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Research Note—

Validation of Single and Pooled Manure Drag Swabs for the Detection of *Salmonella* Serovar Enteritidis in Commercial Poultry Houses

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SUMMARY. Single swabs (cultured individually) are currently used in the Food and Drug Administration (FDA) official method for sampling the environment of commercial laying hens for the detection of *Salmonella enterica* ssp. serovar Enteritidis (*Salmonella* Enteritidis). The FDA has also granted provisional acceptance of the National Poultry Improvement Plan's (NPIP) *Salmonella* isolation and identification methodology for samples taken from table-egg layer flock environments. The NPIP method, as with the FDA method, requires single-swab culturing for the environmental sampling of laying houses for *Salmonella* Enteritidis. The FDA culture protocol requires a multistep culture enrichment broth, and it is more labor intensive than the NPIP culture protocol, which requires a single enrichment broth. The main objective of this study was to compare the FDA single-swab culturing protocol with that of the NPIP culturing protocol but using a four-swab pool scheme. Single and multilaboratory testing of replicate manure drag swab sets ($n = 525$ and 672, respectively) collected from a *Salmonella* Enteritidis–free commercial poultry flock was performed by artificially contaminating swabs with either *Salmonella* Enteritidis phage type 4, 8, or 13a at one of two inoculation levels: low, $\bar{x} = 2.5$ CFU (range 2.5–2.7), or medium, $\bar{x} = 10.0$ CFU (range 7.5–12). For each replicate, a single swab (inoculated), sets of two swabs (one inoculated and one uninoculated), and sets of four swabs (one inoculated and three uninoculated), testing was conducted using the FDA or NPIP culture method. For swabs inoculated with phage type 8, the NPIP method was more efficient ($P < 0.05$) for all swab sets at both inoculation levels than the reference method. The single swabs in the NPIP method were significantly ($P < 0.05$) better than four-pool swabs in detecting *Salmonella* Enteritidis at the lower inoculation level. In the collaborative study ($n = 13$ labs) using *Salmonella* Enteritidis phage type 13a inoculated swabs, there was no significant difference ($P > 0.05$) between the FDA method (single swabs) and the pooled NPIP method (four-pool swabs). The study concludes that the pooled NPIP method is not significantly different from the FDA method for the detection of *Salmonella* Enteritidis in drag swabs in commercial poultry laying houses. Consequently based on the FDA's *Salmonella* Enteritidis rule for equivalency of different methods, the pooled NPIP method should be considered equivalent. Furthermore, the pooled NPIP method was more efficient and cost effective.

RESUMEN. Validación del uso de hisopos de arrastre de gallinaza, individuales y agrupados para la detección de *Salmonella* serovar Enteritidis en casetas avícolas comerciales.

Las muestras de hisopos individuales (cultivadas individualmente) se utilizan actualmente en el método oficial de la Administración de Alimentos y Medicamentos de los Estados Unidos (FDA por sus siglas en inglés) para el muestreo ambiental en casetas de gallinas de postura comerciales, para la detección de *Salmonella enterica* ssp. serovar Enteritidis (*Salmonella* Enteritidis). La FDA también ha concedido la aceptación provisional de la metodología incluidas en el Plan Nacional de Mejora Avícola (NPIP) para el aislamiento e identificación de Salmonella de muestras tomadas del ambiente de casetas de aves de postura. El método NPIP, así como el método de la FDA, requieren del cultivo de hisopos simples para el muestreo ambiental de casetas de aves de postura para *Salmonella* Enteritidis. El protocolo de cultivo de la FDA requiere un de un caldo de enriquecimiento para un cultivo de varios pasos y es más laborioso que el protocolo de cultivo del NPIP, que requiere un caldo simple de enriquecimiento. El principal objetivo de este estudio fue comparar el protocolo de cultivo de la FDA utilizando hisopos simples con el protocolo de cultivo del NPIP pero utilizando un esquema de muestras agrupadas con cuatro hisopos. Se realizaron pruebas en laboratorio único y en varios laboratorios para analizar grupos de muestras de hisopos de arrastre de gallinaza con sus réplicas ($n = 525$ y 672, respectivamente), que fueron recolectados de una parvada de aves comerciales libre de *Salmonella* Enteritidi. Los hisopos se contaminaron artificialmente con *Salmonella* Enteritidis fagotipos 4, 8 o 13a en uno de dos niveles de inoculación: bajo con 2.5 UFC (rango 2.5 a 2.7), o medio con 10.0 UFC (rango 7.5-12). Para cada repetición, se llevó a cabo el análisis un solo hisopo (inoculado), o por conjuntos de dos hisopos (uno inoculado y uno sin inocular) o conjuntos de cuatro hisopos (uno inoculado y tres sin inocular), utilizando el método de cultivo de la FDA o del NPIP. Para hisopos inoculados con el fagotipo 8, el método del NPIP fue el más eficiente ($P < 0.05$) en comparación con el método de referencia para todos los conjuntos de hisopos, con ambos niveles de inoculación. Los hisopos individuales con el método NPIP fueron significativamente mejores ($P < 0.05$) que las muestras agrupadas con cuatro hisopos para la detección de *Salmonella* Enteritidis con el nivel de inoculación bajo. En el estudio en colaboración ($n = 13$ laboratorios) utilizando los hisopos inoculados con *Salmonella* Enteritidis fagotipo 13a, no hubo diferencia significativa ($P > 0.05$) entre el método de la FDA (hisopos individuales) y el método del NPIP con muestras agrupadas (muestras con cuatro hisopos). El estudio concluye que el método del NPIP con muestras agrupadas no es significativamente diferente al

