Interpretations of Antibody Responses to *Salmonella enterica* Serotype Enteritidis gm Flagellin in Poultry Flocks Are Enhanced by a Kinetics-Based Enzyme-Linked Immunosorbent Assay

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Many regulatory and diagnostic programs for the detection of *Salmonella enterica* serotype Enteritidis infection in commercial poultry flocks have relied on rapid Pullorum agglutination tests to screen birds because of the shared antigens of *S. enterica* Enteritidis and *S. enterica* Pullorum and Gallinarum; however, the use of the enzyme-linked immunosorbent assay (ELISA) format affords better analytical sensitivity than crude agglutination tests. In this study, we adapted our earlier conventional indirect ELISA, using gm flagellin as the antigen, to a kinetics-based, computer-controlled ELISA (KELA). The KELA was used to screen for flagellin antibody from three commercial flocks: (i) a large flock involved in a U.S. Department of Agriculture trace back from a human *S. enterica* Enteritidis foodborne outbreak (n = 3,209), (ii) a flock infected with the endemic *S. enterica* Enteritidis serotype but which also had multiple other salmonella serotypes (n = 65), and (iii) an *S. enterica* Pullorum-infected flock (n = 12). The first flock (*S. enterica* Enteritidis prevalence of 2.45% based on culture) provided a field test of the KELA and allowed the calculation of diagnostic sensitivity (D-Sn) and diagnostic specificity (D-Sp). With a cutoff of 10 (used for screening flocks [i.e., high sensitivity]), the KELA has a D-Sn of 95.2% and a D-Sp of 18.5%; with a cutoff of 140 (used in confirmatory flock testing [i.e., high specificity]), the KELA has a D-Sn of 28.0% and a D-Sp of 99.1%. We found that with a cutoff of 60 (D-Sn = 63.4%; D-Sp = 91.6%), we could eliminate reactions in the KELA caused by other non-*S. enterica* Enteritidis salmonellae. The KELA was also compared to two commercial rapid Pullorum tests, the Solvay (D-Sn = 94.9%; D-Sp = 55.5%) and the Vineland (D-Sn = 62.0%; D-Sp = 75.3%).

Infections in domestic poultry with *Salmonella enterica* serotype Enteritidis of many phage types have become increasingly important primarily from the public health standpoint due to the vertical transmission of *S. enterica* Enteritidis in grade A shell eggs (14, 29–31). Since 1990, the National Poultry Improvement Plan has included a provision requiring the testing of poultry breeder flocks for *S. enterica* Enteritidis. The ability to detect *S. enterica* Enteritidis infection on a flockwide basis, and broilers for *S. enterica* Enteritidis intermittently or may eliminate the infection altogether. Serology is the other component of field and laboratory testing of flocks to establish their *S. enterica* Enteritidis status. Because of decreased costs and rapid turnaround time, serology has developed into a promising screening tool for flocks. *S. enterica* Enteritidis is an invasive serotype, and immunoglobulin G (IgG) responses persist in birds that have been infected with *S. enterica* Enteritidis. Therefore, serology would be a superior method to culture for screening birds that are intermittently culture positive or that have eliminated *S. enterica* Enteritidis infection (7–9).

In pilot studies, we developed the *S. enterica* Enteritidis gm flagellin indirect enzyme-linked immunosorbent assay (ELISA) to screen experimentally infected birds and some commercial layer hens (8, 32). gm flagellin is the purified flagellar protein from *S. enterica* Enteritidis (19). In the present study, our goals were (i) to adapt the gm flagellin indirect ELISA to a computer-controlled and very reproducible kinetics-based ELISA (KELA), (ii) to compare the serological re-
sponses of birds naturally infected with S. enterica Enteritidis using two commercially available rapid Pullorum agglutination test antigens versus gm flagellin antigen of the KELA, and (iii) to evaluate the gm flagellin KELA in a field trial using sera from a large S. enterica Enteritidis-infected commercial flock involved in a trace back from an outbreak of human infection, sera from a commercial flock infected with multiple salmonella serotypes, and finally sera from a group of birds naturally infected with Salomonella enterica Pullorum.

MATERIALS AND METHODS

Bacterial strains. S. enterica Enteritidis strain 41 (obtained from M. Opitz [University of Maine] and R. Baker [Cornell University Poultry Science Department]), originally isolated from a laying hen, was used for the production of gm flagellin. This strain had the antigenic formula (1,9,12;g:m) that is characteristic of S. enterica Enteritidis.

