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Evaluation of a serological *Salmonella* Mix-ELISA for poultry used in a national surveillance programme

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SUMMARY

A Mix-ELISA using lipopolysaccharide antigens from *Salmonella enterica* serotype *Enteritidis* and *Typhimurium* was evaluated using samples collected over time in the Danish salmonella surveillance programme for poultry. Serological samples ($n = 42813$) taken from broiler-breeder flocks after a year of bacteriological monitoring with negative results were used for calculating the flock and individual test specificities, which were 0·997 and 0·999, respectively. Layer flocks from the table egg sector were used for calculation of positive predictive values. In the survey, flocks were examined for salmonella by Mix-ELISA and by faecal culture, and in case of a positive result in either of these a repeated, serological testing was performed, and 60 animals were organ-cultured. If one of these samplings was positive, the flock was declared salmonella infected. In a period of 3 months, 35 flocks were found to be positive in the routine samples. Of these, 32 were serologically positive, 2 both serologically and faecally positive and 1 flock only faecally positive. For flocks serologically positive in the surveillance programme, a positive-predictive value of 0·62 for organ culture positivity was found, and while considering serological follow-up samples, the value was 0·95.

INTRODUCTION

Zoonotic salmonella bacteria have always been a threat to human health, but with the concentration of the animal production on fewer and larger production units and a few trade lines, the risk of spreading such agents to a great part of the production has increased dramatically [1]. This, together with the increasing record of bacteria resistant to multiple antimicrobial agents [2], makes it necessary to implement systems for the reduction of zoonotic agents.

In Denmark, a screening programme for control of salmonella in poultry was initiated by law in 1996. The programme was divided into two parts, one comprised the breeder flocks in the broiler production, and the other comprised the table egg sector. The aim was to reduce the salmonella level in broiler flocks to less than 5% and ultimately to eliminate salmonella from meat products and shell eggs.

The programme combines bacteriological and serological examinations. From experimental infections it is known that in the early stage of an infection, before animals have seroconverted, it is possible to isolate salmonella from faeces. Later, during the course of the infection the excretion of bacteria becomes intermittent, although, it is still possible to detect a serological response in a large number of animals [3, 4].

In the initial phase of the programme, a positive serological herd status had to be confirmed by bacteriological isolation of the agent, but in 1998 the

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programme was adjusted for the table egg sector. Thereafter, serologically positive flocks were sampled a second time, and if these samples were serologically positive, the farmer was allowed to sell eggs only for heat-treated products. Furthermore, the sampling frequency was increased from three times a year to once every ninth week.

Numerous ELISAs using different salmonella antigens for detection of salmonella infection in poultry have been described, some of which have been used in national control programmes for salmonella [1, 5, 6]. These assays have often been developed with the aim of detecting infection with a single serotype. However, although *S. Enteritidis* is the predominant serotype in poultry in most countries in Europe and North America, additional serotypes may also cause problems in various countries. Therefore, the development of a Mix-ELISA using antigens from more than one serotype may be a valuable tool in flock control programmes. In Denmark, the two most commonly isolated serotypes from poultry are *S. Enteritidis* and *S. Typhimurium*, and therefore a Mix-ELISA was constructed using lipopolysaccharide (LPS) from both these serotypes.

The aim of the present study was to evaluate the serological Mix-ELISA for salmonella used in the Danish poultry surveillance programme, using the data collected over time in the programme.

MATERIALS AND METHODS

Sampling procedure

Flocks producing table eggs

The flocks were sampled every ninth week. The flock sample comprised of 60 eggs for serological examination and one pool of 60 fresh faecal droppings with a minimum weight of 60 g for bacteriological examination. The farmer was responsible for this sampling. A flock was defined as an epidemiological unit of birds, and more epidemiological units could be situated at the same property. In case of a positive laboratory result (isolation of salmonella or two or more serological reactors, see below), the flock was considered to be suspect, and the official district veterinarian took further samples (60 eggs for serological examination and 60 killed hens for bacteriological examination) for confirmation of the salmonella status. The interval between the routine and follow-up samplings was typically less than 2 weeks. If samples submitted by the official district veterinary officer were positive for salmonella, eggs from the flock were allowed to be sold only for the production of heat-treated products.

Parent layer flocks for the broiler production

The sampling procedure was the same as for the table egg sector, with the modification that the sampling frequency was every fourth week. In flocks with a positive salmonella result, the official district veterinarian submitted 60 killed hens for bacteriological examination. If salmonella bacteria were isolated from these animals, the whole flock was killed.