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método de la FDA para la detección de *Salmonella* Enteritidis en hisopos de arrastre de casetas de aves comerciales. En consecuencia con base en la regla de equivalencia de diferentes métodos para *Salmonella* Enteritidis de la FDA, el método del NPIP con muestras agrupadas debe considerarse equivalente. Además, el método del NPIP con muestras agrupadas fue más eficiente y rentable.

Key words: *Salmonella* Enteritidis, drag swabs, enrichment broth, validation, NPIP method, FDA method

Abbreviations: CFU = colony forming units; FDA = Food and Drug Administration; NPIP = National Poultry Improvement Plan; POD = probability of detection; PT = phage type; TSA = trypticase soy agar

In the United States, *Salmonella* is the leading cause of bacterial food-borne disease, with approximately 1.4 million human cases each year since 1996 (21). It is estimated that 182,060 illnesses due to egg-associated *Salmonella* Enteritidis occurred during 2000 (15). The major food vehicle for *Salmonella* Enteritidis is shell eggs, because 80% of the *Salmonella* Enteritidis outbreaks are when the vehicle is known and approximately 50,000 to 110,000 cases are egg associated in the United States each year (1,15). In 2010, a large *Salmonella* Enteritidis outbreak was associated with consumption of shell eggs, with more than 1,800 reported cases (3).

The most common phage types (PTs) collected from egg-related outbreaks in the northeast United States between 1988 and 1989 were PT8 and 13a, comprising 48% and 20%, respectively (12). PT4 comprised 60% of the PTs in the ecosystem of southern California (13), and it has been the cause of a number of outbreaks of *Salmonella* Enteritidis (2,14,18). PT4 has been the predominant PT in Western Europe and Japan (6,22); however, its prevalence has been declining with the rise of PT14b and PT21. In fact, from 1998 to 2003, the percentage of *Salmonella* Enteritidis isolates identified as PT4 decreased from 61.8% to 32.1%, whereas PT14b and PT21 increased by 276% and 137%, respectively (11).

Salmonella Enteritidis is not considered to be a significant cause of morbidity or mortality in commercial layer flocks in contrast to other avian-adapted salmonellas such as *Salmonella* Gallinarum and *Salmonella* Pullorum (16,17). The major threat to the producer is that the farm could be implicated in a traceback investigation, which may result in diversion of eggs to breaker plants and the associated costs in fulfilling the regulatory compliance. If *Salmonella* Enteritidis can be detected through routine testing of environmental samples, then increased risk factors can be determined and sanitary measures can be placed to help stop the spread of *Salmonella* within the poultry farm.

Detecting *Salmonella* Enteritidis in the environment of laying houses is viewed as an essential component of preharvest biosecurity. Currently the Food and Drug Administration (FDA) method is the official method for testing of *Salmonella* Enteritidis in the environment of laying hens (8). In addition, FDA has granted provisional acceptance of the National Poultry Improvement Plan's (NPIP) *Salmonella* isolation and identification methodology for samples taken from table-egg layer flock environments (20). Both these methods require the environmental sampling and testing of laying houses using single swabs. The NPIP method is simpler to perform, less labor intensive, more efficient, and more cost effective. *Salmonella* negative results using the NPIP method can be achieved within 4 days, which translates to shorter turnaround time that can allow a producer to take action on the farm more quickly. The main objective of this study was to compare the FDA single-swab culturing protocol with that of the NPIP culturing protocol but using a four-swab pool strategy.