Bird sera. Sera from three different groups of birds were tested. The first group consisted of sera from 3,207 commercial layer hens collected by the U.S. Department of Agriculture (USDA) Salomonella Enteritidis (SE) Task Force. This flock was traced as a possible source for a foodborne salmonella outbreak. The second group of 65 sera came from a Northeastern United States commercial layer flock naturally infected with multiple serotypes of salmonella, including S. enterica Enteritidis (27 birds were chosen that were positive in the rapid Pullorum plate test, and 38 birds were chosen that were negative in the rapid Pullorum plate test). The last group of sera was obtained by the USDA from 12 hens culled from a small commercial layer flock shown to be infected with S. enterica Pullorum. All serum samples were shipped to the laboratory on ice packs and stored at −20°C until tested.

Bacterial cultures of organ tissues. For the first group of 3,207 layer hens, the National Veterinary Services Laboratories (NVSL), Veterinary Service, Animal and Plant Health Inspection Service, USDA, Ames, Iowa, cultured the organs of all birds for Salmonella following the USDA SE Task Force protocol, i.e., ovary, oviduct, liver, expressed gall bladder, heart, pericardial sac, and intestine or cecum (33). For the second group of 65 birds, the Task Force's protocol was followed (33), but in addition the intestinal ceca were added to the list and cultured for salmonelle. For the third group of 12 birds, the NVSL followed the National Poultry Improvement Plan protocol for culture of internal organs of the affected birds (34, 35).

Pullorum serological testing. Two commercially available pullorum antigen kits were used to test bird sera, i.e., the S-Solvay-Pullorum Stained Antigen K Polyvalent (Salisbury Laboratories, Charles City, Iowa) and the Vineland Antigen Kit (Vineland Laboratories, Inc., Vineland, N.J.). Testing was performed on glass plates (macroscopic agglutination) and read according to the manufacturers' directions.

gm flagellin production. The method of Ibrahim et al. (19) was used to produce the S. enterica Enteritidis gm flagellin antigen for the KELA. The purity of the flagellin protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% polyacrylamide) by the Laemmli method (23) followed by silver staining (Hi-Ho Silver Stain Kit, Accurate Chemical and Scientific Corp., Westbury, N.Y.). The protein concentration of the flagellin was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif.). Purified flagellin was stored at −70°C until used.

RESULTS

gm flagellin. Silver staining the sodium dodecyl sulfate-polyacrylamide gels revealed a single flagellin band of about 60 kDa. The reported size of gm flagellin is 58.4 kDa, and it is made up of polymers of a single protein (19). Several smaller bands were found at 43 and 15 kDa which were believed to be either minor dissociated proteins of flagellin (19) or to be outer membrane proteins. No lipopolysaccharide (LPS) band of 100-kDa size was found in any of the gels. The protein concentration of the gm flagellin was 750 µg/ml.

SEROLOGY AND ORGAN CULTURE. The results of the Solvay and Vineland Pullorum serologies are shown in Table 1 for the first group of 3,223 commercial birds (note that 3,223 birds were tested versus just 3,207 in the KELA because not all sera tested in the Pullorum serologies were available for the KELA testing). The NVSL found that 79 birds were organ culture positive and 3,144 were culture negative, for an overall prevalence of 2.45%; internal organs but not ceca were cultured from these birds. The Solvay assay had a D-Sn and D-SP of 94.9% and 55.5%, respectively, while the Vineland assay had corresponding results of 62.0 and 75.3%, respectively. For the KELA, multiple cutoffs were evaluated. A cutoff of 10 slope units resulted in a D-Sn of 95.2% and a D-SP of 18.5%, whereas a cutoff of 140 units resulted in a D-Sn of 28.0% and a D-SP of 99.1%.

Based on the culture results, the prevalence of infection in the flocks tested was 2.45%. For this prevalence, the PPVs and NPVs are given in Table 2.

The Pullorum serology for the second group of 65 birds from a breeder flock (flock 2) infected with the endemic serotype S. enterica Enteritidis but which also had other salmonella serotypes revealed that 27 birds were Pullorum serology positive, i.e., internal organs were reared in the Solvay assay, 12 birds reacted in the Vineland assay, and 11 birds reacted in both assays. Internal organs and ceca were cultured from these birds. The salmonella serotypes recovered along with Pullorum and KELA sera are shown in Table 3 for culture-positive birds only. Within this group, the slopes of the Pullorum serology-positive

KELA standard control sera. We prepared four control serum pools, each from approximately 10 birds. Each pool was diluted 1:10 in PBS (pH 7.2), aliquoted in 0.5 ml vials, and stored in sealed vials at −20°C until used. Each pool represented a standard for use in all subsequent runs of the assay. The positive sera originated from birds of a known S. enterica Enteritidis infection status from a commercial flock, and negative sera were from Salmonella-free flocks based on culture. The pools varied in the amount of antibody activity to gm flagellin based on negative, low, medium, and high positive slope values acquired in the KELA. Each standard was run 10 to 15 times in the KELA to determine an average expected activity.

