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The identification of *Salmonella enteritidis*-infected poultry flocks associated with an outbreak of human salmonellosis

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SUMMARY

In the summer of 1991 a human outbreak of *Salmonella enteritidis* infection occurred following a barbecue in which about 100 persons were involved. Eggs, supplied by one or more of 10 different layer farms, were the most probable source of the infection. To identify the *S. enteritidis*-positive flocks, an immunoassay was used to detect salmonella serogroup D-specific antibodies in the yolk of hens eggs. Antibody titres in the eggs from two layer farms, farm A and B, clearly exceeded the titres found in randomly collected eggs. Further investigations on farm A and B yielded high antibody titres in the eggs from flocks A1, A2 and B2, and low titres in the eggs from flock B1. *S. enteritidis* was isolated from the faecal samples of flocks A1, A2 and B2, whereas no salmonella was detected in the faecal samples of flock B1. The flocks present on both farms originated from the same breeder flock.

INTRODUCTION

Salmonella has been recognized for many years as a major cause of acute human gastro-enteritis. Based on sentinel and population studies, it is estimated that the incidence of salmonellosis in The Netherlands amounts to 4500–7500 cases per 10^6 population per year (A. M. M. Hoogenboom-Verdegaal, personal communication). *Salmonella enteritidis* has emerged as a major serotype causing human salmonellosis in several countries [1, 2], particularly in the United States and in Great Britain, where the increase has been dramatic over the past 10 years [3–5]. In The Netherlands about 3000 salmonella-strains isolated from human patients are serotyped annually at the National Salmonella Centre. Analysis shows a near-tenfold increase of *S. enteritidis* from 3·9% of the isolates in 1987 to 34·4% in 1991 (W. J. van Leeuwen, unpublished data) implying approximately 20000–40000 human cases of *S. enteritidis* infection in The Netherlands in 1991. Case control studies on *S. enteritidis* infections suggest shell eggs as an important source of infection [6] and there have been numerous reports of outbreaks of *S. enteritidis* infection associated with consumption of eggs or egg-containing foods [3, 5, 7]. Transovarian transmission of *S. enteritidis* leading to internal contamination of

the egg is now well substantiated [8–10] and the proportion of internally infected eggs originating from naturally infected laying flocks is generally reported as less than 1% [7, 11, 12], although higher proportions have been found occasionally [13, 14]. In The Netherlands, in 1989, a national *S. enteritidis* control programme was implemented in the poultry breeding stock by a joint effort between the government and producers. Main objective of this programme is to deliver chicks free of *S. enteritidis* to layer and broiler farms. It has been claimed that as a result of this programme the contaminated commercial laying flocks have decreased from 17·6% in 1989 to 7·4% in 1990 [15]. However, despite this promising development, the number of *S. enteritidis* infections in man in The Netherlands is still increasing as are outbreaks.

In 1991 following an outbreak of *S. enteritidis* infection which affected about 100 people, and in which eggs were indicated as most probable source of the organism, an attempt was made to trace the offending *S. enteritidis*-infected poultry flocks by using an immunological technique for detecting salmonella serogroup D-specific antibodies in the yolk of the hens eggs.

METHODS

Immunoassay for detecting salmonella serogroup D-specific antibodies in the yolk of hens eggs

