Assessing the Sensitivity of Egg Yolk Antibody Testing for Detecting *Salmonella enteritidis* Infections in Laying Hens

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ABSTRACT The identification of infected commercial poultry flocks has become a pivotal component of efforts to reduce the incidence of egg-associated transmission of *Salmonella enteritidis* to humans. To assess the sensitivity with which testing for specific antibodies in egg yolks can be applied to detect *S. enteritidis* infection in laying chickens, groups of hens were orally inoculated with either 10^3, 10^5, or 10^7 cfu of a phage type 13a strain of *S. enteritidis*. Eggs from these hens were collected for 4 wk after inoculation and yolk samples were tested for antibodies to *S. enteritidis* flagella by ELISA. All hens that were inoculated with 10^7 cfu of *S. enteritidis* were detected as infected by the egg yolk ELISA when eggs were tested individually, as were up to 66 and 35% of hens inoculated with 10^5 or 10^3 cfu, respectively. Even when yolks from infected hens were diluted 1:10 in yolk from uninfected hens, specific antibodies could still be found in eggs from 31% of hens given 10^7 cfu of *S. enteritidis* and 13% of hens given 10^3 cfu. These results demonstrate that egg yolk antibody testing can provide a highly sensitive indication of prior exposure to *S. enteritidis*, and should accordingly be useful for verifying the effectiveness of programs designed to reduce the incidence of *S. enteritidis* infection in poultry.

(*Key words*: *Salmonella enteritidis*, chicken, eggs, antibodies)

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INTRODUCTION

Identifying laying flocks infected with *Salmonella enteritidis* has become an important component of efforts to reduce the incidence of egg-associated illness in humans (Altekruse *et al*., 1993; Henzler *et al*., 1994). Specific serum antibodies have been reported at relatively high titers following either experimental or natural infection of laying hens with *S. enteritidis* (Chart *et al*., 1990; Gast and Beard, 1990; Barrow and Lovell, 1991). Enzyme immunoassays using lipopolysaccharide, flagella, outer membrane proteins, or fimbria as antigens have been applied successfully for detecting *S. enteritidis* infections in chickens (Timoney *et al*., 1990; Kim *et al*., 1991; Cooper and Thorns, 1996). In a study involving commercial breeder flocks in the Netherlands, an ELISA using a flagellar antigen detected *S. enteritidis* infection more often than did bacteriological monitoring of environmental samples (Zijderveld *et al*., 1993).

Antibodies to *S. enteritidis* can also be detected in the yolks of eggs laid by infected hens (Dadrast *et al*., 1990; Nicholas and Andrews, 1991; Desmidt *et al*., 1996), although high titers are attained more slowly in yolk than in serum (Gast and Beard, 1991; Sunwoo *et al*., 1996). In contrast to serum antibody testing, samples for egg yolk antibody testing can be collected very easily and without creating stress for the sampled birds. The frequency of detection of specific antibodies in egg yolks was previously determined to be directly correlated with the isolation of *S. enteritidis* from tissue samples in commercial laying flocks in the U.S. (Gast and Beard, 1991). In the Netherlands, specific egg yolk antibody titers from laying flocks were likewise directly related to the frequency of fecal shedding of *S. enteritidis* (Giessen *et al*., 1992). Testing for egg yolk antibodies using a flagella-based ELISA was shown to be as effective as culturing voided feces for predicting the likelihood of *S. enteritidis* contamination of eggs laid by experimentally infected hens (Gast *et al*., 1997).

Although the practical sensitivity of testing for egg yolk antibodies is limited by the minimum infecting dose of *S. enteritidis* that elicits a consistently detectable yolk antibody titer, this relationship has not previously been defined clearly. Moreover, pooling egg yolks before antibody testing could potentially increase the number of birds represented by each sample, but such pooling could also dilute the antibody concentration and thereby pose a severe challenge to the sensitivity of antibody testing. The objective of the present study was to assess the sensitivity of a flagella-based ELISA for detecting specific antibodies in individual and pooled yolks of eggs laid by groups of hens experimentally

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infected with three different oral dose levels of *S. enteritidis.*

MATERIALS AND METHODS

Experimental Infection of Laying Hens

In each of two replicate trials, 45 Single Comb White Leghorn hens from the specific-pathogen-free flock at our laboratory were housed in single-bird cages in a disease-containment isolation building. In each trial, three groups of 15 hens each were housed in separate rooms. All birds were provided *ad libitum* access to water and an antibiotic-free layer breeder diet (16.7% CP, 2,968 kcal ME / kg, 2.9% Ca, 0.39%P). All hens used were hatchmates and were 32 and 39 wk old at the beginning of the first and second trials, respectively.