Procedure for bacteriological examination

Faecal samples

Approximately 25 g of faecal material was immersed in 225 ml of buffered peptone water (BPW) (Merck 07228). After incubation at 37 °C for 16–20 h, 100 μl of BPW was transferred to 10 ml of Rappaport–Vassiliadis broth with soya (RVS) (OXOID CM 866) and incubated at 42 °C for 18–24 h. 10 μl of RVS was then spread on Rambach agar (Merck 07500) and incubated at 37 °C for 20–24 h. Red colonies were tested serologically [7] with a polyvalent O-antiserum (OM) (all antisera produced by Statens Serum Institut, Copenhagen, Denmark). If a red colony tested negative in OM, up to five colonies were tested for positivity in OM. A colony which tested positive in OM was considered presumptive salmonella. One presumptive colony was transferred to veal infusion broth (Difco 344-17) and incubated at 37 °C until growth. Afterwards, it was transferred to blood agar (OXOID CM 271B) with 5% calf blood (BA) and biochemical test tubes, respectively, and incubated at 37 °C. From BA agglutination with specific O- and H-antigens was performed on indole-negative colonies [7, 8].

Animals

From each of 5 hens out of 60 a cloacal swab and caecal tonsils were pooled and also a pool of a small piece of the liver, the ovarium and the salpinx were made, thus producing 12 intestinal pools and 12 visceral pools. Each of the intestinal and visceral pools was separately immersed in 100 ml of BPW. All bags with BPW were incubated at 37 °C for 16–20 h. Thereafter, 100 μl of BPW was transferred to 10 ml of

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Table 1. *Individual and flock specificity of Mix-ELISA at various numbers of serological reactors and OD percentage values*

	Flock specificity (%)		
	Zero reactors	One reactor	Individual specificity (%)
30 OD %	(644/717)* 89·8	(710/717) 99·0	(42813/42895) 99·8
40 OD %	(677/717) 94·4	(715/717) 99·7	(42853/42895) 99·9

* ( ), negative units/total units.

RVS, and 10 ml of BPW was transferred to 100 ml of selenite broth (SB) (OXOID LP121A). RVS and SB were incubated for 18–24 h at 42 °C and 37 °C, respectively. From SB and RVB, the material was spread on both phenol red/brilliant green agar (OXOID CM 329) and Rambach agar. These were incubated at 37 °C for 20–24 h. Presumptive salmonella colonies were then verified biochemically and serologically according to the same principles as those described for faecal samples.

Serological examination by Mix-ELISA

Lipopolysaccharide (LPS) was purified from formalin killed broth culture as described by Hassan and colleagues [4], from field isolates of *S. Enteritidis* No. 85241/15-16 (O:9, 12) and *S. Typhimurium* No. 3389-1/92 (O:1, 4, 5, 12). Using checker board titration, the optimal dilutions of LPS and serum, giving the highest (positive/negative) ratio for sera from bacteriologically negative and experimentally infected hens, were found. The optimal amount for both LPS antigens was approx. 5 ng per well, and no significant difference in this was observed when combining 5 ng of each antigen per well in the Mix-ELISA.

The LPS was diluted in 0·1 M carbonate buffer pH 9·6 and mixed before application to the ELISA-plate (PolySorb, Nunc, Denmark) with 100 μl per well. After overnight incubation at 4 °C, the plates were emptied and tapped dry. The plates were blocked for 15 min with phosphate buffered saline (PBS), containing 0·05% Tween 20 and 1% BSA (PBS-T-BSA) followed by a single wash with PBS, 0·05% Tween 20 (PBS-T). Serum samples and egg yolk samples were diluted 1:400 in PBS-T-BSA (unpublished observations) and applied to the plates in duplicate with 100 μl per well and incubated at room temperature for 1 h. Following three washes, horseradish peroxidase conjugated rabbit-anti-chick immunoglobulin (IgG) (Ap 162P, Cemicon) diluted 1:10000 in PBS-T-BSA was applied with 100 μl per well and incubated for 1 h at room temperature. After three washes, 100 μl of enzyme substrate (8 mg 1,2 orthophenyldiamine dihydrocloride, 12 ml 0·1 M citrate, pH 5 and 5 μl H_2O_2) was added, with 100 μl per well. After 20 min the colour development was stopped with 100 μl per well of 0·5 M H_2SO_4. The spectrophotometric absorption was read at 490 nm using 650 nm as reference.