For the detection of salmonella serogroup D-specific antibodies in the yolk of hens eggs a sandwich ELISA test as described by Notermans and Heuvelman [16] was used. Wells of polystyrene trays (Dynatech Laboratories cat. no. 001-010-2601) were coated with lipopolysaccharides (LPS) from *S. enteritidis*, extracted by the method of Westphal [17]. To each well 0·1 ml of a solution containing 0·26 μg LPS/ml diluted in 0·07 M phosphate buffered saline (PBS) (pH 7·2) was added. After overnight incubation at room temperature with shaking, the trays were rinsed with PBS containing 0·05% Tween 20 (PBST). Aliquots of 0·1 ml of serial threefold diluted egg-yolk samples (diluted in PBST) were added to the wells. A serial threefold diluted serum sample from a chicken artificially infected with *S. enteritidis* was used as a positive control. The trays were incubated for 90 min at room temperature with shaking. After a further rinse 0·1 ml of a 1 : 10000 diluted (dilution fluid PBST containing 1% (w/v) bovine serum albumin) rabbit-anti-chicken serum conjugated with peroxidase (Rockland cat. no. 203-4301) was added to each well and the trays were incubated for 90 min at room temperature with shaking. After rinsing, the wells were incubated at room temperature with 0·1 ml of a 0·1 M sodium acetate/citric acid buffer containing 0·002% H_2O_2 to which 1 ml of a 42 mM solution of TMB (3,3′,5,5′-tetramethylbenzidine) dissolved in dimethylsulfoxide was added per 100 ml. This reaction was stopped after 5 min by adding 0·05 ml 2 M H_2SO_4 to each well. The reaction product was measured by using a spectrophotometer at 449 nm. The amount of antibody present in yolk samples was expressed as a reciprocal of the dilution (titre) giving an extinction equal to the average extinction value from 10 blank samples + 2·81 × the standard deviation of these blank values (confidence limit 99%). Blank samples consisted of PBST containing 1% bovine serum albumin.

Human outbreak of S. enteritidis *infection*

In July 1991, in The Netherlands, an outbreak of food poisoning occurred following a barbecue in which about 100 people were taken ill. About 20 persons, including 5 children under the age of 5 years, were admitted to hospital and *S. enteritidis* phage type 1 was isolated from their stools. (Phage type 1, according to the Dutch phage set, is identical to phage type 4 of the Colindale system (B. Rowe, personal communication)). Epidemiological investigations strongly suggested that a cake was the vehicle of infection (Inspectorate for Health Protection Leeuwarden, personal communication). All those who became ill had consumed the cake, whereas no one who had not consumed the cake became ill. The cake had been covered by a layer of raw egg material and had been heated in an oven for about 3 min just before consumption. There were no leftovers for examination, but the supplier of the eggs was traced. It appeared that the supplier obtained his eggs from ten different layer farms. From each of these farms 20 eggs were examined for the presence of salmonella, but salmonella was not detected (Inspectorate of Health Protection Leeuwarden, personal communication).

Tracing of S. enteritidis *infected flocks*

Pool-samples of yolks of the above mentioned eggs were made from each farm and examined for the presence of salmonella serogroup D-specific antibodies. If high antibody titres were found in the egg yolk pool sample from a farm, 30 eggs were collected from each laying flock present on this farm and egg yolks were examined individually for the presence of antibodies. In addition, 20 faecal samples were taken from each of these flocks. Each faecal sample consisted of fresh faecal material obtained from 20 different places and was mixed thoroughly. Faecal samples were examined for the presence of salmonella by using a standard method described by ISO 6579. A 25 g portion of faeces was added to 225 ml of buffered peptone water. After incubation for 16–20 h, 0·1 ml of the culture obtained was transferred into 10 ml of selective Rappaport Vasiliadis (RV) medium. After incubation of the broths for 24 h and 48 h the culture in the RV medium was streaked onto brilliant green/phenol red agar. After incubation of the plates for 24 h colonies suspected of salmonella were confirmed biochemically. All isolated strains of salmonella were sero- and phagetyped at the Dutch National Salmonella Centre (W. J. van Leeuwen).

RESULTS

Evaluation of the immunoassay for detecting D-specific antibodies

To evaluate the ELISA for detecting salmonella serogroup D-specific antibodies in the yolk of hens eggs, 574 eggs randomly collected from local shops were tested. The frequency distribution of antibody titres obtained against salmonella serogroup D is presented in Fig. 1. Most titres were around 300.

Identification of S. enteritidis *infected flocks*

Antibody titres in egg yolk pool samples from 8 of the 10 layer farms involved ranged between < 300 and 2700 and thus did not exceed the titres found in yolks of randomly collected eggs. However, in the egg yolk pool samples of two layer

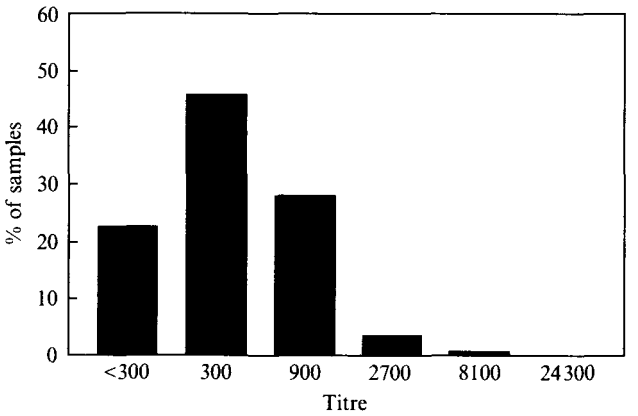

Fig. 1. Antibody titres against salmonella serogroup D in egg yolks of 574 randomly collected eggs.