A phage type 13a *S. enteritidis* strain (reference no. 19299-52-1, originally obtained from C. E. Benson[1]) was prepared as an overnight culture in tryptone soya broth[2] and then diluted to prepare inocula containing approximately 7.5×10^7, 7.5×10^5, or 7.5×10^3 cfu / mL (Gast, 1993). Hens were inoculated by the oral administration of 1 mL of diluted *S. enteritidis* broth culture directly into the crop. Each of the three separately housed groups of hens received a different dose level of *S. enteritidis.*

Detection of Specific Egg Yolk Antibodies

All eggs laid were collected during the week prior to *S. enteritidis* inoculation and for 4 wk after inoculation. Yolk samples from the last egg laid by each hen during each week of collection were diluted 1:5 in phosphate-buffered saline and stored at 4 C for later antibody testing. Thirty yolk samples from eggs laid before *S. enteritidis* inoculation were randomly selected for use as negative controls. These negative control samples and all postinoculation egg yolk samples were tested individually for specific antibodies. To determine whether antibodies could be detected in simulated 5-egg and 10-egg pools, each postinoculation sample was also tested after further 1:5 and 1:10 dilution with a yolk pool prepared from equal portions from each of the 30 negative control samples.

Egg yolk samples were tested for the presence of specific antibodies using an ELISA developed by Holt and Porter (1993) and adapted for use with egg yolks by Gast *et al.* (1997). Purified *S. enteritidis* flagella (at 1 μg / mL) were used as solid-phase detection antigens to bind specific antibodies from yolk samples (tested at a final dilution of 1:250). Monoclonal antibodies specific for chicken IgG (prepared by the authors and used at a 1:40 dilution) and alkaline phosphatase-labeled goat anti-mouse IgG[3] (used at a 1:750 dilution) were added in sequence to allow colorimetric detection. Egg yolk samples from infected hens were classified as positive if their ELISA absorbance values exceeded the mean value for the negative control eggs by more than two standard deviations. Egg yolk antibody titers were determined by the positive:negative ratio method of Briggs *et al.* (1986), using a standard equation derived from preliminary testing of 120 positive egg yolk samples for IgG antibodies to *S. enteritidis* flagella ($\log_{10}$ ELISA titer = [$\log_{10}$ {sample absorbance / negative control absorbance} + 1.546] / 0.654).

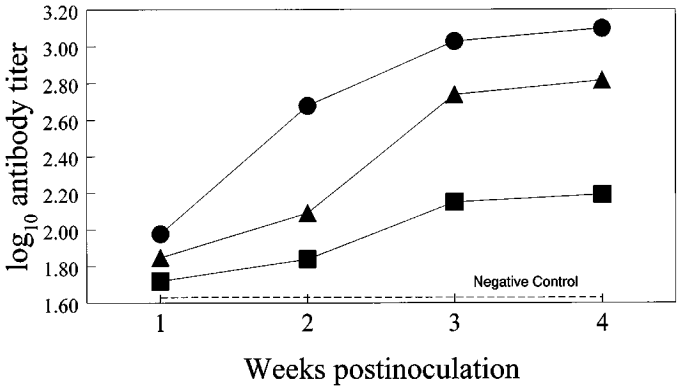

FIGURE 1. Specific antibody titers ($\log_{10}$) in egg yolks from laying hens experimentally infected with *Salmonella enteritidis.* Groups of 30 hens were orally inoculated with doses of either 10^7 (●), 10^5 (▲), or 10^3 (■) cfu of *S. enteritidis.* Eggs collected at weekly intervals were tested for antibodies to *S. enteritidis* flagella by ELISA. The mean antibody titer of 30 egg yolk samples collected before inoculation with *S. enteritidis* is shown as a dashed line.

Statistical Analysis

Significant differences ($P < 0.05$) between means associated with different inoculum doses or sample dilutions were determined by one-way analysis of variance for antibody titer data and by applying Fisher's exact test to data organized into 2×2 contingency tables for antibody detection frequency data (Snedecor and Cochran, 1980). As no significant variation was observed between replicate trials, the results were combined for analysis.

