Development and testing of external quality assessment samples for *Salmonella* detection in poultry samples

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Running headline: External quality assessment samples for *Salmonella* detection.

Key Words: *Salmonella*; Detection; Quality assessment.

Significance and impact of the study:

Salmonella surveillance and control regimes in the European poultry industry and elsewhere require sensitive culture detection of *Salmonella* in environmental samples, including poultry faeces. A ring trial was conducted, and the results highlighted that some of the participating laboratories failed to identify *Salmonella*. This suggests that contaminated frozen faeces cubes could be beneficial to assess proficiency, according to the results of this preliminary study. The data obtained in this study can be used as an indication for the design of realistic external quality assurance for laboratories involved in official testing of *Salmonella* in poultry flocks.

Abstract

Salmonella-contaminated poultry house dust, plus 10 g chicken faeces inoculated with *Salmonella* Enteritidis and then frozen for storage and transport, were used as candidate external quality assurance test samples. Variations in faeces sample preparation, storage and culture were examined initially. This indicated that, within modest limits, the age of the inoculating culture and of the faeces did not affect detection, nor did swirling the pre-enrichment culture or extending its duration. Under optimal conditions of preparation and storage, *Salmonella* numbers of 70 colony-forming units (cfu) and above were reliably detected at the originating laboratory.

A ring trial was performed, involving 13 external UK laboratories plus the originating laboratory. Faeces samples inoculated with *Salmonella* Enteritidis were frozen, transported and tested by the ISO 6579:2002 (Annex D) method. Detection by the originating laboratory was consistent with the previously-established lower limit for reliability of 70 cfu. However, the sensitivity of detection by the external laboratories was apparently poorer in several cases, with significant inter-laboratory variation seen at the lowest inoculum level, using Fisher's exact test. Detection of *Salmonella* in poultry house dust appeared to be more sensitive and uniform among laboratories.

Introduction

The control of *Salmonella* in farmed poultry in the European Union has in recent years been subject to harmonised control programmes. Testing for laying flocks has required composite faeces samples or boot swab (bootsock) environmental samples to be submitted for culture using the standard method of ISO 6579:2002 (Annex D) (Anon. 2007). *Salmonella* concentrations in faecal and environmental samples from sub-clinical flock infections may often be low (Wales et al. 2006), and the sensitive isolation of *Salmonella* from such samples requires consistent and careful technique (Carrique-Mas and Davies 2008).

Recent data from laying hens in most European countries indicate a low prevalence of *Salmonella* serovars Enteritidis and Typhimurium (SE and ST) infection (Defra 2012). This suggests that the egg industry has made substantial progress in *Salmonella* control in recent years, which corresponds with a major reduction in SE phage-type 4 infection in the human population (O'Brien 2013). There is, however, a need to demonstrate that further progress in the identification and elimination of flock infections is not being limited by a low sensitivity for detection of *Salmonella* in samples sent to testing laboratories. Consequently, it is important to utilise realistic external quality assessment (EQA) procedures.

It is recognised that *Salmonella* organisms in artificially-contaminated control samples may be easier to culture than *Salmonella* present in real faecal matrix, partly due to the stress imposed by conditions in the sample matrix and the presence of certain substances and competing organisms that are inhibitory for the growth of *Salmonella* (Baggesen et al. 2007). In faeces, boot-swab and dust samples submitted under national control programmes, vastly outnumbering Enterobacteriaceae is considered to be the main limiting factor in *Salmonella* detection. Simpler control samples may be necessary to ensure consistency and repeatability amongst samples, but it is desirable that there be an EQA sample that reliably tests the sensitivity and specificity of *Salmonella* detection from environmental (including faeces) samples.

The present studies aimed to develop a candidate sample type, inoculated with known or closely-estimated numbers of SE, for suitability as an EQA sample for culture by ISO 6579:2002 (Annex D). The sample was further evaluated for optimal transport and handling conditions, and then used in a ring trial of *Salmonella* detection involving thirteen external laboratories recruited on a voluntary basis. These laboratories were all situated in the UK, as was the organizing laboratory.