Table 1. *Antibody titres against salmonella serogroup D in pool samples of egg yolks* (n = *20*) *from the layer farms supplying the eggs*

Farm	Antibody titre	Farm	Antibody titre
A	> 24300	F	300
B	> 24300	G	300
C	2700	H	300
D	900	I	< 100
E	300	J	< 100

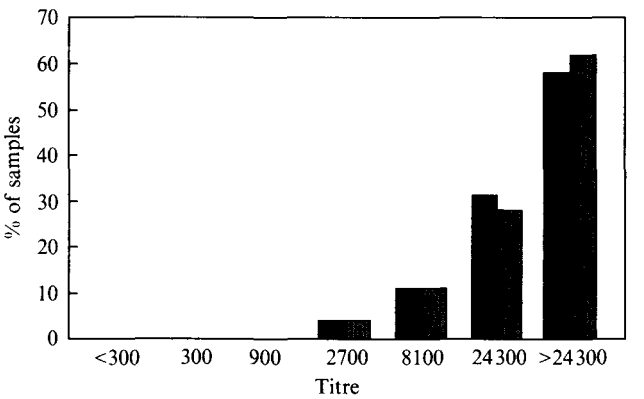

Fig. 2. Antibody titres against salmonella serogroup D in eggs (n = 30) from flock A1 (■) and in eggs (n = 30) from flock A2 (▨) on farm A.

farms, farm A and farm B, an antibody titre of > 24300 was demonstrated (Table 1). On farm A two laying flocks (indicated as flocks A1 and A2) were present in two separate free-range litter houses. These flocks consisted of 6500 birds each at an age of 49 weeks. Antibody titres in 60 egg yolks from these two flocks ranged between 2700 and > 24300 (Fig. 2), and thereby clearly exceeded the titres established in the randomly collected eggs (Fig. 1). In flock A1 salmonella was detected in 12 out of 20 samples of faeces. *S. enteritidis* phage types 1 and 16 (Dutch phage set) were isolated from 10 and 2 samples respectively (Table 2). In flock A2 salmonella was detected in 16 out of 20 samples of faeces. In this flock

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Table 2. *Occurrence of* S. enteritidis *in faecal samples from laying flocks on farms A and B*

Flock	No. of samples	No. salmonella positive	Sero- and phagetype (no. of samples)
A1	20	12	*S. enteritidis* pt. 1 (10)
			S. enteritidis pt. 16 (2)
A2	20	16	*S. enteritidis* pt. 1 (13)
			S. enteritidis pt. 16 (2)
			S. enteritidis pt. 18 (1)
B1	20	0	
B2	20	2	*S. enteritidis* pt. 1 (2)

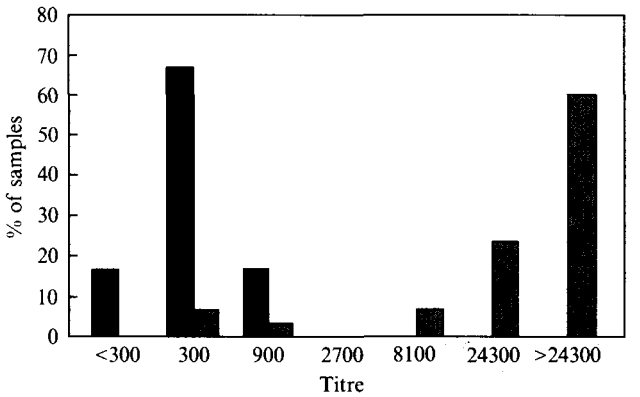

Fig. 3. Antibody titres against salmonella serogroup D in eggs ($n = 30$) from flock B1 (■) and in eggs ($n = 30$) from flock B2 (▨) on farm B.

