



A final project report for:

The Food Standards Agency

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FSA Project Reference B15003:

**Recommendations on the best practical procedures to sample and test poultry flocks for *Salmonella*.**

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## **Report format**

This document is the technical report for FSA Project B15003.

The report is organised into 3 main sections namely:

Section 1. An overall summary of the project and its findings written in non-technical language as a lay-person's overview.

Section 2. A report describing the practical, farm-based sampling aspects of this study which has been prepared in a format suitable for publication in a peer-reviewed journal.

Section 3. A literature review summarising key scientific publications.

## Section 1: Layperson's summary

## Layperson's Summary

### Background and Objectives

A survey undertaken by the FSA in 2004 showed that 5.7% of raw chicken meat offered for retail sale to British consumers was contaminated with *Salmonella*. Although this was a significant improvement over previous surveys, it still means that more than 1 in 20 chickens purchased in the UK have the potential to cause an outbreak of food poisoning. The FSA have an obligation to protect consumers from foodborne illness and are therefore keen to further reduce *Salmonella* contamination of chickens. One way that this can be achieved is to test flocks on farm to determine whether they are infected with *Salmonella*. When *Salmonella*-positive flocks are identified, these can be slaughtered separately so that the risk of cross-contaminating *Salmonella*-free carcasses from is minimised. The purpose of this study was to find the most effective way to sample poultry on-farm so that an accurate determination of the *Salmonella* status of flocks can be made. The results can then be used to provide appropriate guidance to the poultry industry.

### Approaches

Review of previously-gathered information and published literature for on-farm *Salmonella* sampling and theoretical appraisal of sampling methods.

In 2003, the FSA funded a study (reference code ZB00023) to gather information on the *Salmonella* testing and scheduling practices used by the UK poultry industry. The first part of this study, was to update these findings and include additional information on the practices followed by smaller-throughput producers and slaughterhouse operators. The findings from this part of the study showed that, although a large percentage of British birds are tested for *Salmonella* before slaughter, there was no standard method in use either for the collection of the test samples or their analyses in the laboratory. It was found that the sampling and analyses methods used for on-farm *Salmonella* detections were chosen largely for historical reasons and specific assessments were rarely made regarding the sensitivity of these method.

To find out how the sampling and testing practices in use in the UK related to other EU countries and the USA; guidelines for *Salmonella* testing and scheduling produced in these other countries were obtained and concise comparative summaries were written. Some of the methods used in other countries (e.g. milk-

soaked gauze drag swabs) were discounted for routine use in the UK on the grounds that they were impractical.

A literature review was also undertaken which identified and summarised over 40 key scientific publications. Although a number of publications described experiments that compared how the method of sampling related to *Salmonella* incidence, it was unclear from the scientific literature which sampling method was most sensitive for the detection of *Salmonella* in flocks.

#### Practical evaluation of sampling and testing procedures

Chicken houses on four *Salmonella*-positive commercial farms were visited and samples were collected from the floor litter by boot swabs, sock swabs, and direct sampling. Dust was also collected; both as an individual sample, and in combination with boot swab samples. Samples were analysed for the presence of *Salmonella* and the most effective sampling method determined. When the total number of positive detections were counted, direct litter sampling and diluent-moistened boot swabs were equally as effective as sampling methods for the detection of *Salmonella*. Further statistical analyses of the results revealed that litter sampling and boot swabs were significantly most effective for sampling on two of the farms visited. These methods however were not significantly better (or worse) than the other sampling methods at the remaining two farms that were sampled.

#### **What it means and why it's important**

The method that is used to sample broiler houses on-farm for the presence *Salmonella* in chickens can influence the laboratory analysis results. This study has shown that boot swabs and direct litter sampling were statistically significantly the most effective for sampling for *Salmonella* at broiler houses on two farms. Both are cost effective methods for the sampling of broiler houses and for these reasons either of these methods is recommended for broiler house sampling. Adopting these methods allows cost-effective detection of *Salmonella*-positive flocks by abattoirs. Improvements to slaughterhouse scheduling for *Salmonella*-positive flocks and prevention of the cross contamination of chicken carcasses during slaughter and processing is likely to lower the risk of *Salmonella* from chickens causing foodborne illness to British consumers.

## Section 2: Farm-based sampling studies

**A comparison of the effectiveness of different sampling methods to determine the *Salmonella* infection status of broiler flocks on farm.**

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## Abstract

Broiler houses on four *Salmonella*-positive commercial farms were visited and samples were collected from the floor litter by boot swabs, sock swabs, and direct sampling. Dust was also collected both as an individual sample and in combination with boot swab samples. Samples were analysed for the presence or absence of *Salmonella* after enrichment. The most effective sampling method was determined on a farm-by-farm basis as well as across all farms. On two farms, boot swab and directly-sampled litter had significantly higher detections than the other sampling methods. At the remaining two farms there were no significant differences between any of the sampling methods. Overall, the highest number of total detections for *Salmonella* was from directly-sampled litter and diluent-moistened boot swabs samples. However, when the results were analysed *en masse* there was a highly significant sampling-method-by-farm interaction ( $P < 0.001$ ) which confounded the identification of significant differences between any of the sampling methods ( $P = 0.122$ ).

## Introduction

Although the incidence of foodborne illness caused by *Salmonella* has been declining slowly in the UK since the early 1990s (Adak *et al.* 2002), *Salmonella* are still responsible for more than 11% of the presentations of foodborne bacterial gastroenteritis in Britain (Adak *et al.* 2002). Traditionally in the UK, poultry are regarded as a significant reservoir of *Salmonella* (Davies *et al.* 2003; Davies and Wray 1996). Poultry infected with *Salmonella* shed the bacterium into their faeces (Ekperigin and Nagaraja 1999) and a recent survey of UK farms reported that 17% of freshly deposited poultry wastes contained *Salmonella* at up to  $2.2 \times 10^4$  CFU g<sup>-1</sup> waste (Hutchison *et al.* 2004). The detection methods used for this survey were however quantitative, lacked enrichment, and no special consideration was made to sample birds at 3 weeks of age when *Salmonella* shedding into faeces is highest (Gradel *et al.* 2002). Thus it is probable that the actual prevalence of sub clinical infection of UK broilers by *Salmonella* is significantly higher than was reported (Hutchison *et al.* 2004).