Materials and Methods

Inoculum and matrix

The *Salmonella* Enteritidis (SE) strain (S711/08) used was isolated from faeces collected from a caged layer house. An aerobic culture (18h, 37 °C, Buffered Peptone Water medium [BPW; Merck 1.07228.0500]) was held at room temperature for varying times, as detailed below, then diluted in quarter-strength Ringer's solution and added to the test matrix. Quantification using serial dilution and a spread plate method (Nutrient Agar) was performed on all cultures before dilution.

Fresh naturally-pooled chicken faeces were obtained from tested *Salmonella*-free farms that had participated in Animal Health and Veterinary Laboratories Agency (AHVLA) studies, or from *Salmonella*-negative AHVLA birds. All batches of faeces were tested for *Salmonella* before use by the culture technique described below. Faeces were stored at 4 °C for various specific periods, as detailed below, before use.

Culture of samples

Qualitative culture was by a modification of the ISO 6579:2002 (Annex D) method, with Rambach agar being used as the sole final plating medium. This technique has, in the current protocol, been shown to provide a sensitivity for detection of *Salmonella* that is equivalent to the ISO method (Carrique-Mas and Davies 2008). Samples (10 g) were pre-enriched in 225 ml buffered peptone water (BPW) for 18 ± 2 h at 37 °C. An aliquot (0.1 ml) of the BPW culture was inoculated onto modified semi-solid Rappaport Vassiliadis (MSRV; Mast DM440D) plates containing 0.01% novobiocin and incubated for 24 and 48 ± 3 h at 41.5 °C. A 1 µl loop from the edge of the opaque growth zone was inoculated onto Rambach agar (Merck 1.07500.0002) and incubated for 24 ± 3 h at 37 °C. The identity of presumptive *Salmonella* colonies from the Rambach plates was confirmed by a slide agglutination test. For semi-quantitative isolation, decimal dilutions were prepared in BPW and after incubation overnight at 37°C the isolation method described above was followed.

Candidate EQA matrix studies

Stationary culture was held for several days at room temperature, then diluted to high (10^2 to 10^3 cfu.ml⁻¹) and low (10^1 to 10^2 cfu.ml⁻¹) concentrations. Aliquots (20 µl) were added into an indentation in approx 10 g of poultry faeces in ice cube tray compartments. The actual concentration of *Salmonella* was determined by spread plating 20 µl of the dilutions used, in quadruplicate. The trays were frozen at -80 °C, then the faeces cubes were transferred from the tray to zip-seal plastic bags and held at -80 °C until they were thawed (at room temperature), each dispersed in BPW and cultured as previously described. In addition, an extended pre-enrichment phase in BPW (28 h) and swirling of the pre-enrichment culture just before subculture to MSRV were included as trial variations on the standard culture procedure.

Sample holding and handling studies

Experiments varying a number of factors in differing combinations were performed. Factors subjected to defined alterations were as follows:

- Age of faeces matrix
- Age of inoculating culture
- Storage time of frozen inoculated matrix
- Storage temperature profile of inoculated matrix
- Storage conditions to simulate transport to external laboratory: bags versus bottles, water ice versus dry ice chilling

- Time plus temperature immediately prior to culture, to simulate handling in the destination laboratory

Ring trial

Sixteen external UK laboratories were contacted, and eleven participated in the first round of trials. For the second round, three of these eleven declined to take part but three new ones volunteered to join, making a total of eleven external laboratories in this round also. Participating laboratories were blinded to the number of positive samples and to the expected concentration of *Salmonella*. Cubes of inoculated faeces were prepared and held at -80 °C for 1 to 3 days in Euro-tubes (screw capped polypropylene containers; Scientific Laboratory Supplies CON8512). The faeces used were assessed for *Enterobacteriaceae* and total viable count (TVC) before and after the freezing procedure, via serial dilution and aerobic culture on violet red bile glucose agar (Oxoid CM485 VRBGA) and 5% sheep blood agar, respectively.