Stopping the colour development of the ELISA-plates after a fixed number of minutes rather than at a predetermined OD-reading of a reference serum caused the ELISA-plates to have some variation in the OD-readings. In order to compensate for this, all results on each ELISA-plate were calibrated according to the deviation in the OD-reading of the reference sera from a predetermined set of average standard values for the reference sera. This yielded calibrated sample OD-values. Further, the calibrated sample OD-values were expressed as calibrated sample OD-percentages (cal. OD %) by defining an OD % scale by setting 2000 milliabsorbances to 100 OD % and 38·5 milliabsorbances to 0 OD %. In accordance to preliminary results, a flock in the surveillance programme was declared serologically positive if it had two or more reactors $>$ 40 cal. OD %.

Calculation of specificity

Broiler breeder flocks were used for calculation of the specificity of the Mix-ELISA. Flocks having remained bacteriologically negative for more than 1 year were selected, and only serological results obtained after a year of bacteriological negativity were included in the calculations. This method of selecting flock samples for calculations meant that a varying number of consecutive samplings from each flock, or its successor, were included in the calculations. Flock

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specificities were calculated as the number of serologically negative flock samples divided by the total number of serological flock samples.

The individual sample specificity was calculated as the number of serologically negative individual samples divided by the total number of serological samples.

Predictive values

The table egg layer flocks were used for calculation of positive predictive values. Samples were included over a 3-month period from the introduction of the Mix-ELISA as a definitive test. If one of the routine samples (faeces or serological samples) was positive, organs from 60 animals were cultured and 60 samples tested serologically. The positive predictive value was calculated as the percentage of serologically positive routine flock results being also organ culture positive.A second positive predictive value was calculated as the percentage of flock results having been found serologically positive in both the routine samples and the follow-up samples and also being organ culture positive.

Logistic regression was used to investigate the relationship between the number of serological reactors (at least two, i.e. for seropositive flock samples) and the probability of at least one positive organ culture result in the subsequent examination. The calculation was carried out in SAS 6.10 using the logit link function in PROC GENMOD.

RESULTS

Specificity

The demand that a broiler breeder flock should have remained bacteriologically negative and participated in the surveillance programme for at least 1 year, reduced the number of participating localities from a total of 164 to 130 flocks. The 717 submissions from these localities were used for the calculation of the specificities shown in Table 1. For a cut-off at 40 cal. OD%, a change in the accepted number of seroreactors from zero to one reduced the frequency of serologically false positive flock sample results by a factor of 18·6 (Table 1), whereas it only reduced the number of serological positive table egg flocks less than threefold (18·9 to 7·8%) (Table 2).

Table 2. *The frequencies of serologically positive table egg flock results at various cut-off values*

	Number of positive flocks (%)	
	Zero reactors	One reactor
30 OD%	28·4	10·2
40 OD%	18·9	7·8

Surveillance results

In a 3-month period, 40 flocks were organ cultured. Of these, 32 flocks were organ cultured solely due to a serologically-positive routine result, 2 flocks due to positive samples in both the serological and faeces routine samples, and 1 flock solely due to a faeces-positive routine result. Of the remaining 5 flocks, 3 flocks were cultured due to trace-back of human infections, and 2 flocks due to a salmonella positive flock on the same property.

The 34 serologically positive flock results in the routine sampling and the serological follow-up results compared to the organ culture results, are presented in Table 3. For flocks serologically positive in the routine samples, a positive-predictive value of 0·62 (21/34) for organ culture positivity was found. When considering the serological follow-up samples, the value was 0·95 (18/19). Only one flock was declared salmonella infected due to combined serological results without bacteriological verification. From Table 3 the reproducibility of a positive routine result on the positive follow-up result can be calculated to 56%.

The salmonella serotypes and phage types for the 21 culture-positive flocks are presented in Table 4. Nineteen of the salmonella flock isolates had O-factors represented in the Mix-ELISA antigen. In one flock, serologically positive with 3 reactors in the routine samples and with 10 reactors in the follow-up samples, *S. Infantis* (O:6, 7) was found in the organs and *S. Livingstone* (O:6, 7) in the faeces, hence the O-factors from the isolated bacteria did not match the Mix-ELISA antigens.The predicted probability of a positive organ culture result as a function of the number of seroreactors and the 95% confidence limit for this probability, is illustrated in Figure 1. As the number of seroreactors increased from 2 to 25 there was a strong increase in the probability of being organ culture positive; for higher numbers of seroreactors the probability of positive organ culture was almost 100%.