The *Salmonella* status of broiler flocks is important for white meat slaughterhouses since the slaughter and processing of *Salmonella*-positive flocks before uninfected flocks has been shown to cause cross contamination of carcasses prior to retailing (Olsen *et al.* 2003). If the foodborne incidence of *Salmonella* in the UK is to continue to fall, it is important that the *Salmonella* status of broiler flocks is determined accurately. *Salmonella* detection is a pre-requisite for the identification of risk factors that are associated with bird colonisation and infection, and essential for the ultimate eradication of this zoonotic agent. A number of authors have, however, commented on the inconsistencies of *Salmonella* detection on farm when using different sampling protocols (Byrd *et al.* 1997; Skov *et al.* 1999a) and it is now widely acknowledged that the method used for sampling can have a significant influence on the detected prevalence of *Salmonella* in broiler flocks (Gradel *et al.* 2002; Kwon *et al.* 2000; Skov *et al.* 1999b).

When *Salmonella* are sampled in broiler houses, it is important that a method which obtains a representative sample of the entire house is used. One of the reasons for this requirement is related to the uneven distribution of *Salmonella* through broiler houses, and in particular floor litter (Hayes *et al.* 2000). When bulk litter samples collected from the floor of an entire broiler house were compared with small-area targeted samplings from the same house, Hayes *et al.* (2000) found that the percentage moisture content and water activity of the samples were not related. Environmental conditions favourable

for *Salmonella* were therefore not uniform in the litter of any of 86 houses that were sampled during their study (Hayes *et al.* 2000).

The sensitivities of various sampling methods have been studied previously in order to determine the most appropriate for *Salmonella* detection in broiler houses (Byrd *et al.* 1997; Gradel *et al.* 2002; Hayes *et al.* 2000; Heyndrickx *et al.* 2002; Skov *et al.* 1999a). However it is still unclear which method offers the best performance because these studies are not directly comparable with each other. In particular the laboratory analyses methods used to analyse the samples were not standardised across these studies and are likely to influence detection efficiency. Thus the purpose of this study was to practically evaluate a number of sampling protocols described previously as appropriate for *Salmonella* sampling in broiler houses; analyse these samples using a standard, traditional microbiological analysis method and determine the most sensitive method.

## Materials and Methods

### Study Farm Recruitment and House Preparation

Study farms were identified as *Salmonella*-positive by the routine monitoring undertaken as standard practice by slaughterhouse companies. A separate confirmation sample was collected and analysed as described below by one of our laboratories to ensure that the house contained *Salmonella* before scheduling an on-farm sampling visit. All of the houses sampled had the capacity to contain at least 20,000 birds and all had wall-mounted exhaust fans. Houses were divided into 10 separate sections for the purposes of sampling. Sections were defined to ensure each contained exhaust fans and ledges which had accumulated dust. In total, four houses on four separate farms were sampled for this study.

### Sample Collection

Five different sample methods were used and five replicates of each sample were collected from each of the 10 sections in each house samples. Collections were from litter using boot swabs (Arnolds, Shrewsbury, UK), 20cm-length sock swabs (Tubigrip Size D; Brosch, Peterborough, UK), and direct grab sampling of litter using gloved hands as described below. Dust was also collected as a simple sample and also in combination with boot swabs as described below.

Litter samples were collected using a fresh pair of disposable plastic gloves for each sample. Separate pinches of litter ( $n=25$ ) of between 4 and 6 g in mass were collected randomly from the section being sampled.

Boot swab sampling involved using a fresh pair of polythene overboots (Arnolds) for each sample. Boot swabs were worn on top of the overboots and moistened with 5 ml of maximum recovery diluent [MRD; Oxoid, Basingstoke, UK]. Exactly 100 steps were made randomly around the section being sampled and each sample comprised a pair of boot swabs.

Dust samples were collected using a new pair of disposable plastic gloves for each sample. Pinches of dust (between 250 and 600 mg) from fans, horizontal ledges, cabling, electrical control boxes, drinker and feed equipment external surfaces and

rafters was collected. Each sample comprised as many pinches of dust as was required to generate a 200 ml volume. Dust from space heaters was not collected.

Combination boot swab and dust samples were taken as described above. The boot swabs and 200 ml of dust were combined at the laboratory to generate the combination sample.

Sock swabs were also taken using a fresh pair of polythene overboots for each sample. As before 100 steps were taken randomly around each sector sampled. After each 25 steps however, the swabs were turned 90° to ensure the entire area of the sock was used to collect the sample. Each sample comprised a pair of Tubigrip socks.

### **Sample Storage Before Analysis**

Samples were kept cool and away from direct sunlight during sample collection and the 1-2 hrs needed for transit to the laboratory. In order to mimic the conditions that samples would be subjected to if they had been collected by farm staff and posted to a laboratory, storage after arrival in the laboratory and before sample processing commenced was in a cool (unrefrigerated) environment of 12-16°C.

### **Analyses of Samples for *Salmonella***

Subsamples of 25 g (litter), 15 g (dust), 2 socks and/or 2 boot swabs were generated and enriched in 225 ml volumes Buffered Peptone Water for 18 hours at 37° C. Enrichment broth (200 µl) was inoculated into an MSR/V (semi-solid Rappaport Vassiliadis; Difco BD, Oxford, UK) plate and incubated in a moist incubator at 42°C for 24 - 72 h. Motile (>20mm spreading) colonies were streaked onto XLD (xylose-lysine-desoxycholate agar; Oxoid) and BGA (brilliant green agar; Oxoid) plates.

### **Confirmation for *Salmonella***

Colonies were confirmed according to ISO 6579 (ISO 6579 2002). In addition, presumptive colonies were screened by direct agglutination with polyvalent 'H' or 'O' antisera (Pro-lab, South Wirral, UK). Biochemical confirmation of *Salmonella* was conducted using API 20E (BioMérieux, Basingstoke, UK).

### **Statistical Analyses**

For each farm, the sampling methods were compared using a linear logistic regression model that included terms for sector and method. Chi Squared or Fisher's exact tests

(StatsDirect, Cheshire, UK) were used as appropriate to test significance of *Salmonella* presence-absence. A combined analysis of variance (ANOVA) was undertaken for the four farms. Each farm was treated as randomly selected from a larger population of infected farms. For ANOVA, the proportion of positive detections in each sector ( $p$ ) were transformed to arcsin (square root ( $p$ )) in order to remove any inequalities in variance due to the different values of  $p$ . For all tests, a  $P$  value of  $<0.05$  was used to determine the significance of differences.