Standardization of data. The expected slopes for the standards were used in development of a standard curve for each run of the assay. The standard curve was generated by linear regression of the expected slope values against the slope values obtained in that run for the respective standards. A normalized slope for each test sample was then derived from the standard curve, allowing direct comparison of data from run to run (3, 20).

KELA cutoff. Because the frequency distribution of the organ culture-positive and -negative birds for the first group of birds (flock 1) was not normally distributed, the selection of an optimal cutoff point between positive and negative sera was not intuitively obvious. Diagnostic sensitivity (D-Sn) and specificity (D-SP) estimates were determined for various cutoffs in the KELA based on the "gold standard" of 3,128 culture-negative and 79 culture-positive birds (flock 1). A cutoff at the lower end of the scale of KELA slope values gives a high sensitivity estimate that is particularly useful in a screening assay. Conversely, a cutoff at the upper end of the KELA slope scale gives a high specificity estimate that is useful in a confirmatory assay (13).

Predictive values. For the first flock of 3,207 birds, the positive predictive value (PPV) and the negative predictive value (NPV) were calculated from the test's sensitivity, specificity, and disease prevalence levels according to the following formulas: PPV = (Prev)(D-Sn)/[(Prev)(D-Sn)+ (1−Prev)(1−D-Sp)]; and NPV = (1−Prev)(D-SP)/[(1−Prev)(D-SP)+(Prev](1−D-Sn)]; where Prev is prevalence.

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Serological diagnoses of Pullorum disease (S. enterica Pullorum) and fowl typhoid disease (S. enterica Gallinarum) in poultry have utilized agglutination tests, i.e., the serum plate agglutination test (Pullorum test) and macroscopic tube agglutination tests; these screening tests detect predominantly IgM antibody to cell wall (LPS) antigens (10, 38). For the serological screening of other Paratyphoid salmonella infections in poultry, e.g., S. enterica Enteritidis infections, surveillance programs have taken advantage of the fact that S. enterica Pullorum and S. enterica Enteritidis share cell wall antigens (both are found in Salmonella serogroup D and share antigens 9 and 12); the use of the Pullorum rapid serum plate agglutination test to detect cross-reacting IgM antibody in S. enterica Enteritidis-infected flocks was used in early S. enterica Enteritidis surveillance programs (15, 22). However, IgM responses are short-lived compared to IgG responses, and diagnosticians quickly learned that there was poor correlation between the Pullorum serum plate test to cell wall antigens and S. enterica Enteritidis-positive cultures (22). Consequently, there has been controversy over the utility of the Pullorum rapid plate tests for S. enterica Enteritidis diagnosis (7, 12, 15, 22, 29, 38).

Titters to S. enterica Enteritidis cell wall antigens may persist for many weeks (8, 16, 26). Salmonella cell wall antigens have the disadvantage of lacking analytical specificity, i.e., they cross-react with antigens shared by related gram-negative bacteria, e.g., the serogroup B S. enterica Typhimurium and the serogroup D S. enterica Pullorum and Gallinarum and S. enterica Enteritidis. In contrast, researchers have shown that the antibody response to salmonella gm flagellin occurs earlier than responses to cell wall antigens (40), peaks early, and within 10 weeks reverts to low levels (2). The advantage of the gm flagellin antigen is that it is shared by no other salmonellae invasive for chickens (i.e., possesses analytical specificity) and is found on only a few salmonella serotypes (Menston, Derby, and Montevideo) that occasionally cause transient intestinal infections (32). Thus, gm flagellin would be useful to differentiate infection by related serogroup B and D infections in poultry (8). Also, gm flagellin is highly antigenic and is readily recovered in relatively pure form from the surfaces of the salmonella bacteria; it would be inexpensive to produce for widespread use.

