experimental and field conditions elicits circulating antibodies with O-antigen specificity (7,8). While LPS antibodies are important, flagellar antibodies are considered by some investigators as more serotype specific (19,20). However, high levels of LPS antibodies in cattle up to one year after inoculation (5), points towards LPS ELISA as a useful method to trace previous infections in herds.

The method applied here for determination of the cut-off levels has been successfully used by others (21). This method allows calculation of more than one cut-off value, facilitating a flexible ELISA application. For the purpose of the present study, the cut-off values were set to obtain the best possible herd specificity and herd sensitivity. While in the indirect ELISA the best cut-off level was achieved by choosing an OD of 0.3, it was necessary to use a cut-off level as high as 50% in the blocking ELISA to obtain 100% specificity, leading to a relatively lower percentage of seropositive animals in comparison to the indirect ELISA.

Another consideration in choosing a high cut-off level in the blocking ELISA was the high level of background reaction in some of the herds from Sealand. The higher concentration of test serum (1:10) applied in the blocking ELISA in comparison to the indirect ELISA (1:800) may have affected the level of background reaction. Consequently, the cut-off level of the blocking ELISA was chosen so as to take account of this high background found in herds from Sealand, and to achieve the highest herd specificity correlated to culturing.

The main reason for developing an O:9 blocking ELISA was its potential to distinguish between infections caused by *S. dublin* and *S. typhimurium* as the major serotypes isolated from cattle in Denmark. However, due to the lack of a large series of *S. typhimurium* infected herds at the time of this study, it was not practical to assess the serogroup D_1 specificity of the blocking ELISA. This aspect is currently under investigation and will be reported.

In general, there was a significant ($p < 0.001$) correlation between the two tests ($rs = 0.46$). Five percent of animals from the endemic area of Jutland were seropositive in both tests, while as many as 17% were seropositive in the indirect ELISA.

Calves in herds from Sealand had an overall lower level of background reaction than heifers and cows. Perhaps, the higher level of background reaction found in the older age groups was due to previous or present infections with some food-borne salmonella serotypes, giving rise to low levels of cross-reacting antibodies. This emphasizes the need to use the highest age-related cut-off level in screening tests, where information is missing on the age of the cattle being tested. In contrast, calves in infected herds had higher, though not significant, levels of antibodies to LPS than heifers and cows. Another study found a comparable level of ELISA titers in calves and cows in an infected dairy herd (22).

A small percentage of cattle recovered from salmonellosis become latent carriers (4,23). Carrier cattle can react strongly towards the LPS antigen (9), as some of the animals

did in the present study (Fig. 2 and Fig. 3). This may be an indication of the presence of carrier animals in some of the herds. However, not all high-reactors can be considered carriers, as some of the animals may be convalescents. Another study (9) showed that LPS ELISA could not distinguish carrier cattle from uninfected convalescent herd-mates, since antibodies to LPS can be detected long after the infection is cleared (5). Therefore, correlation of seroreactivity to culture results is being investigated in a longitudinal study in our laboratory.

A drawback of the present study was the wide gap in time of blood sampling (1 yr) following outbreak of salmonellosis in some herds. For practical reasons, it was not possible to collect the samples within a shorter period after the outbreak. This may explain the large variation in the number of seropositive animals among the herds in Jutland. Similarly, follow-up studies by others show a substantial decrease in the number of seropositive animals approximately four months after outbreak of salmonellosis (22). It is therefore necessary to include the number of animals in a herd to be certified on the basis of careful calculation of sample size as a function of expected prevalence, herd size and test sensitivity (24). Here, it must be noticed that higher average herd size in the infected herds from Jutland was in favor of the high herd sensitivity obtained in the present study.

In conclusion, the results indicate that it is possible to apply LPS ELISA in screening of dairy herds. A large screening program would map out endemic areas with varying degrees of salmonella infection. As the next step, the ELISAs can be used in periodic certification of dairy herds for salmonella. This may reduce cattle trade as a risk factor of spreading salmonella infections. Finally, the ability of LPS ELISA in eradication programs through identification of carrier cattle can be investigated.

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