## Results and Discussion

Extensive sampling of populated houses for several hours agitates poultry and has welfare implications; thus all sampling was undertaken after birds had been cleared from the house and before the house had been cleaned and sanitised. It has been shown previously that *Salmonella* numbers in litter are highest when birds are 2-3 weeks old (Gradel *et al.* 2002; Heyndrickx *et al.* 2002). However when sampling known-positive houses, there are advantages to sampling after house clearing. Principally, lower *Salmonella* numbers make it more likely that differences between sampling method sensitivities can be detected. Although this strategy was successful for three of the four farms sampled, an average of 93% of the samples collected from farm 3 contained *Salmonella* (Table 1). Such a high percentage of *Salmonella*-positive samples makes it difficult to detect any differences between the five sampling methods used.

When farms were analysed individually to determine if there were significant differences between the sampling methods, two farms (Table 1; Farms 1 and 4) showed highly significant ( $P < 0.001$ ) differences as *Salmonella* was more frequently detected with direct litter and boot swab sampling rather than the sock and dust methods. At farms 1 and 4, the combination boot swab and dust sample was not significantly different ( $P = 0.442$  and  $P = 0.374$  respectively) from the simple boot swab method. At farm 4 only, *Salmonella* was more frequently detected by sampling than combination boot swab and dust sampling ( $P = 0.037$ ).

The total number of *Salmonella*-positive samples at farm 2 was four; which was significantly lower ( $P < 0.05$ ) than at the other farms. All of the positive samples at farm 2 were either simple dust samples ( $n = 3$ ) or a combination boot swab and dust sample ( $n = 1$ ). Although such a low frequency of *Salmonella* detection makes interpretation of these results difficult, we noted that higher frequency of *Salmonella* in dust markedly contrasted to the results obtained for farms 1 and 4 and thus warrants further discussion. Gathering of dust can be difficult to standardise because of its non-uniform distribution in houses. In order to obtain a sample of sufficient mass to process in the laboratory we collected dust from a variety of sources including roof beams and rafters. Results from dust in places that are difficult to clean such as rafters should be treated cautiously. If all of the dust is not completely removed during between-flock shed cleaning then it is likely that dust from poorly-cleaned sheds provides a historical snapshot of the *Salmonella* status of the shed rather than the status of the current or

most-recently housed flock. It is unclear whether dust was genuinely the best sampling method for *Salmonella* detection on farm 2; or whether the flock that was removed was not infected and the dust collected contained *Salmonella* from a previously-infected flock housed in the shed. The low number of detections and the lack of *Salmonella* in the litter points strongly towards the latter.

Previously Kwon et al (2000) have compared the effectiveness of sampling litter and dust removed from house air extraction systems in four pens artificially contaminated with *Salmonella*. Since dust is derived from all areas of the broiler house (Kwon *et al.* 2000), collection of samples from fans had the potential to provide a reliable indicator of the housed flock infection status. *Salmonella* detection was by PCR which makes it difficult to directly compare results with this study. The reported results showed dust sampling was less effective than litter sampling and the authors speculated that the role of PCR inhibitors in dust required further investigation in order to clarify the reasons for their observation.

The results were also analysed *en masse* using a combined ANOVA for the four farms. Results were treated as randomly selected from a larger population of infected farms. There was a highly significant sampling method by farm interaction ( $P < 0.001$ ). Because of this, the overall test for differences between sampling methods when the results were combined from all farms, was not significant ( $P = 0.122$ ).

Farm	Litter	Sample collection method (% positive detections)				Average % detections
		Boot swab	Combined dust and boot swab	Dust	Sock swab	
1	54	58	50	30	16	41.6
2	0	0	2	6	0	1.6
3	92	98	92	92	94	93.6
4	94	84	76	60	52	73.2
Total	240	240	220	188	162	
Mean	60	60	55	47	41	

Table 1 A summary of percentage positive *Salmonella* detections from broiler houses sampled by direct litter sampling, boot swabs, a combination of boot swabs and dust, dust sampling and sock swabs. The total number of samples collected from each farm by each sampling method was 50.

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## Section 3: Literature Review of Relevant Previously-Published Material

## EXECUTIVE SUMMARY

There is not an industry-standard protocol for on-farm scheduling and testing of poultry for *Salmonella* prior to slaughter. However, there are a small number of sampling methods that are routinely used overseas and which have been previously assessed as fit for purpose. The sampling methods that appear to be most favoured are drag swabs and boot swabs. There is currently not enough information available to determine if either of these sampling methods offers a significant advantage in sensitivity over the other. Practically however, boot swab sampling is easiest and cheap to undertake. A small-scale investigation will be undertaken as part of this study to compare boot swabs, sock swabs, dust and litter sampling across a range of farms to unequivocally determine the method best suited for *Salmonella* detection in broiler houses.

There are a large and diverse range of microbiological analyses methods that have been used for the detection of *Salmonella* in broiler-derived samples. Within the EU the two most commonly encountered analyses methods for broiler house samples are ISO 6579 and method No 71 (developed originally by the Norwegian government). The diversity of analyses methods in the EU is most likely a consequence of EU directive 92/117/EC. In the United States, the USDA have a standard method described as part of their National Poultry Improvement Plan (NPIP). Within the published scientific literature a large range of methods have been described.

There is currently no standard, or widely adopted method in the UK or EU for sampling and scheduling of broilers for *Salmonella* prior to slaughter. The methods currently in place in the UK are used for historical reasons and their relative performances have never been scientifically evaluated.

