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Simple and rapid methods for detecting *Salmonella enteritidis* in raw eggs

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Abstract

The Centers for Disease Control and Prevention estimates there were 300,000 cases of *Salmonella enteritidis* (SE) in 1997. Egg products were associated with many of the cases. To address this problem, many producers implemented flock surveillance of the SE situation at their facilities. A rapid and simple method for detecting SE from poultry samples is critical for the effective implementation of such testing strategies. A lateral flow device for the detection of SE utilized in this study was manufactured by Neogen, Lansing, MI. The test panel is a presumptive qualitative test system that detects only members of Group D1 *Salmonella* species. A series of studies were conducted to optimize the test procedure for raw eggs with different sample preparations. A novel antigen extraction method was developed for use with the test panel kit. The detection limit of the test panel kit was increased approximately tenfold when the extraction method was used. Detection of SE was 100% in raw egg pools inoculated with 10 SE cells per ml of egg and incubated at a 1:10 ratio in buffered peptone water (BPW) or tetrathionate brilliant green broth (TBG) for 24 h at 37 °C. The developed lateral flow test kit could provide a simple, rapid, and inexpensive method for egg producers and processors to test specifically for *Salmonella* group D1 serovars, such as SE, in egg samples.

Keywords: *Salmonella enteritidis*; Rapid detection; Egg

1. Introduction

Salmonella enteritidis (SE) emerged as a major cause of human salmonellosis in the United States throughout the 1980s and 1990s. Of the 360 SE outbreaks with a confirmed source, 279 (82%) were associated with raw or undercooked shell eggs between 1985 and 1998 (CDC, 2000). Grade-A shell eggs have been attributed to the human salmonellosis problem due to the ability of SE to infect ovarian tissues and to be deposited into the developing egg. It is difficult to detect flocks or individual birds that may be infected with SE and lay contaminated eggs since laying hens seldom show any clinical symptom of SE infection. Studies indicate that the percentage of infected eggs is extremely low and

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sporadic with most contaminated eggs having small number of SE (Humphrey, 1994). Culturing environmental samples or bird tissues is not sufficient for detecting all birds laying SE infected eggs. The only method of directly proving that a flock is laying SE-contaminated eggs is by culturing the organism from eggs, although the percentage of infected eggs is extremely low.

Conventional methods for detection of salmonellae, including SE, from eggs take 5–7 days and are labor-intensive, involving isolation of the organism using pre-enrichment as well as selective enrichment procedures and serological confirmation tests. Detection of small numbers of SE in inoculated pools of egg contents was successful using direct plating of incubated egg pools onto agar plates, although sensitivity was enhanced with enrichment (Gast, 1993). Supplementing pools of egg contents with iron in the form of ferrous sulphate and concentrated enrichment broth has been suggested to improve detection of SE from raw eggs without using enrichment broth (Gast and Beard, 1992; Cudjoe et al., 1994; Gast and Holt, 1998). More rapid methodologies also have been developed using ELISA and PCR techniques (Holt et al., 1995; Woodward and Kirwan, 1996). In contrast to conventional methods, these tests can detect SE in 2 days. However, they are not free of drawbacks. The tests involve time-consuming enrichment incubations, exhibit varying degrees of cross-reactions and both systems have been known to produce false positive reactions. The sensitivity of rapid detection methods for SE in eggs was substantially decreased due to the interference of egg contents (Brigmon et al., 1995). Cudjoe et al. (1994) found that the more viscous, undiluted mixtures of eggs showed the highest bead loss compared with diluted samples when immunomagnetic beads were applied to recover SE from raw eggs. We have been collaborating with Neogen, Lansing, MI, to develop an antibody-based lateral flow assay (LFA) system to rapidly detect Group D *Salmonellae* from poultry samples. The current study was undertaken to evaluate a simple and rapid lateral flow device for its ability to detect SE (a group D) and not other *Salmonella* serovars from eggs. We also examined the factors that either enhance or decrease the detection capability of the test panel. Direct detection of SE from egg without enrichment broth using a novel antigen extraction method is also described.