MATERIALS AND METHODS

Collection of manure drag swabs from poultry laying houses. The sampling of drag swabs was performed according to the FDA Guidance Document for sampling and testing for *Salmonella* Enteritidis (10). The drag swab consisted of sterile 12 ply gauze pads (10 cm × 22 cm when unfolded; Johnson & Johnson Nu Gauze, 10.2 cm × 10.2 cm, four-ply; Skilman, NJ). Each swab was prepared aseptically using latex gloves and moistened with canned skim milk immediately before sampling. Throughout the study, drag swabs were collected per the FDA rule from a cage-free house with concrete floor and side curtains that was previously known to be *Salmonella* Enteritidis–free based on flock monitoring history. Using a sterile technique, the moist gauze pad was attached to the alligator clip, which was attached to one end of the rod. Next, the individual walked through the house "dragging" the gauze pad in the house environment where the most manure was present. Moist drag swabs were labeled as sets of single-, two-swab, and four-swab pools in separate Whirl-Pak® bags (Fisher Scientific, Fair Lawn, NJ) and placed in a cooler with gel packs until the required numbers of swabs were achieved for each set of single-, two-, and four-swab pools. Whirl-Pak bags were placed in a cooler and transported to the laboratory. Both the metal rod and the alligator clip were cleaned and disinfected between sampling by being wiped down with alcohol-soaked gauze pads.

Bacterial strains and test inoculum preparation. Two *Salmonella* Enteritidis PTs obtained from the FDA (PT4, no. 421 chicken liver, Scotland; and PT8, no. 13183 chicken breast, Iowa) and a third PT (PT13a, identified as Iso-001, poultry environment) from the National Veterinary Services Laboratories, Ames, IA, were used for inoculating swabs.

The medium dose (range 7.5–12 CFU/ml) and low dose (range 2.5–2.7 CFU/m) of PT4, 8, or 13a were prepared as previously described (4,5). A single colony of *Salmonella* Enteritidis (PT4, 8, or 13a) was streaked onto a trypticase soy agar (TSA) plate and incubated at 35–37 C for 18–24 hr. Using a cotton swab applicator stick, a few colonies were picked up to make a cell suspension with 2.5 ml 0.85% normal saline and adjusted to the O.D. 0.5 McFarland Standard. From this suspension, a 1 to 10 dilution with 0.85% normal saline from 10^{-1} (1/10) to 10^{-6} (1/1,000,000) was prepared. To determine the CFU/ml, 100 µl from 10^{-5} diluent was transferred onto a TSA plate and spread by Hockey stick. This procedure was repeated on three TSA plates, and plates were incubated at 37 ± 2.0 C for 18–24 hr. The average plate count of the three plates after 24 hr gave the CFU/sample. The "medium-level" inoculants were prepared by making 1 to 50 dilutions from 10^{-5} diluents.

Table 1. Trial 1: Sample matrix and enrichment scheme for NPIP culture method.

No. of replicates	Inoculum	No. of swabs/bag[A]	Enrichment (ml)	Enrichment (hr)
50	PT 4	1	100	20–24
50	PT 4	2	200	20–24
50	PT 4	4	300	20–24
25	Uninoculated	1	100	22–24
50	PT 8	1	100	20–24
50	PT 8	2	200	20–24
50	PT 8	4	300	20–24
25	Uninoculated	1	100	20–24
50	PT 13a	1	100	20–24
50	PT 13a	2	200	20–24
50	PT 13a	4	300	20–24
25	Uninoculated	1	100	20–24

[A]One drag swab = ~25 g.

Table 2. Trial 2: Sample matrix and enrichment scheme.

Analysis method	No. of replicates	Inoculum	No. of drag swabs[A]	Enrichment broth	Enrichment time (hr)	Secondary enrichment time (hr)
FDA	50	PT 8	1	100	22–24	22–24
	50	PT 8	4	300	22–24	22–24
	25	Uninoculated	1	100	22–24	22–24
NPIP	50	PT 8	1	100	22–24	
	50	PT 8	4	300	22–24	

[A]One drag swab = ~25 g.