1.1	INTRODUCTION .....	3
1.2	PREVIOUSLY-DESCRIBED SAMPLING METHODS .....	3
1.2.1	<i>Drag swabs</i> .....	4
1.2.2	<i>Socks and overshoe sampling</i> .....	4
1.2.3	<i>Grab handful litter sampling</i> .....	4
1.2.4	<i>Faecal sampling</i> .....	5
1.2.5	<i>Other sampling methods</i> .....	5
1.2.6	<i>Comparative sampling studies</i> .....	5
1.2.7	<i>Sampling protocol conclusions</i> .....	8
1.3	LABORATORY METHODS .....	8
1.3.1	<i>Comparative analyses studies</i> .....	11
1.3.2	<i>Analyses protocol conclusions</i> .....	11
1.4	SAMPLING AND SCHEDULING IN EUROPE .....	12
1.4.1	<i>Cyprus</i> .....	14
1.4.2	<i>Germany</i> .....	14
1.4.3	<i>The Netherlands</i> .....	15
1.4.4	<i>Sweden</i> .....	15
1.4.5	<i>Norway</i> .....	16
1.4.6	<i>Denmark</i> .....	16
1.4.7	<i>France</i> .....	16
1.4.8	<i>Ireland</i> .....	17
1.4.9	<i>Belgium</i> .....	17
1.5	SAMPLING AND SCHEDULING IN THE USA .....	19
1.5.1	<i>American National Poultry Improvement Plan</i> .....	19
1.5.2	<i>On farm sampling for chickens, turkeys, waterfowl, exhibition poultry and farmed game birds</i> .....	19
1.5.3	<i>Environmental swabs</i> .....	19
1.5.4	<i>Cloakal swabs</i> .....	20
1.5.5	<i>Drag swabs</i> .....	20
1.5.6	<i>Chick box papers</i> .....	21
1.5.7	<i>NPIP-approved method for microbiological examination of samples</i> .....	21
1.6	REFERENCES .....	23

## **1.1 Introduction**

Accurate determination of the *Salmonella* status of broilers on a flock basis is an essential prerequisite for identifying those risk factors that are associated with bird colonisation and infection, and the ultimate irradiation of this zoonotic agent. Flock infection status may also be important for white meat slaughterhouses since processing of positive flocks after uninfected birds has been inferred (Olsen *et al.* 2003) and also discounted (Heyndrickx *et al.* 2002) as influencing the contamination of carcasses prior to retailing.

A number of authors have commented on inconsistencies of *Salmonella* detections using different sampling protocols (Byrd *et al.* 1997; Skov *et al.* 1999a). Such comments have led to a number of comparative publications detailing the performance of different sampling methods (Gradel *et al.* 2002; Kwon *et al.* 2000; Skov *et al.* 1999b). The purpose of this review is to summarise relevant literature and theoretically evaluate previously-described sampling and analyses protocols.

## **1.2 Previously-described sampling methods**

When sampling for *Salmonella* in broiler houses, it is important that a method which obtains a representative sample of the entire house is obtained. One of the most important reasons for this requirement are related to the uneven distribution of *Salmonella* through broiler houses, and in particular floor litter (Hayes *et al.* 2000).

Hayes *et al.* (2000) concluded that the overall percentage moisture content and water activity of litter was not related with samples obtained from small-area targeted samplings from the same house. Thus environmental conditions favourable for *Salmonella* were not uniform in the litter of any of 86 houses that were sampled during their study. The authors speculate that for this reason, in positive houses, it may be possible to determine “hotspot” areas most likely to contain *Salmonella*.

A similar hypothesis, which attempted to identify regions of broiler houses which did not contain *Salmonella*, (i.e. coldspots) had been investigated previously with limited success (Opara *et al.* 1992a). The Opara *et al.* (1992) study compared the (measured) *Salmonella* status of broiler houses with a number of environmental parameters including litter pH, water activity, and temperature. The study sampled litter from wet (i.e. near drinking nipples) and visibly dry regions of the broiler house. Significant correlations between house status and litter physicochemistry. For both

wet and dry litter, water activity was related with *Salmonella* detection. For dry litter, lower moisture levels were associated with absence of *Salmonella*. *Salmonella* absence was also correlated with high ammonia and pH levels in wet litter.

Thus an ability to obtain a sample which is representative of the entire broiler house is important. One of the reasons for an inconsistency of *Salmonella* detection when containing different sampling protocols is likely to be linked with a variable ability for obtaining representative house samples (Skov *et al.* 1999b; Skov *et al.* 1999a).

### **1.2.1 Drag swabs**

Drag swabs are ~60cm<sup>2</sup> pieces of gauze that are tied to lengths of string and used by walking around flock houses, dragging the swabs through the floor litter. Swabs can either be used dry or can be moistened with water, MRD, or skimmed milk powder.

Typically, drag swabs tend to be used in pairs with a single pair used for up to 500 birds in a house and two pairs being used for more than 500 birds (Caldwell *et al.* 1994; Opara *et al.* 1992a; Opara *et al.* 1992b; Opara *et al.* 1994; USDA Animal and Plant Inspection Service 1997). Each drag swab pair should be dragged through the litter for at least 15 minutes. In addition to the commonly-used method of sampling, drag swabs have also be rubbed over large areas (~10%) of the house such as nest boxes that are frequently visited by birds.

### **1.2.2 Socks and overshoe sampling**

Socks, boot swabs or overshoes are terms used generally lengths of elasticated tubular stockings which are worn over the samplers footwear. The swabs are generally used dry, and samples are collected by simply walking through the broiler house to be sampled wearing the overshoes. A recent study by Heyndrickx *et al.* (2002) collected samples simply using clean footwear and also sampled from in-house feed and water points. Comparisons with overshoes and collection of caecal droppings were also undertaken. The study concluded that the most efficient method for sampling for *Salmonella* was using overshoes (Heyndrickx *et al.* 2002).

### **1.2.3 Grab handful litter sampling**

Litter sampling is a blanket term for samples that consist of faecal material, litter, dust or floor litter surface. Most commonly, such samples are collected by walking

through the broiler house and collecting a number of grab handfuls of material using a stomacher bag or sterile equivalent turned inside out over the hand used for taking samples. The European Directive 92/117/EEC specifies that 60 is an appropriate number of handfuls to collect for a flock size of larger than 500 birds. Although the directive also specifies appropriate locations for sample collection, a main drawback of using this method of sampling is that it is fairly subjective (Section 1.4). The location of physical collection is subject to the whim of the sampler. Furthermore much of the instructions specified by European Directive 92/117/EEC are open to interpretation and thus this method is likely to suffer from low reproducibility.

#### **1.2.4 Faecal sampling**

A study aimed at defining the risk factors associated with *Salmonella* infections in Danish broilers used faecal sampling to assess flock infection status. For each flock 12 samples were collected at 3 weeks. Each sample comprised five fresh faecal samples. Such a sampling regime will detect an infected flock with 95% confidence provided that individual bird infections have a prevalence higher than 5%, the sampling are truly random, and the sensitivity of the microbiological method used is 100% (Skov *et al.* 1999a).