2. Materials and methods

2.1. Bacterial strains

S. enteritidis phage type 13a (SE, serotype O: D1, 9, 12; H: f, g, t), *Salmonella kentucky* (SK, sero type O: C_3 (8), 20; H: phase1-i, phase 2-z), and *Salmonella typhimurium* (ST, sero type O: B 1, 4, 5, 12; H: phase1-i, phase 2-1, 2) were obtained from stocks kept at the Southeast Poultry Research Laboratory, Athens, GA (SEPRL). Purity of the cultures was confirmed using serotyping (Difco Laboratories, Detroit, MI). Cultures were grown in tryptic soy broth (TSB; Difco) and viable counts were obtained by plating tenfold serial dilutions of broth cultures onto nutrient agar (Difco) and incubating the plates at 37 °C overnight. Whenever necessary, cultures were diluted with phosphate buffered saline (PBS, pH 7.2) as needed and counts were made as above.

2.2. Homogenized egg preparation

Eggs were collected from the SEPRL specific-pathogen-free flock and were soaked in 70% isopropanol (Sigma, St. Louis, MO) for 1 min and air-dried to disinfect the shells before they were broken. The egg contents (10 eggs) were pooled and homogenized for 30 s in a Stomacher Model 400 Lab Blender (Tekmar, Cincinnati, OH). The blended egg contents were kept at 4 °C before used for experiments.

2.3. Lateral flow assay (LFA)

A portion (100 μl) of samples was placed into the round sample port of the test device, initiating a lateral flow through a reagent zone containing specific anti-*S. enteritidis* antibodies conjugated to colloidal gold particles. The antibody used in the device was developed in our lab and described in a previous study (Holt et al., 1995). Test results were interpreted as positive or negative, scored on a scale from 0 (−) to 3 (+++) in proportion to the color intensity determined visually using SE pure culture,

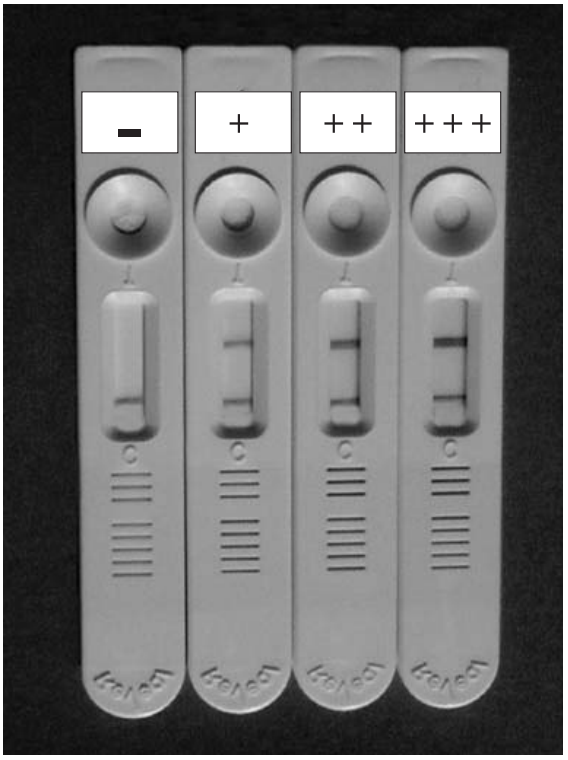

Fig. 1. The scale of band intensity. −: Negative control with 1×10^8 CFU/ml of *S. typhimurium*; +: positive with 1×10^7 CFU/ml of *S. enteritidis*; ++: positive with 5×10^7 CFU/ml of *S. enteritidis*; +++: positive with more than 1×10^8 CFU/ml of *S. enteritidis*.

after 20 min from the addition of the sample culture (Fig. 1).