One swab from each set of drag swab samples was spiked with 0.5 ml of the medium dose (150 µl of the stock suspension was added to 7.35 ml normal saline for a final volume of 7.5 ml). The "low-level" inoculants were prepared by making 1 to 20 dilutions from 10^{-6} diluents. One swab from each of the set of drag swab samples was spiked with 0.5 ml of the low-level dose (400 µl of the stock suspension was added to 7.6 ml normal saline for a final volume of 8 ml).

Inoculations of swabs. Single or sets of two (one inoculated and one uninoculated) or four (one inoculated and three uninoculated) swabs were inoculated with medium dose $\bar{x}$ = 10.0 CFU (range 7.5–12) or low dose $\bar{x}$ =2.5 CFU (range 2.5–2.7) of PT4, 8, or 13a, following Tables 1, 2, and 3. All swabs were then stored at 4 C for 48 hr allowing for inoculated swabs to age.

Single laboratory validation. Trial 1 (NPIP method), consisted of 450 sets of drag swabs (= 25 replicates × 2 levels of inoculations × 3 matrices [single-, two-, and four-swab pools] × 3 PTs [4, 8, and 13a]) and 75 uninoculated single swabs (controls) (= 25 swabs for each PT). For inoculation of swabs, medium dose $\bar{x}$ = 9.5 CFU (range 7.5–12) and low dose $\bar{x}$ = 2.6 CFU (range 2.5–2.7) were used. All swabs in this trial were tested by the NPIP method (Table 1).

In trial 2, 200 sets of drag swabs were used (25 replicates × 2 levels of inoculations [medium and low] × 2 matrices [single- and four-swab pools] × 2 methods of analysis [FDA and NPIP]). The swab sets were inoculated with PT 8, medium dose $\bar{x}$ = 10.0 CFU (9.6–10. 4) or low dose $\bar{x}$ = 2.5 CFU (2.5–2.7). Twenty-five sets of single uninoculated swabs were tested by the FDA method, as negative controls (Table 2).

Collaborative study and test portion distribution. Media and reagents were purchased from common sources and shipped to participants that were outside the FDA laboratory network or laboratories that did not have in-house media preparation capabilities. Each of the 14 collaborators received blinded inoculated 24 single- and 24 four-swab pool (one inoculated and three uninoculated) sets of eight low dose (2.5 CFU), eight medium dose (10.2 CFU) of PT13a, and eight uninoculated (48/laboratory, total). Culture slants of *Salmonella* Dublin and *Escherichia coli* were also sent as positive and negative controls to each participating laboratory. Participants were instructed to use the specified analysis protocol for each method (8,21), following Fig. 1 and Table 3. The samples were packed with cold packs and shipped on Monday for overnight delivery according to the category B Goods shipment regulations set forth by the International Air Transport Association. Each laboratory received the samples on Tuesday, and the analysis began on Wednesday. Samples were coded with initial of the FDA or NPIP method followed by a number (1–24, for each method) and affixed to the sample bag. Laboratories were not aware of the inoculation status of the swabs. The laboratories were asked to document the conditions of the samples as they received them, and no problems were reported.

Isolation and identification of *Salmonella* Enteritidis. Preparation of test portions and sample enrichment procedures were carried out at the host laboratory following Fig. 1 and Tables 1 and 2 and following Table 3 at each collaborative laboratory. Isolation and identification of *Salmonlla* Enteritidis for the FDA method (8) and the NPIP method (20) were performed as previously described.

The diagnostic sensitivity (proportion of samples testing positive, e.g., no. positive/no. tested) for each method was calculated (7) by inoculation level and PT for each pool size. A summary measure of diagnostic sensitivity for each method (combining all inoculation levels and PTs) was also reported. The summary measure of diagnostic sensitivity was compared (19) for each pool size using the Z-test for comparison of two proportions.

RESULTS AND DISCUSSION

Results of single-laboratory replicates of the single, pair, and four-swab pool tested by the NPIP method are shown in Table 4. The overall diagnostic sensitivity of the NPIP method was 87.3% on broth samples from single-swab inoculations (range: 72%–96% dependent on PT and inoculation level). The overall diagnostic sensitivity of the NPIP method did not decrease significantly (P = 0.15) when applied to broth samples incubated with two swabs (of which one was contaminated: SE = 81.3%) compared to broth samples incubated with a single swab (SE = 87.3%). Similarly, the overall diagnostic sensitivity was not significantly lower (P = 0.06) when applied to broth samples incubated with four swabs (of which one was contaminated: SE = 79.3%) compared to broth samples incubated with a single swab (SE = 87.3%). The overall diagnostic sensitivity of the NPIP method did not differ significantly (P = 0.66) in broth samples incubated with two-swab pools (SE = 81.3%) compared to broth samples incubated with four-swab pools (SE = 79.3%).