#### **1.2.5 Other sampling methods**

A number of other, less-commonly encountered, sampling methods have also been described. Kwon *et al.* (2000) compared the effectiveness of sampling litter and dust removed from house air extraction systems in four pens artificially contaminated with *Salmonella*. Since dust is derived from all areas of the broiler house (Kwon *et al.* 2000), collection of samples from fans had the potential to provide a reliable indicator of the housed flock infection status. The reported results were disappointing however; aerosol sampling was less effective than litter sampling when *Salmonella* detection was by conventional microbiological methods.

#### **1.2.6 Comparative sampling studies**

A study by Skov *et al.* (1999) compared four different sampling methods. The methods studied were pooled samples taken from five areas of each broiler house. Samples were collected from each of the five areas of each house as 12 pools composed of 5 grab handfuls of soiled litter from each area, a single drop paper from

each area, five pairs of sampling socks used for each area, and a single set of sampling socks used for each area. The five pairs of socks, five paper sheets and 60 grab handfuls all detected the same prevalences of *Salmonella* infections if the prevalence was at least 5.6% (at least 15 positive pooled samples). In flocks with an estimated incidence of 1.4% (no more than four positive pooled samples), a good correlation between the five pairs of socks and 60 grab handfuls was observed (Skov *et al.* 1999b). A single pair of socks was less effective than the other sampling methods for detection of *Salmonella* even at high prevalences of almost 10%. The study concluded that using five pairs of socks and 60 pooled grab samples were equally sensitive for detecting *Salmonella*. Additional analyses were undertaken to determine the reproducibility. The reproducibility calculated was similar for five socks, five papers and 60 grab handfuls, although the larger number of samples analysed for handfuls (12) mean that overall this method had lowest variation. (Variation was calculated as the square root of standard deviation from the highest detection).

Direct litter sampling by grab handful compared less favourably with drag swabs in a study by Hayes *et al.* (2000). Sensitivities from the 86 houses sampled were calculated as 92.3% and 65.8% for drag swabs and litter samples respectively (Hayes *et al.* 2000).

The Heyndrickx *et al.* (2002) has been described in Section 1.2.2 and concluded that the most efficient method for sampling for *Salmonella* was using overshoes (Heyndrickx *et al.* 2002).

Byrd *et al.* (1997) directly compared the efficiencies of *Salmonella* recovery between drag swabs soaked in double-strength reconstituted skimmed milk and unmoistened dry drag swabs. Previously it had been independently reported that dry swabs were significantly more (Opengart *et al.* 1991) as equally efficient (Davison *et al.* 1995) and less efficient (Opara *et al.* 1994) at recovering *Salmonella* from broiler houses. The results of the study showed that for each of the 30 houses sampled, *Salmonella* isolations were two-fold more frequent for milk-soaked swabs. Furthermore, the numbers of *Salmonella* recovered were also significantly ( $P < 0.05$ ) elevated when milk-soaked swabs were used.

Double strength skimmed milk was also found to be the most effective moistening agent for drag swabs in a separate study by Opara *et al.* (1994). A comprehensive

range of possible alternatives of saturation diluents were evaluated in this study including double strength skimmed milk (2XSM), 2% (w/v) buffered peptone water (BPW), Carnation<sup>®</sup> evaporated skimmed milk (CSM), physiological saline (PS), distilled water (DW), and unmoistened dry swabs (UDS) (Opara *et al.* 1994). When CSM was compared with 2XSM, 2XSM was found to be more effective at recovering *Salmonella*. When 2XSM was compared with the remaining diluents, the results, ranked from most effective to least effective, were 2XSM, PS, BPW, DW, DD.

An earlier study undertaken by Opara *et al.* (1992) compared the performance of four diluents and assessed their ability to preserve *Salmonella* sampled onto drag swabs. The diluents assessed were 2XSM, BPW, a modified Cary-Blair transport medium (CB) and a lactose broth (LB). *Salmonella* detection was by antibody capture and selective plating, and 2XSM had the highest level of recovery of *Salmonella* after prolonged storage under a variety of conditions (Opara *et al.* 1992b).

A more recent comprehensive study by Gradel *et al.* (2002) compared different sampling procedures and also the time of sampling for detection of *Salmonella* in 41 broiler houses. Sampling methods evaluated were five pairs of socks analysed as five individual samples, two pairs of socks analysed as a single sample and 60 grab handfuls analysed as a single sample. The study found that two pairs of socks with pooled analyses had comparable sensitivity for *Salmonella* detection with the 60 grab handfuls analysed as a single sample. Both sampling methods could reliably detect a prevalence of 5%. Comparison of sampling times using 5 pairs of socks showed that sampling when the birds were 3 weeks old was most effective for detection of *Salmonella*. Sampling a few days before slaughter had a lower detection efficiency. The authors were unable to tell if *Salmonella* had difficulty surviving in older more soiled litter or if the shedding pattern for the birds had altered as they matured (Gradel *et al.* 2002).

Caldwell and colleagues investigated the statistical likelihood of *Salmonella* detection using four drag swabs (Caldwell *et al.* 1994) at 31 individual farm locations. The swabs were analysed individually allowing an assessment of probabilities of detection using three, two and a single swab. In general, increasing the number of swab assemblies caused increased detection. The authors assumed that using four swabs accurately detected 100% contamination in houses. In houses occupied by birds, a single assembly detected 53% of the four swab total, two swabs detected

78% and three swabs detected 83%. Similar detections were observed for empty (between flock) houses.

To date there does not appear to be a study which compares directly the relative effectiveness' of socks and drag swabs. These sampling methods appear to consistently achieve higher detections of *Salmonella* than broiler sampling, dust sampling or direct faecal sampling. Thus, as part of this study, a comparison of the two methods will be undertaken in order to assess the relative effectiveness of each method.

### **1.2.7 Sampling protocol conclusions**

Although a number of comparative publications have attempted to rank the sensitivities of different sampling methods, it is still unclear which method offers the best performance. A likely explanation for the often-conflicting conclusions which have been drawn by a number of studies is that the laboratory analyses method used to analyse the samples is likely to have an effect on detection efficiency. However there is no standardised analyses method and different workers use different media combinations and incubation temperatures.

The two sampling methods that have consistently produced favourable results are socks and drag swabs moistened with 2XSM. Previous studies have determined that 4 drag swabs (Caldwell *et al.* 1994) and 5 pairs of overshoes (Gradel *et al.* 2002) are effective for measuring house status. Gradel *et al.* (2002) also reports enhanced *Salmonella* detection for samples collected when birds are 3 weeks old. Thus as part of this study we will undertake comparisons between socks and drag swabs in order to determine which is most sensitive. Experience with these sampling protocols is also important for assessing the practicalities and costs associated with each method.