After the rapid Pullorum assays to screen flocks for infection with S. enterica Enteritidis, the next improvement in S. enterica Enteritidis diagnosis was the IgG ELISA (8, 11); ELISAs are inherently more analytically sensitive than macroscopic agglutination tests, such as the Pullorum test for detecting IgG responses. Other researchers have shown that gm flagellin is a useful antigen in the ELISA. Van Zijdeveld et al. showed that an indirect gm flagellin ELISA was better than an indirect ELISA, using S. enterica Enteritidis LPS to detect S. enterica Enteritidis-infected chicks and adult hens (36). In contrast to our work, they used only experimentally infected birds and thus had no results available from any field studies; field studies would be needed for D-Sn and D-SP determinations in a real-world situation. They also tested a blocking ELISA using gm flagellin which had better analytical specificity than the indirect gm flagellin ELISA. A field study in Holland by this group further supported the usefulness of the flagellin ELISA over culture for the detection of S. enterica Enteritidis infection in commercial flocks (37). Using the gm flagellin KELA, we took part in a World Health Organization-sponsored inter-laboratory comparison of different ELISA antigens to determine if birds were S. enterica Enteritidis infected (11); specific-pathogen-free chicken sera were tested from S. enterica Enteritidis-infected birds having a high antibody titer to S. enterica Enteritidis cell wall antigens, medium S. enterica Enteritidis titer, or specific-pathogen-free, uninfected birds with low titers to S. enterica Enteritidis. Also, sera from S. enterica Enteritidis-infected commercial birds having high S. enterica Enteritidis cell wall titers and uninfected commercial birds with low titers were tested in the gm flagellin KELA. In all cases, the KELA accurately determined the infection history of the birds (11).

We used the gm flagellin antigen coupled with a rigorous method in the KELA to normalize data and thus were able to reduce the effects of variables, such as ambient temperature,
We calculated results from a standard curve generated by four serum controls using linear regression analysis. This approach is more precise than that used in most indirect ELISA assays because it does not rely on the use of only a single serum control to which all test sample values are normalized. KELA data are thus more consistent from day to day and from run to run.

Table 3 shows the high level of analytical specificity (lack of cross-reactivity) of the gm flagellin KELA compared with the Pullorum serologies. This commercial flock, flock 2, was infected with endemic S. enterica Enteritidis but also had other salmonella serotypes present in the flock. The Heidelberg and Typhimurium serotypes share antigen 12 with S. enterica Enteritidis, as does the nonmotile serotype 4,12:–:–, but yet birds infected with these serotypes had undetectable levels of gm flagellin response at a cutoff of 60 in the KELA (NPV = 98.9% [Table 2]). The one S. enterica Enteritidis culture-positive bird failed to react in the KELA; it is assumed that the lack of KELA response is related to the stage of infection of the bird, i.e., perhaps the bird had just been exposed to S. enterica Enteritidis and had not yet developed an immune response to the S. enterica Enteritidis gm flagellin. It is also possible that this bird may not have become infected, depending on the S. enterica Enteritidis strain’s inherent virulence and infectious dose, etc. Rather, the culture isolation of S. enterica Enteritidis from this bird’s cecum may have reflected merely environmental exposure. The Pullorum serology from this bird would appear to somewhat support this latter assumption, i.e., perhaps no Pullorum response reflects no infection, whereas a chronically infected bird would be expected to have developed some long-lasting S. enterica Enteritidis cell wall antibody that would be detectable at least at a low level in the Pullorum assay. The six positive samples found on the Pullorum serology for this bird were not further tested, but future studies may include the use of other seasons and environments.

Table 4 records the results of the third flock of birds infected with the endemic Pullorum serotype (of unknown S. enterica Enteritidis status) and tells us more about the analytical specificity of the gm flagellin assay. Birds 3, 9, 11, and 12 all had low responses except for bird 4, which was classified as negative due to the high specificity of the KELA.
KELA slopes and negative Pullorum serologies, suggesting lack of antibody to gm flagellin in Pullorum-infected birds; with a cutoff of 60 in the KELA, all but one bird (bird 4) would be considered gm flagellin negative. Bird 4 also did not react in the Pullorum test but was positive in the KELA. It is unknown if S. enterica Enteritidis was recovered from bird 4 to account for the positive KELA result. It is also unknown if S. enterica Pullorum was ultimately recovered from every one of the 12 birds at the NVSL. False-negative and false-positive Pullorum test results are problematic (38). The other birds tested with the KELA from this flock all had low KELA slopes in the negative range.