In a second trial the FDA and the NPIP methods were compared using PT8 at two levels of inoculums (Table 5). The overall diagnostic sensitivity for single swabs for the FDA method was 40% (range 24%–56%) and the four-swab pools 34% (range 20%–48%). The diagnostic sensitivity for the NPIP method for the single swab was 94% (range 88%–100%) and for the four-swab pools was 76% (range 60%–92%). Overall, the NPIP method of single swabs performed significantly better than its four-pool swabs ($P < 0.05$). For all swab sets and all inoculations the NPIP method had higher diagnostic sensitivity than the FDA method ($P < 0.05$).

Table 3. Collaborative study: Sample matrix and enrichment scheme.

Analysis method	No. of replicates/lab	Inoculum	No. of swabs per bag[A]	Enrichment broth (ml)	Enrichment time (hr)	Second enrichment time (hr)
FDA	16	PT 13a	1	100	22–24	22–24
	8	Uninoculated	1	100	22–24	22–24
NPIP	16	PT 13a	4	300	22–24	
	8	Uninoculated	4	300	22–24	

[A]One drag swab = ~25 g.

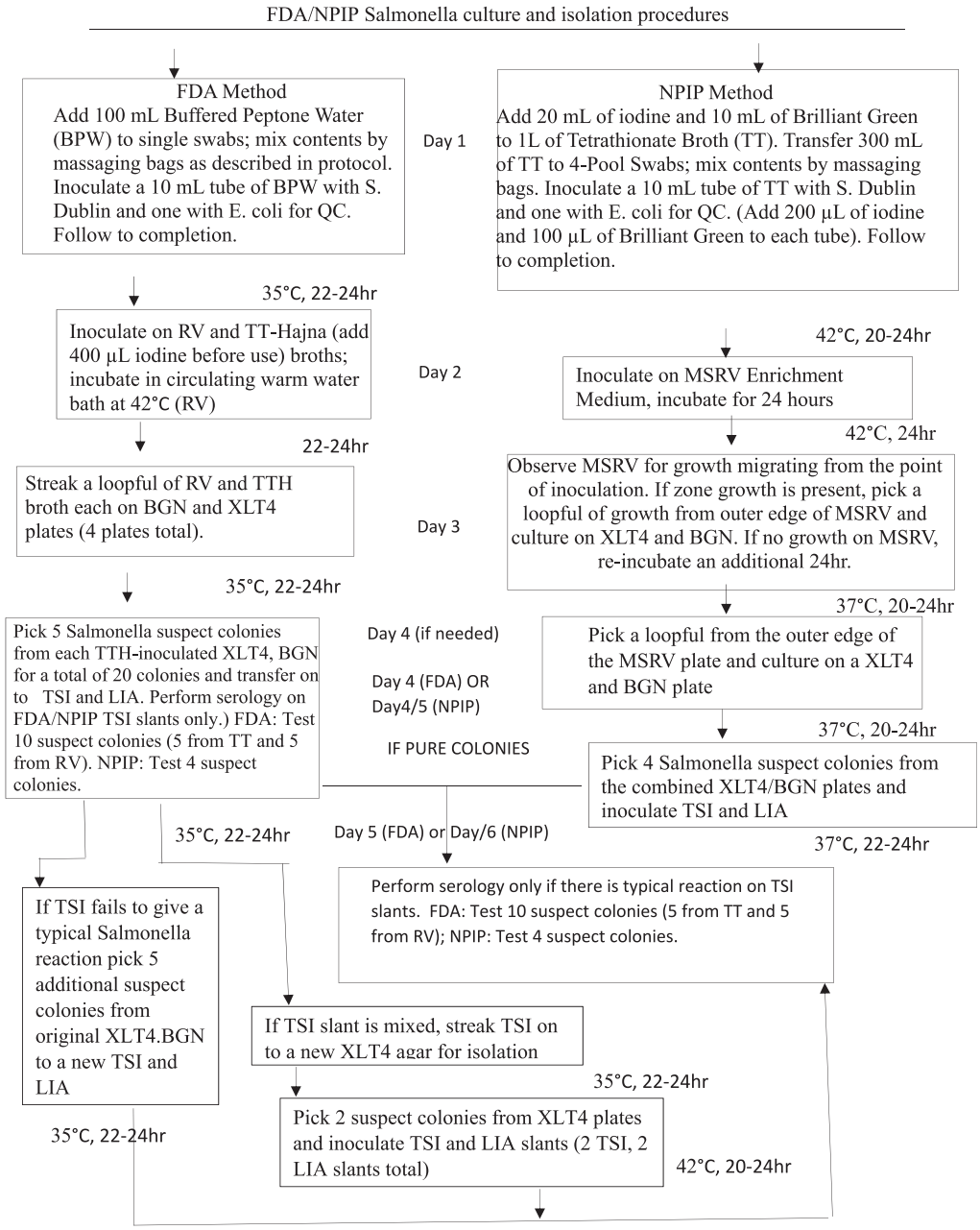

Fig. 1. FDA/NPIP salmonella culture and isolation procedures.

Table 4. Measures of diagnostic sensitivity for NPIP methods of detecting *Salmonella* Enteriditis in environmental swabs inoculated with either low (2.5–2.7 CFU) or medium (7.5–12 CFU) levels and incubated in one-, two-, or four-swab pools.

			Swabs in pool (%)		
Inoculum	Phage type	No. tested	Single	Pair	Four-pool
Low (2.5–2.7 CFU)	4	25	72.00	64.00	68.00
	8	25	84.00	72.00	72.00
	13	25	88.00	88.00	80.00
Medium (7.5–12 CFU)	4	25	92.00	88.00	88.00
	8	25	92.00	80.00	76.00
	13	25	96.00	96.00	92.00
Low and medium combined	4, 8, and 13a	150	87.33	81.33	79.33

Thirteen individual collaborative laboratory results are summarized in Table 6. Data from laboratory 1 were not included for analysis because of laboratory error. The overall diagnostic sensitivity for the FDA method single-swab and the NPIP four-swab pools was

Table 5. Comparison of diagnostic sensitivity for NPIP and FDA methods of detecting *Salmonella* Enteriditis PT8 in environmental swabs inoculated with either low (2.5–2.7 CFU) or medium (9.6–10.4 CFU) levels and incubated in one- or four-swab pools.