2.4. Variables affecting sample flow on panel

2.4.1. Dilution of egg contents with PBS

Egg contents (900 ul) were artificially seeded with 10^9 cell of SE (100 ul) to obtain a final concentration of 10^8 cells/ml. Undiluted, two-, four-, and tenfold dilutions of the inoculated egg contents were prepared in PBS and 100 ul of each sample was administered to the test panel to find the optimum running ability of the samples.

2.4.2. Antigen extraction with a mixture of fatty acids

A mixture of fatty acid (v/v) was prepared by combining 2 parts of oleic acid (Aldrich, Milwaukee, WI) and 1 part of caprylic acid (Sigma). One milliliter of the inoculate egg contents and 0.5 ml of the fatty acid mixture were stirred together for 1 min with an applicator stick or vortexed for 30 s in a 1.7-ml Eppendorf centrifuge tube to allow the acid to react and form an ester with reactive OH groups. The reaction forms water as a by-product and at the same time breaks up the structure of the egg contents as it releases the bacteria into the aqueous phase. The reaction is near completion when the mixture becomes viscous, generally between 1 and 2 min at room temperature. The tube is then centrifuged for 5 min at $10{,}000 \times g$ in a microcentrifuge. Centrifugation forms an oily layer with yellow carotenes and oil solubles on the top, a soapy hard layer of esters in the middle, and a clear aqueous layer with soluble bacterial antigen and a pellet of bacterial debris on the bottom (Fig. 2). The aqueous layer containing soluble antigen was removed with a 1 ml syringe and then 100 ul of the solution was applied into the test panel port.

2.5. Detection limit of the test panel

The homogenized egg contents (900 ul) were inoculated with 100 ul of serially diluted SE to make final concentration of 10^5–10^8 cells/ml, diluted with 9 ml PBS, and tested on the panel as described previously. A second 1 ml aliquot of egg contents containing each

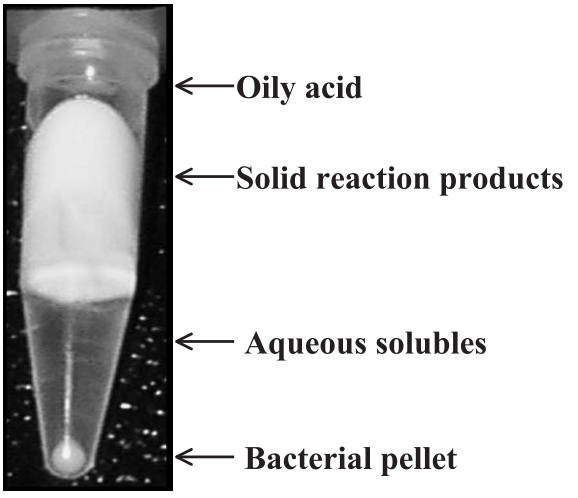

Fig. 2. Extraction of *S. enteritidis* antigen from whole egg contents.

SE concentration was used for antigen extraction and tested on the panel as explained previously.

2.6. LFA combined with enrichment method

Homogenized egg samples (100 ml) were artificially inoculated with 100 ul suspensions of SE cells to make final a concentration of approximately 10 cells/ml. The spiked samples were mixed with buffered peptone water (BPW) giving egg to broth ratios of 1:2, 1:4 and 1:10, and further stomached as described before. After 24 h incubation at 37 °C, 100 ul of each broth culture treatment was applied onto the test panel and the results were determined after 20 min.

2.7. Specificity and detection capacity of LFA combined with different enrichment media

Twenty five milliliters of homogenized eggs were artificially inoculated with 10 cells of SE in 100 ul PBS and mixed with 225 ml of either BPW or tetrathionate brilliant green broth (TBG; Difco). The control samples received 100 ul of sterile PBS. The egg and enrichment broth mixtures were incubated at 37 °C and sampled at 24 and 48 h, respectively. The lateral flow assay was performed as described previously using 100 ul of the individual enrichment broth cultures from each sampling time. For the tetrathionate enrichment broth samples, vortexing and autoclaving steps were applied to determine if the extra steps could improve the sensitivity. Two other *Salmonella* serovars, SK and ST, were used to examine the specificity of the test panel using the same procedure as described before.