For dispatch, Euro-tubes were inserted into Bio-Bottles (Bio-Bottle New Zealand Ltd.), packed in dry ice in insulated containers, and sent by courier to arrive at the laboratory by 10 am the following day. *Salmonella*-positive dust samples were also sent to participating laboratories in the same consignments but at ambient temperature. These had been collected in fattening turkey houses and contained 1 to 10 cfu.g⁻¹ *Salmonella* Derby, determined by semi-quantitative culture (Wales et al. 2006).

Prepared sample sets were randomised for destination and were labelled for postage by staff unrelated to the project. Laboratories were given handling instructions (available on request from the authors) and instructed to process the samples according to their normal procedures, which may have included serovar determination, on the day of receipt. Two sample sets were retained by AHVLA, packaged with dry ice in the same manner and for the same duration as the dispatched samples, and were processed for culture on the same day as samples were processed in the external laboratories.

Two rounds were conducted:

Round 1. One set containing ‘high’ (four cubes, 340-440 cfu per cube) and ‘low’ (eight cubes, 34-48 cfu per cube) numbers of SE, plus blanks (two cubes, no *Salmonella* added) were dispatched to each laboratory. Two such sets were processed at AHVLA.

Round 2. One set containing ‘high’ (four cubes, 1700-2100 cfu per cube) and ‘low’ (eight cubes, 170-210 cfu per cube) numbers of SE, plus blanks (two cubes, no *Salmonella* added) and two dust samples (5 g each) were dispatched to each laboratory. Two such sets and three sets of dusts were processed at AHVLA.

Fisher’s exact test was used to compare the proportions of missed positives among the laboratories, including the reference samples at AHVLA, for each of the ‘high’ and ‘low’ contamination sets in each round.

Results and discussion

An ideal EQA sample in the context of poultry environmental *Salmonella* detection would have a quantified number of viable *Salmonella* cells and would present challenges to successful isolation that are similar to field samples, particularly in terms of *Salmonella* stress, competing organisms and inhibitory substances. As most environmental poultry samples are either faeces or contain faecal elements, it was decided that a realistic challenge to isolation would require the EQA sample to be based upon a faecal matrix.

Findings from the matrix and handling studies are summarised in Table 1. Dry ice showed a clear advantage over water ice in preserving viability during simulated transport. Under simulated dry ice transport, inocula of 72 cfu per cube and higher were consistently recovered, using inoculum cultures varying in age between one and 14 days and in faeces between three and 24 days old. At the lower levels of inocula, aged faeces was associated with a greater recovery of *Salmonella* than was apparent with three-day-old faeces, under the experimental conditions used in this study, with a limited number of replicates.

One particular challenge with environmental samples is to recover damaged or stressed *Salmonella* cells amidst large numbers of other aerobic bacteria. The culture process used in the present study (Anon. 2007) uses a non-selective aerobic pre-enrichment stage that is intended to allow the recovery and multiplication of *Salmonella*, particularly damaged or slow-growing cells, without deleterious overgrowth of competing micro-organisms (Edel and Kampelmacher 1973). Optimising this balance between target and non-target organisms requires careful technique, which is why modest departures from standard procedure at this stage were examined for their effects on the eventual recovery of *Salmonella* from the samples. In the present study, minor deviations from the pre-enrichment protocol (swirling/shaking BPW or prolonging incubation up to 28 h) did not reduce the frequency with which *Salmonella* was recovered from the EQA samples. This is in contrast to deleterious effects upon *Salmonella* detection of such deviations from standard procedure observed by one author (RD) with some environmental samples (Davies and Wray 1996).

Poultry faeces inoculated directly with quiescent (beyond the lag phase) *Salmonella* culture appeared to be a suitable EQA preparation. They were associated with reliable detection of *Salmonella* after inoculation of about 70 cfu or more per cube plus frozen storage, using the standardised culture protocol practised by all the participating laboratories (Table 2). A heavy competing microflora of *Enterobacteriaceae* and other organisms was demonstrated even after freeze-thawing. In samples received for routine testing within four days of collection it is expected that *Enterobacteriaceae* and other viable bacteria would be present in substantial amounts, making these EQA samples a realistic substitute for such naturally-contaminated samples.