		Single swabs (%)		Four swab pools (%)	
Level of inoculum	No. tested	FDA	NPIP	FDA	NPIP
Low (2.5–2.7 CFU)	25	24	88	20	60
Medium (9.6–10.4 CFU)	25	56	100	48	92
Combined low and medium	50	40	94	34	76

Table 6. Comparison of diagnostic sensitivity for FDA and NPIP methods of detecting *Salmonella* Enteriditis PT13a in environmental swabs inoculated with either low (2.5 CFU) or medium (10.2 CFU) levels and incubated in one- or four-swab pools.[A]

Level of inoculum	No. of SE positive swabs/total swabs (%)	
	FDA single swabs	NPIP four-swab pool
Low (2.5 CFU)	81/104 (78)	79/104 (76)
Medium (10.2 CFU)	91/104 (87.5)	100/104 (96)
Low and medium combined	172/208 (83)	179/208 (86)

[A]We analyzed 624 sets of swabs at 13 participating laboratories, 24 sets of swabs/lab for each test method: 416 inoculated (at two levels of CFU) and 208 uninoculated sets of swabs (negative controls).

83% (range 78%–87.5%) and 86% (range 76%–96%), respectively, and there was no statistical difference between the two methods at low-level inoculation or when data were combined from the low and medium levels of inoculations ($P < 0.05$).

Overall, the NPIP method performed remarkably well considering that the method required only up to four *Salmonella* suspect colony picks compared to 20 *Salmonella* suspect colony picks as required by the FDA method. The FDA Final *Salmonella* Enteritidis rule states that if other methods are at least equivalent to the FDA method in accuracy, precision and sensitivity for the detection of *Salmonella* Enteritidis, that they may be used instead of the specified method (9). This study has shown that there is no significant difference between the sensitivity of four-swab pools of environmental drag swabs analyzed by the NPIP culture method and single swabs analyzed individually by the FDA culture method for the detection of *Salmonella* Enteritidis from poultry laying houses.

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