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Table 3. *Serological flock results for seropositive routine samples and their follow-up samples compared to organ culture results*

	Routine samples	Follow-up samples	
Organ culture	Serologically pos.	Serologically pos.	Serologically neg.
Positive	21	18	3
Negative	13	1	12

Table 4. Salmonella *serotype and phage type for 21 organ culture positive flocks*

No. of flocks	Serotype	Phage type
1	Enteritidis	1
2	Enteritidis	4
4	Enteritidis	6
7	Enteritidis	8
1	Enteritidis	21
1	Enteritidis	23 and 8
1	Enteritidis	25
1	Infantis and (Livingstone)*	
1	Typhimurium	193
1	Pullorum	
1	Untypeable	

* The parentheses indicate that the serotype was found in faeces.

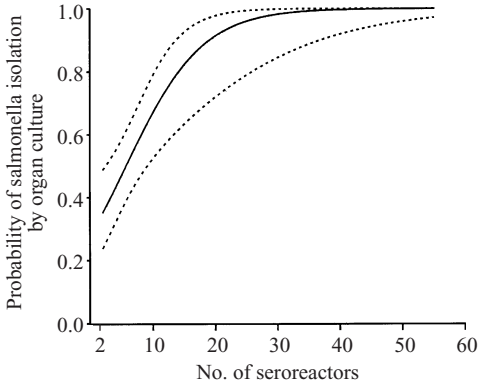

Fig. 1. Probability of salmonella isolation by organ culture related to the number of serological reactors. The dotted line gives the 95% confidence interval.

DISCUSSION

The selection of a rather high serological cut-off was decided for this surveillance programme due to the elevated background found in production flocks compared to SPF animals [9] and the wish to reduce the number of false positive test results. The elevated background may be caused by otherwise undetected levels of salmonellae and/or by other *Enterobacteriaceae* encountered by the animals during their lifetime [9, 10].

Strong serological reactions caused by bacteria normally found in chickens do not seem to play a significant role in unspecific reactions in the LPS ELISA methods [9]. The high specificity found in this investigation indicates that cross-reactions are insignificant at the chosen cut-off level. Invasive salmonella serotypes carrying the same O-factors as used in the Mix-ELISA antigens may, however, give strong serological reactions [9, 11].

The low number of faeces culture positive flocks compared to organ culture positive flocks is in agreement with findings by others (unpublished observations). In the early stages of infection, salmonella is shed in the faeces, and after the initial phase most animals clear themselves of the bacteria. However, a few animals become carriers and shed bacteria intermittently. Gast and colleagues [12] found a high predictive value of both positive faeces samples and antibody titres for hens laying *S. Enteritidis* contaminated eggs. Little is known about salmonella infections in naturally infected flocks but the time course and horizontal transmission may vary depending on the route and burden of salmonella introduced [13].

Most flock infections verified by organ culture were found by suspicion due to seropositivity, but it is questionable whether it is possible to control salmonella satisfactorily solely by bacteriological methods [14]. The serological positive predictive value of 0·62 for organ culture positivity is low, however, two serologically positive flock results in an interval of 14 days, increases the positive predictive value to 0·95.

The low reproducibility (56%) of serologically positive routine results on the positive follow-up results may be a consequence of low infection prevalences and a combination of uneven distributions of the infection in the flocks and non-random selection of the samples. A sample size of 60 eggs should with a confidence limit of 95 detect an infection

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prevalence of 5%. It has in some cases been observed that in routine samples submitted in boxes with two egg-trays of 30 eggs, that one tray might contain a high number of serological reactors while the other was negative, thus indicating an uneven distribution of the infection and a non-random sampling. This reduces the sensitivity of the serological test, but apparently also brings its performance closer to the sensitivity of the organ culture test. An increase in the sample size for organ culture would undoubtedly increase the positive predictive value of the serological routine samples. The positive predictive value is also influenced by the salmonella serotype and its ability simultaneously to provoke an immune response and persist in the host. In this investigation, no records were available about antibiotic treatments of flocks which may also reduce the likelihood of cultivating salmonella from seropositive flocks.

In conclusion, the Mix-ELISA described in the present paper has proved a valuable tool in the surveillance and control of salmonella infections in Danish poultry production. At the chosen cut-off value the recorded specificity of the test was shown to be very high, assuring farmers and producers against truly negative flocks being destroyed due to false positive test results. The validity of the recorded relatively low predictive value of the Mix-ELISA in pointing out infected flocks may be debated, as it is well known that the sensitivity of bacteriological culture methods is low especially in late stages of flock infections with invasive serotypes, although bacteriological examinations may be advantageous in the early stage of flock infections. As suggested, the inclusion of a greater number of animals for bacteriological culture from suspected flocks may partly solve the problem and further work on the surveillance results from the Danish salmonella control programme is in progress to clarify this matter.

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