Variations in preparation and handling were assessed in order to define suitable conditions to be used in the Ring trial. Within tested limits (one to 14 days), the age of the inoculating culture did not appear to affect the likelihood of subsequent detection, but the age of the faeces into which it was inoculated was more critical. This may reflect changes in the microflora and chemistry of the faeces. Dry ice packaging for simulated transport appeared to preserve low-inoculum samples as well as

storage in a -80 °C freezer, whereas packing in water ice was associated with inadequate preservation.

For the ring trials, all control samples were correctly identified as *Salmonella*-negative, and all contaminated dust samples (Round 2) were correctly identified as *Salmonella*-positive. Other results are summarised in the Figure and in Table 2. At the lowest contamination level (34-44 cfu per cube before freezing) there was inconsistent isolation of *Salmonella*, including from the reference samples at AHVLA. Improved recovery was seen with most laboratories, including the reference samples, at the next contamination level of 170-210 cfu per cube. Therefore, the numbers of *Salmonella* in the cubes appear to have been in a suitable range for testing the sensitivity of detection among the laboratories. Furthermore, in both rounds there were laboratories that detected as many or more low-concentration positive cubes than did the originating laboratory, indicating that preservation of *Salmonella* viability during transport was good.

Laboratory 1 in Round 2 recovered *Salmonella* infrequently from both 'high' and 'low' concentration cubes. However it was found to have mishandled the samples by delaying the start of the examination and storing the test materials in a refrigerator for 24 hours. These results were therefore excluded from the statistical analysis. This analysis yielded evidence of significantly differing proportions of missed positives between laboratories with the 'low' contamination samples in Round 1 ($P < 0.01$). In the other three cases (Round 1 'high' and Round 2 'high' and 'low') differences between all laboratories were not found to be significant. It is logical that inter-laboratory variation will be highest when test samples contain numbers of *Salmonella* that are near the lower limits of detection. However, with samples containing numbers of *Salmonella* 10 to 20 times higher than this, there was still variability in detection. Although these variations were not statistically significant, the statistical analysis had limited power owing to low numbers of samples per laboratory, and such inconsistency remains a potential concern. Further assessment of laboratory performance using the present approach would usefully include more samples stored in dry ice and then processed for detection at the originating reference laboratory, to provide more baseline data against which to compare the sensitivity and variability of detection in other laboratories.

For some laboratories (2, 8 and AHVLA) the proportion of positive results correlated positively and well with the number of inoculated *Salmonella* cells per cube, but for others (e.g. Laboratory 3) there was poorer correlation between these values. The reason(s) for this are unclear, but it may be that some elements of sample handling or processing were being inconsistently performed. Indeed, five of the 14 test laboratories that participated in either or both of the rounds failed to isolate *Salmonella* from one or more of the two most heavily contaminated sets of faeces samples, whilst *Salmonella* was consistently isolated from the corresponding reference samples at AHVLA. However, it cannot be excluded that the some of the inconsistency within the results was observed due to the inhomogeneous nature and to the small number of the samples tested.

Dust samples with low numbers of *Salmonella* (10 to 50 cfu) were correctly reported as positive in all cases. This sensitivity to *Salmonella* in dust may be in some measure a consequence of lower numbers of competing microflora in dust, compared to faeces (Sommer et al. 2012). Alternatively, inhibitory substances (such as bacteriocins) present in faecal culture but less so in dust may

adversely affect the culture of *Salmonella*, and more markedly in laboratories with non-optimised sample handling and processing.

In conclusion, poultry faeces inoculated with *Salmonella* and then frozen in cubes appeared to be a suitable preparation to act as EQA samples for *Salmonella* detection from poultry environmental samples, given suitable (dry ice) refrigeration during transport. This sample type revealed variations in sensitivity and consistency among testing laboratories nominally using the same procedures, although specificity was 100%. It appears there is scope for further optimising of standard *Salmonella* isolation techniques in some laboratories, and suitable EQA samples will have a role in this process.