3. Results and discussion

The minimum concentration of SE to generate a positive band on the test panel was approximately 10^7 cell/ml in pure culture (Fig. 1) and no cross-reactivity was detected with two other *Salmonella* serovars, ST and SK in pure culture (Data not shown). Since eggs are an important source of human food-borne SE infections, the detection of the organism directly from egg samples was primary on the list of tasks. One variable factor, which is crucial for the success of the test panel, is the ability of the sample to be effectively wicked down the membrane within the panel so that the organism present in the sample can interact with the detection antibodies. Whole egg does not effectively transit the panel due to the viscous nature of the sample (Table 1). Progressively diluting the egg with PBS reduces the viscosity of egg contents and allows the more effective wicking of sample. A 1:10 dilution of sample with PBS worked the best. However, the intensity of the positive band appeared weaker as the dilution was increased resulting in low sensitivity to approximately 10^8 cell/ml. Similar drawbacks of immunoassays as a result of high viscosity of egg were reported in a previous study (Brigmon et al., 1995) when they used ELISA to detect SE in eggs. In that study, a minimum of 10^7 cells/ml were required for a positive reaction with 10% homogenized whole eggs

Table 1
Direct detection of *S. enteritidis* from whole egg contents using dilution technique with PBS and antigen extraction using fatty acid mixture (2 parts of oleic acid and 1 part of caprylic acid)

	Whole egg	Egg/PBS (1:2)	Egg/PBS (1:4)	Egg/PBS (1:10)	Egg/acid[a] (2:1)
Band intensity[b]	−	++	++	+	+++
Sample running performance[c]	poor	not good	good	very good	very good
Controls[d]	−	−	−	−	−

Egg contents were artificially spiked with SE at 10^8 SE cells per ml and serially diluted with PBS.

[a] The inoculated egg contents (1 ml) were combined with the fatty acid mixture (0.5 ml).

[b] Band intensity: −: negative, +: weak positive, ++: strong positive, +++: very strong positive (Fig. 1).

[c] Poor: liquid did not wick through to the bottom of the membrane; not good: it required more than 5 min to get all liquid to the bottom and the bars (positive and control lines) were not clear; good: liquid wicked through to the bottom, but the lines were not clear; very good: liquid wicked through readily and lines were clear to read.

[d] Samples not inoculated with *S. enteritidis*.

Table 2
Detection limit of direct detection of *S. enteritidis* from whole egg contents using dilution technique with PBS and antigen extraction with fatty acid mixture

Number of cells (CFU/ml)	Methods	
	Dilution technique	Fatty acid extraction
10^8	+++	+++
10^7	+	+++
10^6	–	++
10^5	–	+