## **1.3 Laboratory methods**

There are a large number of published analyses methods for the enriched detection of *Salmonella* including a horizontal International Standards method (ISO 6579 2002). Although these methods do include well characterised biochemical protocols such as antibody based detection, molecular protocols such as PCR- and blotting-based detection in addition to traditional culture methods, only culture methods will

be assessed as part of this document. The reasons for restricting the scope of the discussion are on the basis of costs involved. Traditional culture methods cost between 5 and 25 times less than biochemical or molecular protocols (Direct Laboratories- Commercial Testing Internal Costings). Furthermore, the equipment costs associated traditional culture methods are far lower than those required for PCR and ELISA. Thus traditional microbiological analyses represent less of a financial burden for poultry producers and processors and on these grounds are most appropriate for assessment.

Although biochemical and molecular protocols have been excluded on the grounds of cost, both of these method types do offer the principal advantage of dead cell detection when compared with traditional culture. Since *Salmonella* levels have been shown to peak in broiler houses when birds are 3 weeks old (Gradel *et al.* 2002), sampling and testing which is undertaken after this time would likely be more sensitive if a non-viable detection method was used (Kwon *et al.* 1999).

In general, most traditional culture-analyses methods can be broken down into four main stages. These are:

1. Pre-enrichment which includes the initial sample collection and storage in a weak-nutrient diluent or culture medium.
2. Selective enrichment either by direct inhibition of background flora, or selection on the basis of other characteristics such as motility.
3. Detection by plating onto a selective media.
4. Confirmation by biochemical profiling and serological/phage typing.

A selection of the most commonly-encountered media that have been used for each of these stages is summarised as Table 2.

Pre-enrichment	Selective enrichment	Detection	Reference
2 X skimmed milk	Modified Tetrathionate broth (MTB)	xylose lysine tergitol 4 (XLT4) agar and Brilliant Green Agar+N	(Hayes <i>et al.</i> 2000; Opara <i>et al.</i> 1992b; Opara <i>et al.</i> 1992a; Opara <i>et al.</i> 1994)
Buffered peptone water (BPW)	Rappaport Vassiliadis soy peptone broth (RVSPB)	XLD and one other agar	(Chadfield <i>et al.</i> 2001; Nordisk Metodikkommitte for livsmedel 1999; Skov <i>et al.</i> 1999a; Skov <i>et al.</i> 1999b)
BPW,	MSRV	MacConkey agar (MC)	(Read <i>et al.</i> 1994)
BPW	Tetrathionate Brilliant Green broth (TBGB)	(XLT4) agar and Brilliant Green Agar+N	(Read <i>et al.</i> 1994)
BPW	RV or Diasalm	MSRV or XLD	(Heyndrickx <i>et al.</i> 2002)
None (saline diluent for transit)	Tetrathionate broth (TB)	BGA +N	(Byrd <i>et al.</i> 1997; Caldwell <i>et al.</i> 1994)
2% peptone	Universal Pre-enrichment broth (UPEB)	BGA + N + Nal*	(Kwon <i>et al.</i> 2000)
BPW	RVSPB	Rambach Agar (RA)	(Gradel <i>et al.</i> 2002; Gradel <i>et al.</i> 2003)

Table 2: A selection of media used for enrichment and detection of *Salmonella* from broiler house samples

Abbreviations used: N- Novobiocin, Nal- Nalidixic acid

\* was used to recover a Nalidixic acid-resistant strain introduced into the broiler house.

### **1.3.1 Comparative analyses studies**

Heyndrickx et al (2002) undertook an assessment of different enrichment media for *Salmonella* as part of a larger study aimed at identifying routes of *Salmonella* contamination of poultry meat. The types of samples collected from broiler houses that are relevant for this report were caecal droppings and overshoes. Samples were collected from 18 flocks of birds. Sample culture was after either inhibition enrichment in Rappaport Vassiliadis (RV) or motility enrichment in either Diasalm or modified semisolid RV and plating onto XLD. Overall sample collection in buffered peptone water (BPW) followed by selective enrichment in RV (0.1 ml of BPW sample in 10 ml final volume RV) with a 24hr incubation was the most effective (100% sensitive) at detecting *Salmonella* in all of the sample types tested. The recovery results for broiler house samples were not reported.

Comparisons of *Salmonella* enrichment exclusively from broiler litter for two culture methods was undertaken by Read *et al.* (1994). The methods that were compared were:

Method 1: pre-enrichment in BPW, selective motility enrichment in MSR/V and plating onto MacConkey agar (MC)

and

Method 2: enrichment in tetrathionate brilliant green broth (TGB) followed by dual plating onto xylose lysine tergitol 4 agar (XLT4) and brilliant green agar with novobiocin (BGAN).

Method 1 resulted in significantly higher isolation of *Salmonella*, was cheaper in terms of media cost and less labour intensive (Read *et al.* 1994). Read et al (1994) also underscore the importance of laboratory analyses costs to the broiler industry.

### **1.3.2 Analyses protocol conclusions**

As discussed in section 1.2.7, the effectiveness of the analyses method that is used will depend on the type of sample being analysed. Although there are a number of studies that have compared sampling methods, we were unable to find any studies

that compared directly the effectiveness of different analyses methods on samples collected using a single sampling method. Most of the previously-reported sampling studies have used in-house laboratory methods, and thus there are a wide range of potential methods available. The scope of this study however allows for a narrow range of methods to be assessed practically. The two methods that will be used are the standard ISO method (ISO 6579 2002) and a method that has been trialled favourably by a number of EU member state government agencies including the UK Veterinary Laboratories Agency. The reasons for these methods being selected are that the ISO method is a horizontal method which has been shown previously to perform well on a range of sample types. The VLA-supplied method has been shown to be effective at isolating *Salmonella* from broiler litter.

#### **1.4 Sampling and Scheduling in Europe**

EU council directive 92/117/EEC is concerned with measures for the protection of products of animal origin from *Salmonella* and applies to all EU member states. The directive is limited to breeding flocks of at least 250 birds and includes generalised outlines for sampling and analyses for *Salmonella*. A summary of the sampling required for compliance with 92/117/EC is shown as Table 3 and Table 4.