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Conflict of interest

No conflict of interest exists.

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Table 1: Recovery of *Salmonella* from faeces artificially inoculated with *Salmonella* Enteritidis then frozen as cubes in ice trays and subjected to various storage and simulated transport conditions.

Conditions	Age of culture ^a (days)	Age of faeces (days)	Inoculum (cfu per cube)	Standard culture ^b	Extended pre-incubation ^c
A: Freezer -80 °C bagged, 96 h	1	24	97 – 111	4/4 ^d	4/4
			7 – 17	4/4 ^d	4/4
	14		114 – 137	4/4 ^d	4/4
			8 – 14	3/4 ^d	3/4
B: ‘A’ plus simulated transport in bags on water ice packs 25 h, then 2 h bench time ^e .	1	24	97 – 111	0/4	0/4
			7 – 17	0/4	0/4
	14		114 – 137	1/4	1/4
			8 – 14	0/4	0/4
C: ‘A’ plus simulated transport in bags on dry ice 25 h, then 2 h bench time ^e .	1	24	97 – 111	4/4	4/4
			7 – 17	1/4	1/4
	14		114 – 137	4/4	4/4
			8 – 14	3/4	3/4
D: ‘A’ modified to 18 h, to Euro-tubes ^f , freezer -80 °C 30 min, then simulated transport with tubes bagged on dry ice 26 h, then 2 h bench time ^e	11	3	101 – 134	4/4 ^d	ND
			7 – 13	0/2 ^d	ND
E: ‘D’ modified by putting Euro-tubes ^f in Bio-Bottles ^g not bags for transport.	11	3	101-134	4/4 ^d	ND
			7-13	1/10 ^d	ND
F: In trays, ‘A’ modified to 18 h, to Euro-tubes ^f , freezer -80 °C 3 h, then 2 h modified bench time ^h	6	20	360 – 640	4/4	4/4
			72 – 128	4/4	4/4
			36 – 64	4/6	5/6
G: ‘F’ modified by inserting simulated transport with tubes in Bio-Bottles ^g on dry ice 21.5hr, before bench time	6	20	360 – 640	4/4	4/4
			72 – 128	2/2	2/2
			36 – 64	5/8	5/8

ND = Not done

^a Overnight culture held at room temperature for the given number of days before dilution in Ringer's solution to form the inoculum for faeces.

^{b, c} Thawing of cube followed by pre-enrichment in buffered peptone water for 18 h (b) or 24-28 h (c), then culture as described in the text.

^d Repetitions done with shaking or swirling of pre-enrichment mix yielded the same results.

^e One hour in packaging without dry ice on the workbench followed by one hour outside of packaging in a petri dish on the workbench.

^f 60 ml polypropylene screw-capped tubes.

^g Screw-capped high-density polyethylene bottles.

^h Two hours in Euro-tube without dry ice on the workbench.

Table 2: Results of bacterial counts performed on faeces used as matrix in the ring trials

		Total viable count (cfu.g⁻¹)	<i>Enterobacteriaceae</i> count (cfu.g⁻¹)
Round 1	Before freezing	3.6 x 10 ⁹	1.6 x 10 ⁹
	After thawing	1.6 x 10 ⁹	7.6 x 10 ⁸
Round 2	Before freezing	2.6 x 10 ⁹	1.8 x 10 ⁷
	After thawing	2.1 x 10 ⁹	1.2 x 10 ⁶

Quoted *Salmonella* counts are from samples before freezing. Results from laboratories that participated in both rounds are shown as black bars. R1 and R2 were reference samples, retained frozen at the source laboratory and cultured according to the standard protocol on the same day as the distributed samples.

* Laboratory 1 was found to have mishandled samples in Round 2, which may have contributed to relatively poor performance.

Figure: Findings from a two-round ring trial of *Salmonella* isolation from artificially contaminated poultry faeces, distributed as frozen cubes.