S. enteritidis phage types 1, 16 and 18 were isolated from 13, 2 and 1 samples respectively (Table 2). On farm B two laying flocks (indicated as flocks B1 and B2) were present in two separate free-range litter houses. These flocks consisted of 7000 birds each and were 23 and 34 weeks of age respectively. The results of flock B2 were comparable to that of flocks A1 and A2. Although a few of the 30 eggs from this flock showed an antibody titre < 2700, titres in the larger part of the eggs ranged between 8100 and > 24300 (Fig. 3). In 2 of 20 samples of faeces from this flock *S. enteritidis* phage type 1 was detected (Table 2). In contrast to the results of flock B2, antibody titres in 30 eggs from flock B1 all ranged between $<$ 300 and 900. Salmonella was not isolated from the faecal samples of this flock. The laying flocks present on farm A and B were of the same breed and originated from the same breeder flocks.

DISCUSSION

In this study *S. enteritidis*-infected laying flocks were successfully identified after a human outbreak of *S. enteritidis* by using an immunoassay for detecting salmonella serogroup D-specific antibodies in the yolks of hens eggs. Of the 10 layer farms supplying the suspected eggs two farms with *S. enteritidis*-infected flocks were identified. As both these laying flocks had the same origin, *S. enteritidis* may well have been transmitted vertically from an infected breeder flock to its progeny [18]. The results of this study suggests that eggs originating from these

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laying flocks were involved in the outbreak as the source of infection. In all probability, the heating of the cake covered by the raw egg material was insufficient to kill off all salmonellae, and there were enough surviving organisms to infect the consumers. The outcome of this study emphasizes once more the role of hens eggs in the human *S. enteritidis* problem.

In The Netherlands the national *S. enteritidis* control programme has resulted in a reduction of *S. enteritidis* contamination in the poultry breeding stock to a low level [19]. This result implies that almost all 1-day-old chicks delivered to layer and broiler farms are free of *S. enteritidis*. If the transmission of *S. enteritidis* is mainly vertical from generation to generation, the programme should lead to a reduction in the number of *S. enteritidis* positive flocks in the commercial laying stock. However, the number of cases of *S. enteritidis* in humans in The Netherlands is still increasing and eggs continue to play an important role as vehicle and source of infection. One explanation might be that a significant number of laying flocks become infected with *S. enteritidis* from environmental sources. Insufficient cleaning and disinfection of poultry houses may play an important role and transmission of the organism by the feed cannot be excluded. Thus, the detection of positive laying flocks in addition to the testing of breeding stock should lead to further reductions in the numbers of contaminated poultry flocks and hopefully also the incidence of human salmonellosis.

Identification of *S. enteritidis*-infected laying flocks by the culture of litter samples or cloacal swabs is expensive and time consuming. Immunological methods are probably more appropriate for this purpose. Serology can be used to screen flocks for the presence of *S. enteritidis* [20]. However, this method is also labour intensive and can be stressful to birds in production. Recent work has suggested the usefulness of a LPS-specific ELISA in screening egg yolks from laying flocks for evidence of infection with *S. enteritidis* [21–23]. This technique relies on the passage of antibody from infected birds into the egg yolks. Since the birds may carry a range of salmonella serotypes, antibody responses to different types may be invoked and different antibodies may appear in the egg yolks. By using a LPS-specific ELISA for the detection of antibodies to *S. enteritidis* false positive reactions may be obtained as cross-reactions with antibodies to other salmonellae possessing identical O-antigens may occur. Thus, for evidence of an actual infection with *S. enteritidis* positive ELISA results should always be confirmed by bacteriological tests. In this study, *S. enteritidis*-positive laying flocks were successfully identified by using a salmonella serogroup D-specific ELISA. The antibody titres in both the egg yolk samples and the individual eggs from the *S. enteritidis* positive flocks were much higher than those found in randomly collected eggs. Thus, the immunological detection of antibodies in egg yolk is an effective tool for screening laying flocks for *S. enteritidis*-infection. Moreover, the high levels of antibodies in the eggs from infected birds make it possible to use the pool sampling technique, which saves time and labour.

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