RESULTS

Significant increases in specific egg yolk antibody titers were observed for all three *S. enteritidis* dose levels within 3 wk of inoculation (Figure 1). At 1 wk postinoculation (PI), the mean $\log_{10}$ titers ranged only from 1.72 to 1.98 in eggs from hens infected with the three different doses of *S. enteritidis* and did not differ significantly from the mean titer for the 30 negative control eggs (1.63). However, by 2 wk PI, the mean antibody titer in eggs from hens inoculated with 10^7 cfu of *S. enteritidis* had risen to 2.68 and was significantly greater than the mean titers among negative control eggs ($P < 0.001$) or among egg from hens inoculated

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[3]Calbiochem Corp., La Jolla, CA 92037.

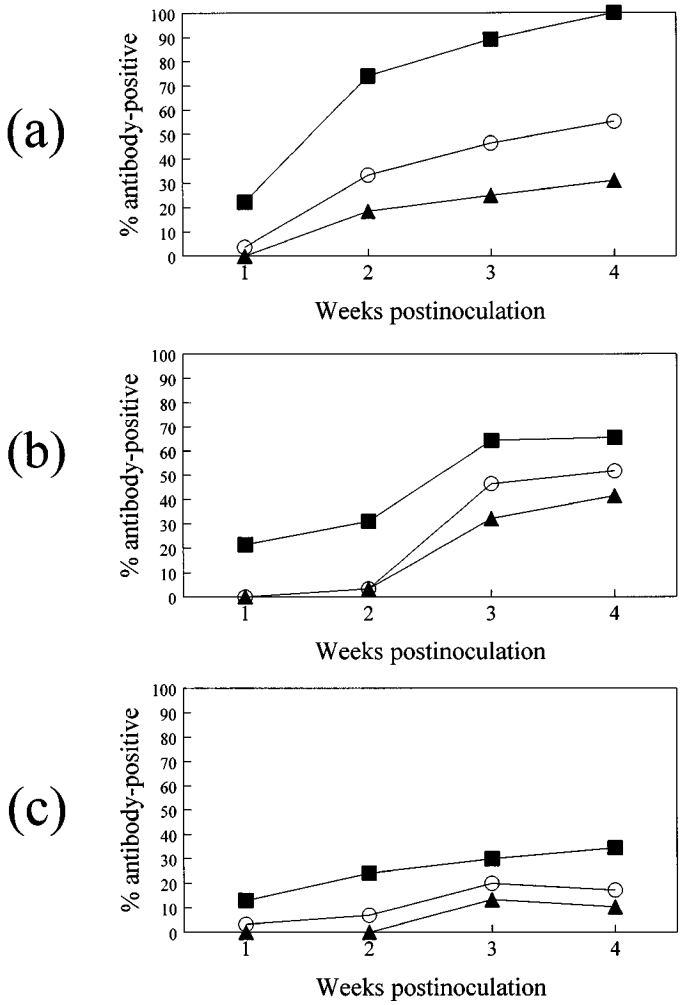

FIGURE 2. Frequency of detection of specific antibodies in egg yolks from laying hens experimentally infected with *Salmonella enteritidis.* Groups of 30 hens were orally inoculated with doses of either 10^7 (a), 10^5 (b), or 10^3 (c) cfu of *S. enteritidis.* Eggs collected at weekly intervals were tested for antibodies to *S. enteritidis* flagella by ELISA. Each sample was assayed individually (■) and after 1:5 (o) and 1:10 (▲) dilution in egg yolks from uninfected hens.

with either 10^5 ($P < 0.05$) or 10^3 ($P < 0.001$) cfu of *S. enteritidis.* The mean antibody titer in eggs laid by hens inoculated with 10^5 *S. enteritidis* (2.09) had also increased significantly ($P < 0.05$) in comparison to the negative control eggs by 2 wk PI. By 3 wk PI, the mean antibody titer in eggs from hens infected with 10^3 *S. enteritidis* (2.15) had also reached a level that was significantly higher ($P < 0.01$) than that of the negative control eggs. At 4 wk PI, eggs from hens inoculated with either 10^7 or 10^5 cfu of *S. enteritidis* exhibited significantly ($P < 0.01$) higher mean antibody titers (3.10 and 2.82, respectively) than were observed in eggs from hens inoculated with 10^3 cfu (2.19).