Bird age	Sampling method
1 day	“Samples from lining papers in chick boxes” and “carcasses of dead chicks”
4 weeks	“Pooled faeces samples made up of separate samples of fresh faeces each weighing not less than 1 g and taken at random from a number of sites in the” [building or group of buildings] “in which the birds are kept”

Table 3: Sample collections and bird ages for compliance with EU directive 92/117/EC in breeding flocks

Number of birds	Number of samples to be collected
1-24	Equal to the number of birds up to 20
25-29	>20
30-39	>25
40-49	>30
50-59	>35
60-80	>49
90-199	>50
200-499	>55
>500	60

Table 4: Number of samples to be collected for ranges of bird numbers for compliance with EU directive 92/117/EC in breeding flocks

Examination of samples for *Salmonella* is similarly general and should be by “tried and tested national methods which afford guarantees laid down in EU commission decision 89/610EEC”. The guarantee conditions laid down in decision 89/610EEC are similarly general and do not list specific analyses-method details.

Thus, although there are a specific set of EU criteria aimed specifically at detection of *Salmonella* infections in poultry breeding flocks, the criteria are general and subject to a variety of interpretations in individual member states. For these reasons there exists a variety of sampling and analyses methods within the EU. Standardised methods based on EU directive 92/117/EC outline methodologies for *Salmonella*-free breeding stocks have not be carried further downstream into the production birds. Thus currently there are a range of protocols operating within the EU for broilers. This section of the report is a summary of the different sampling and scheduling methods currently advised nationally by different member states within the EU.

#### **1.4.1 Cyprus**

In Cyprus around 10% of broiler flocks are sampled for *Salmonella* 15 days before slaughter (Christos Hadjipapas Animal Welfare MOA Cyprus, personal communication). Samples are pooled faecal material composed of up to 60 grab handfuls of at least 1g each, a sampling regime indistinguishable from EU directive 92/117/EC for breeding flocks. Samples in Cyprus are tested using the International Standards *Salmonella* method (ISO 6579 2002) in independent government-operated laboratories.

#### **1.4.2 Germany**

When asked about government or other national procedures regarding pre-slaughter *Salmonella* scheduling and testing, Germany provided two documents written in German (Ellerbroek *et al.* 2001; Wichmann *et al.* 2001). Translation of key parts of these documents revealed that they were papers from a German journal called “Meat Research and Development”. The documents detail the occurrence of *Salmonella* in German poultry in:

A. Large sized broiler houses and broiler slaughterhouses  
and

## B. Small establishments with on-site slaughter and marketing facilities

Sampling in broiler houses was by grab handfuls of litter and analyses was by ISO 6579 and plating onto Rambach agar. Although these papers report the prevalence of *Salmonella* in sampled broiler houses in Germany in 1999, they do not appear to be national guidance issued by the German government. Thus it seems likely that aside from EU commission directive 92/117/EEC, no formal guidance exists in Germany for the scheduling of broiler sampling for *Salmonella* prior to slaughter.

### 1.4.3 The Netherlands

The Netherlands has specific sampling requirements for broilers when they are first introduced into a farm. Forty soiled lining papers (5cm x 5cm dimension) from transit crates are tested from each batch of new birds. 2 weeks before slaughter, 2 pairs of overshoe samples are taken per flock. Both types of samples are processed for *Salmonella* according to a method standardised across the Dutch *Salmonella* programme. Briefly, samples are diluted in BPW and incubated for 24h at 37°C. Aliquots (1ml) are then plated onto MSR/V agar and incubated for 24-48h at 41.5°C. Presumptive positive isolations are cultured on BGA and confirmed by serological testing (Nico Bolder, Wageningen University NL, personal communication).

### 1.4.4 Sweden

Although Sweden has a relatively small poultry industry [<50 million broilers annually (National Veterinary Institute *et al.* 1995)] authorities have had a formalised *Salmonella* control programme since 1995. The programme covers all live poultry including laying hens, broilers, turkey, geese and ducks from breeders through to consumed product. Sampling for *Salmonella* in broilers occurs 1-2 weeks before the birds are scheduled for slaughter. A choice of sampling methods is allowed. Thirty dead birds, sampled by culturing excised caecums, are required for large (>499 birds) flocks; a reduced sampling of 10 broiler caecums is allowed for small flocks (<500 birds). Caecums derived from five turkeys, ducks or geese are required. Faecal samples are collected as described in EU commission directive 92/117/EEC. Large flocks and non-broiler poultry require 60 faecal samples, small flocks require only 30 samplings. Laboratory analysis takes place in government-approved laboratories using a method which is “as sensitive as the International Standards reference method (ISO 6579 2002)“.

### **1.4.5 Norway**

The Norwegian *Salmonella* control programme for live poultry covers laying hens, broilers and all other meat production poultry from elite flocks to commercial stock. The programme is very similar to that described in section 1.4.4 for Sweden; although the programme was implemented a year earlier in 1994. Much of the text in the Norwegian Salmonella Control Programme is repeated verbatim, and thus it seems likely that there was strong collaboration between Norway and Sweden when implementing their respective programmes. Consequently there are no significant differences between Sweden and Norway and for the remainder of this report the two programmes will be referred to collectively as the Nordic programmes.

Although a choice of analyses microbiological methods are allowed for the Nordic programmes, in reality, most detection is undertaken using “method 71” (Nordisk Metodikkommitte for livsmedel 1999). Briefly a sample is pre-enriched in an equal volume of phosphate-buffered peptone water at 37°C for 18 h. An aliquot (0.1 ml) is transferred to pre-warmed RV broth for 24h at 42°C before plating onto XLD plus one other appropriate agar and incubation at 37°C for 24h.

### **1.4.6 Denmark**

The Danish sampling and scheduling schemes are tightly linked with a country-wide initiative to irradiate *Salmonella* from all foodchain livestock. A set of generalised scheduling conditions exist which have been written to cover both laying and broiler poultry (Wegener *et al.* 2003) and are summarised as Table 5.

### **1.4.7 France**

The documents submitted by France were again generated for compliance with EU directive 92/117/EC. Samples which are allowed in France are shipping crate lining papers soaked in 2x BPW, swabs soaked in single strength BPW, chicks, or faecal materials .