while only 10^4 cells/ml of SE in pure culture were necessary for ELISA detection. This suggests that the albumen and lipids in yolk may act as blocking agents inhibiting antibody–antigen binding reactions in the ELISA. Extraction of bacterial antigen from egg contents using the organic fatty acid mixture provided excellent running capability and a stronger positive signal on the test panel kit (Table 1). The detection limit of the test kit increased more than tenfold up to 10^6–10^5 cells/ml in whole egg contents using the acid extraction technique compared with tenfold PBS dilution (Table 2). Egg pools inoculated with 10 cells/ml of SE were detected as SE-positive on the test panels after 24 h incubation at 37 °C using both the antigen extraction method with fatty acid mixture and the dilution technique with PBS (1:10, Table 3). It is noteworthy that the direct antigen extraction method generated a stronger signal than the dilution technique (Table 3). This technique could lead to relatively rapid and inexpensive detection of SE in whole eggs by saving enrichment media and reducing the incubation time. However, it has been reported that some SE strains did not multiply above 10^6 cells/ml in liquid whole egg after 24 h incubation at 37 °C without iron supplementation (Gast and Holt, 1995). Therefore when liquid whole eggs are incubated without enrichment broth, a longer incubation time and iron supplementation would be required to allow SE to multiply to reach the cell density necessary for efficient detection. Although egg contents provide excellent nutrients for SE growth, use of enrichment broth helps maximize SE growth (Gast and Holt, 1998). Dilution of inoculated whole egg contents with buffered peptone water broth or tetrathionate broth at various ratios increased the number of SE well above the detection limit of the test panel kit after incubating for 24 h at 37 °C. The optimum results after 24 h enrichment with the panel kit were accomplished when the inoculated whole egg mixtures were incubated in BPW at 1:10 ratio without further dilution with PBS after 24 h enrichment (Table 4). However, we found that when BPW was used, a slight, but potentially problematic, reaction occurred in samples inoculated with SK or ST when 24 h (very faint positive) and 48 (weak positive) incubation at 37 °C were applied (Table 5).

Table 3
Comparison of detection capability of sample preparation methods

Samples	Methods	
	Dilution technique	Fatty acid extraction
Uninoculated eggs	– (3/3)	– (3/3)
Inoculated eggs[a]	+ (10/10)	+++ (10/10)

[a] Egg contents were artificially inoculated with 10 CFU/ml of *S. enteritidis* followed by incubating 24 h at 37 °C while control samples were not inoculated ($n = 10$).

Table 4
Comparison of detection capability of enrichment methods in conjunction with sample running

	Whole egg	Egg/BPW[a] (1:2)	Egg/BPW (1:4)	Egg/BPW (1:10)
Band intensity	–	+++	+++	+++
Sample running performance	poor	not good	good	very good
Controls	–	–	–	–

[a] Various dilutions of liquid whole egg samples were prepared using buffered peptone water and artificially inoculated with 10 *S. enteritidis* cells per ml followed by incubating 24 h at 37 °C while control samples were not inoculated ($n = 10$).

Table 5
Comparison of detection capability of different enrichment media

	SE		SK		ST	
	24 h	48 h	24 h	48 h	24 h	48 h
Control	–	–	–	–	–	–
BPW	+++	+++	+	+	+	+
Control	–	–	–	–	–	–
TBG	+++	+++	–	–	–	–

Liquid whole egg samples artificially inoculated with 10 CFU/ml of *S. enteritidis* (SE), *S. kentucky* (SK), *S. typhimurium* (ST) were diluted with buffered peptone water and tetrathionate brilliant green (1:10) followed by incubating 24 and 48 h at 37 °C while control samples were not inoculated ($n = 10$).

No false positive samples were observed when TBG was used instead of BPW after 24 and 48 h incubation, respectively (Table 5). Therefore, TBG appears superior to BPW in terms of specificity. This detection was diminished if the tetrathionate samples were shaken while dramatically enhanced if the samples were autoclaved prior to application to the panel (Data not shown). The presence of the two other *Salmonella* serovars in the mix along with SE did not diminish the detection of the SE (data not shown).

The current study showed that a simple and rapid test panel kit could be successfully used to detect SE in eggs in conjunction with various enrichment methods. A novel method for extracting antigen out of egg contents has potential to increase the sensitivity of the test panel kit or other rapid detection systems by avoiding the viscous nature of egg contents and reduce costs of detection of SE by avoiding an enrichment broth. The advantages of the extraction procedure are (1) to provide effective wicking of sample, (2) to generate very clear diagnostic bars (the positive bar) on the strip and (3) to increase sensitivity by avoiding dilution of egg contents in PBS. The developed lateral flow test kit could provide a simple, rapid, and inexpensive method for egg producers and processors to test specifically for *Salmonella* group D1 serovars, such as SE, in egg samples.

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