The frequency of detection of specific egg yolk antibodies by ELISA (at a level more than two standard deviations greater than the mean value for the negative control eggs) was affected by both the dose of *S. enteritidis* administered to the hens and by the dilution of yolk samples into simulated egg pools (Figure 2). Among eggs laid by hens inoculated with 10^7 cfu of *S. enteritidis,* 22.2% yielded positive ELISA results when tested individually at 1 wk PI, 74.1% at 2 wk, and 100% at 4 wk (Figure 2a). However, when yolk samples from these hens were diluted before testing, specific antibodies were seldom detected at 1 wk PI and at maximum frequencies of 55.2 and 31.0% (for the 1:5 and 1:10 dilutions, respectively) at 4 wk PI. The frequency of antibody detection in individually tested eggs from these hens was significantly ($P < 0.023$) greater than in yolk samples diluted 1:5 (2 to 4 wk PI) or 1:10 (1 to 4 wk PI).

The frequency at which specific antibodies were detected in individually tested eggs laid by hens inoculated with 10^5 cfu of *S. enteritidis* rose from 21.4% at 1 wk PI to 65.5% at 4 wk (Figure 2b). None of the diluted yolk samples from these hens were antibody-positive at 1 wk PI, but by 4 wk PI antibodies were found in 51.7 and 41.4% of yolk samples diluted 1:5 and 1:10, respectively. Antibodies were detected significantly ($P \leq 0.032$) more often in individually tested egg yolks from these hens than in yolk samples diluted 1:5 (1 to 2 wk PI) or 1:10 (1 to 3 wk PI).

Specific antibodies were found in only 12.9% of individually tested egg yolks at 1 wk after hens were inoculated with 10^3 cfu of *S. enteritidis* and in 34.5% at 4 wk (Figure 2c). Antibodies were seldom detected in diluted egg yolk samples from these hens during the first 2 wk PI, and were found at peak frequencies of only 20.0% (1:5 dilution) and 13.3% (1:10 dilution) at 3 wk PI. Significantly ($P \leq 0.028$) more individually tested egg yolks were ELISA-positive at 2 and 4 wk PI than were yolk samples diluted 1:10.

DISCUSSION

Significant increases in specific egg yolk antibody titers between 1 and 3 wk PI were associated with all three *S. enteritidis* inoculum doses in the present study. The majority of hens that were given 10^7 cfu of *S. enteritidis* could be detected as infected by the egg yolk ELISA within 2 wk of inoculation. Each 100-fold dilution of the inoculum dose resulted in an approximately one-third reduction in the maximum frequency at which egg yolk antibody testing identified infected hens. Nevertheless, the observed sensitivity of detection of *S. enteritidis* infection by the egg yolk ELISA in the current experiments was greater than was achieved by culturing voided feces from hens infected with similar inoculum doses in a previous study (Gast, 1993).

Approximately 33, 66, and 100% of hens infected with 10^3, 10^5, and 10^7 cfu of *S. enteritidis* produced detectable levels of egg yolk antibodies in the present study. Accordingly, sampling 30 egg yolks by ELISA should consistently detect infection at incidences of about 10, 5, and 3% for the three dose levels, respectively. Although detection sensitivity could be improved by testing more egg yolks, the cost of performing enzyme immunoassays might become prohibitive for large samples sizes.

Pooling egg yolk samples severely reduced the frequency of antibody detection in this study, but more than 10% of the samples were still found to be positive even when eggs from hens inoculated with only 10^3 cfu of *S. enteritidis* were mixed into simulated 10-egg pools. Pooling samples could permit large number of egg yolks to be tested using an affordable number of ELISA plates and reagents.

Indirect confirmation of the potential sensitivity of egg yolk antibody testing has also been provided by reports that indicate considerable success in using this approach to detect naturally occurring *S. enteritidis* infections in commercial poultry (Nicholas and Andrews, 1991; Giessen *et al*., 1992; Desmidt *et al*., 1996). Detectable levels of antibodies have been found in eggs from experimentally infected hens for at least 7 wk after inoculation (Gast and Beard, 1991) and in serum for at least 27 wk (Barrow and Lovell, 1991). By thus providing a very sensitive record of past exposure to *S. enteritidis*, egg yolk antibody testing can be used to verify the efficacy of microbial quality assurance programs for laying flocks.

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