The usefulness of the KELA depends on the selection of a cutoff point that is suitable for the intended purpose: a low cutoff leads to a high D-Sn with a low D-Sp (useful for a screening assay), while a high cutoff results in a low D-Sn and a high D-Sp (confirmatory assay). The gm flagellin KELA was designed for use on a flockwide basis (not for individual birds), such as when a flock is involved in a trace back from a human outbreak of foodborne disease. In light of the diagnostic sensitivity and specificity problems of the Pullorum tests, a more sensitive and specific assay was needed for assigning a flock infection status. The gm flagellin KELA with two cutoffs, one for screening (we typically use a slope of 10) and another for confirmation (use a slope of 140), was thus conceived. Also, if one uses a single cutoff of 60 slope units, the KELA gives a better D-Sp (91.6%) than currently is available in the Solvay and Vineland assays (55.5% and 75.3%, respectively). This enhanced specificity results in a predictive value for a positive KELA test result that is two to three times higher than that of the other assays (at a 2.45% prevalence of infection). Table 2 shows that birds with KELA slope units greater than about 200 units have a PPV of 84.4%, whereas the Pullorum assays have PPVs of from about 5 to 8% (data not shown in Table 1 but can be calculated).

The D-Sn of KELA with a cutoff of 10 slope units (95.2%) is about equivalent with that of the Solvay assay (94.9%) but much better than the Vineland assay (62.0%). With this cutoff, the NPV of a KELA test result, with a prevalence of 2.45%, is 100%. With a cutoff of 60, we have shown that the gm flagellin KELA has a greater D-Sp than either of the two commercially available rapid Pullorum slide tests (Tables 1 and 2); the KELA also has a greater D-Sn than the Vineland but not the Solvay Pullorum test (the Solvay test’s increased sensitivity may be attributed to its higher false-positive rate); this agrees with earlier work by Timoney et al. (32). It also corroborates the work of Gast and Beard (18) and Mutalib et al. (25) who showed that the Solvay antigen was more sensitive than the Vineland antigen, but the Solvay antigen also had a higher false-positive rate than the Vineland antigen (44.5% versus 24.7% in this study).

Even though serology is a quicker method to establish the infection status of a flock, national regulatory agencies usually require bacterial culture to provide the definitive evidence of flock status. Antemortem bacterial cultures of cloacal swabs, eggs, and environmental swabs and postmortem cultures of internal tissues (ovary, oviduct, liver, gall bladder, heart, pericardial sac, and sometimesecum) are fairly sensitive techniques for S. enterica Enteritidis detection on a flockwide basis (33, 34). S. enterica Enteritidis is often found in extraintestinal sites in the bird, e.g., ovary, oviduct, liver, spleen, (5, 27, 29) in addition to the intestinal tract. However, some birds’ ceca (intestines) are colonized by S. enterica Enteritidis, while other internal tissues (e.g., ovary and oviduct) are not (6, 7, 38). This fact has consequences for regulatory programs such as that conducted by the USDA SE Task Force (begun in 1990 by Veterinary Service, Animal and Plant Health Inspection Service, USDA) whose field protocol does not require the testing of any intestinal or cecal tissues (33). Spent hen surveys from areas of the country where S. enterica Enteritidis is endemic have shown a high prevalence of S. enterica Enteritidis cecal carriage in hens (17, 39). Truly infected birds having antibody to S. enterica Enteritidis but culture negative (by the SE Task Force protocol), could account for some of the relatively high KELA slopes among culture-negative birds. Misclassification of bird infection status slightly reduces the calculated D-Sp of the KELA and also has an impact on the D-Sn. Other possible reasons for negative salmonella cultures are that individual birds excrete S. enterica Enteritidis intermittently or birds eliminate the infection altogether; in all of these cases, culture results may appear negative but bird sera may actually possess antibodies to gm flagellin and to other cell wall antigens of S. enterica Enteritidis. Were the birds classified correctly for infection status, the performance characteristics of the KELA would improve.

In summary, the gm flagellin KELA is a useful flock screening tool for use in commercial poultry flocks to detect S. enterica Enteritidis infection. The antigen is relatively easy to produce, and the KELA format provides excellent within-run and between-run reproducible testing. The antigen also confers improved analytical specificity to serology, making it useful to distinguish S. enterica Enteritidis from other salmonella infections. With the use of a competitive KELA format, perhaps that analytical specificity can be enhanced even further. The gm flagellin KELA will be a useful complement for state flock surveillance and testing programs and for screening flocks involved in trace backs from human foodborne outbreaks.

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