Acceptable laboratory analyses in France are well defined. Pre-enrichment is in buffered peptone water for 8-24h at 37°C±2°C. Enrichment is in a choice of selective media for 18-24h. Media choices include modified RV-Soya peptone supplemented with malachite green and magnesium chloride (42±1°C), selenite cysteine (37±2°C)

or Muller Kaufmann Tetrathionate broth ( $37\pm 2^{\circ}\text{C}$ ) or through motility through semi-solid media. Isolation is by plating on Rambach agar, a sorbitol MacConkey-style agar, XLT4 or TSI for up to 48h at  $37\pm 2^{\circ}\text{C}$ . Presumptives are tested by biochemical profiling and serological confirmation.

#### **1.4.8 Ireland**

Aside from standard outline legislation generated for compliance with EU directive 92/117/EC and prevention of infections in breeding birds, the Irish Department of Agriculture and Food does not appear to have issue guidelines to determine broiler flock status on-farm prior to slaughter monitoring. The Irish however have issued control measures for processing plants. During processing, carcass samples are taken after evisceration and at least one sample from each houseful of birds is required. In the event of a positive isolation at the plant, trace-back to flock, hatchery and breeding flock is undertaken. Prior to restocking the house, extensive cleaning and disinfection is undertaken (Bernard Bulser, DAF, Ireland, personal communication).

#### **1.4.9 Belgium**

In keeping with most other EU member states, Belgium has sufficient legislation and guidance to comply with EU directive 92/117/EC, but nothing further with respect to pre-slaughter scheduling and testing in production flocks. The methods are similar to those described for other member states (sections 1.4.3 and 1.4.7).

Stage of Production	Age or Frequency	Samples Taken	Analysis method
Control rearing stations, broiler and egg sector	Day-old Chickens	10 samples of crate material, 20 dead or destroyed chickens	Microbiological
	1wk	40 dead chickens	Microbiological
	2wks	2 pairs of sock samples	Microbiological
	4wks	60 faecal samples	Microbiological
	8 wks	2 pairs of sock samples	Microbiological
	2 weeks before moving	60 faecal samples and 60 blood samples	Microbiological / serological

Table 5: Salmonella surveillance of the broiler and egg production, Denmark (adapted from Wegener *et al.* 2003)

## **1.5 Sampling and Scheduling in the USA**

### **1.5.1 American National Poultry Improvement Plan**

In 1997 the Animal and Plant Health Section of the United States Department of Agriculture (USDA) launched a voluntary code of best practices called the National Poultry Improvement Plan (NPIP) along with some auxiliary provisions (USDA Animal and Plant Inspection Service 1997). The program is aimed principally at breeding flocks and multiplier flocks and thus is analogous to EU directive 92/117/EC. The plan was designed to lower the incidence of Salmonella in poultry generally and so NPIP covers poultry raised for both eggs and meat. There are specific sections that cover sampling and analyses methods individually for chicken and turkey broilers on-farm. The sampling and analyses methods in the NPIP document are far more detailed than those outlined in the EU counterpart document.

### **1.5.2 On farm sampling for chickens, turkeys, waterfowl, exhibition poultry and farmed game birds**

Although turkeys are listed separately from chickens in the NPIP guidelines, samples for both types of bird are collected in a similar manner and are thus discussed together.

The USDA specify four types of samples that can be collected in order to ensure NPIP compliance. The four types of samples are environmental, cloacal, drag swabs and chick papers and for some of these sample types a choice of collection method is described.

### **1.5.3 Environmental swabs**

Environmental samples are analogous to the grab handful samples discussed in section 1.2.3 of this report. Samples can be collected into broth or alternatively dry containers.

Broth collections involve the use of sterile enrichment broth such as Hanja or Mueller-Jauffmann Tetrathionate Brilliant Green, is used. The guidelines strictly specify the dimensions for the broth container (1-2cm diameter, 15-20 cm length) and

the approximate volumes to be used (2/3 of the container full, ~15ml media). A single sample should be collected for every bird pen or for every 500 birds. Samples are collected using a cotton-tipped swab. The swab is repeatedly drawn through fresh faeces, diarrheic (sic) droppings and areas frequently visited by the birds such as roosts, feed and water troughs. Periodically the material collected is transferred into the media by swirling the swab inside the tube. The recommendations state that a single sample should ideally contain 1-2g of litter per 10-20ml of media. The broken-off swab is included as part of the sample.

Dry container sampling consist of several specimens taken from “representative locations in the pen or house”. Samples should vary in “type and consistency” At least 10g of material should be collected for each sample using a sterile tongue depressor or similar. Dry sampling is more time consuming than swab sampling because a larger number of samples have to be collected when compared to broth sampling. Dry sampling requires 5 samples be collected for up to 500 birds, 10 samples for 500-2500 birds and 15 samples for more than 2500 birds. Laboratories can pool samples from houses with larger bird numbers, but a minimum of 5 still need to be processed from each house or pen.

#### **1.5.4 Cloakal swabs**

It is unlikely that cloakal swabbing is widely used in the US. The NPIP guidelines state that each bird in the flock should be sampled or a minimum of 500 birds for large flocks. Each bird is sampled using a cotton swab which is inserted into the cloaka. This form of sampling is time-consuming and also expensive to analyse since samples are pooled into batches of 5 and an individual swab is used for each bird.

#### **1.5.5 Drag swabs**

The most common form of sampling used for NPIP-compliance are drag swabs. The description given in section 1.2.1 and that given in the NPIP document are indistinguishable.

For shipping to the lab, drag swabs from chicken and turkey houses are refrigerated in polythene bags at 2-4°C. For turkey swabs, 5 ml of double strength skim milk should be added to the bag during transit.

### **1.5.6 Chick box papers**

Chick box papers are also an approved method for determination of the *Salmonella* status of a flock. Again a choice of methods are described. Either the papers can be collected aseptically and submitted directly to the laboratory for analyses, or the papers can be moistened with double strength skim milk and rubbed with milk-soaked drag swabs. Sending papers directly to laboratories is most common because it is less time consuming, and no refrigeration is required during transit to the lab. Swabs from papers need to be bagged in polythene bags with additional volumes (5-10 ml) of double-strength skim milk added to each and samples kept chilled at 2-4°C for transit to the analyses lab. The only reason that producers would use drag swabs to sample papers is if transit to the laboratory was delayed. The use of milk allows samples to be frozen in order to extend storage of the sample.

### **1.5.7 NPIP-approved method for microbiological examination of samples**

A single approved laboratory method is allowed for analyses of environmental samples. A summary overview is detailed as Figure 1.

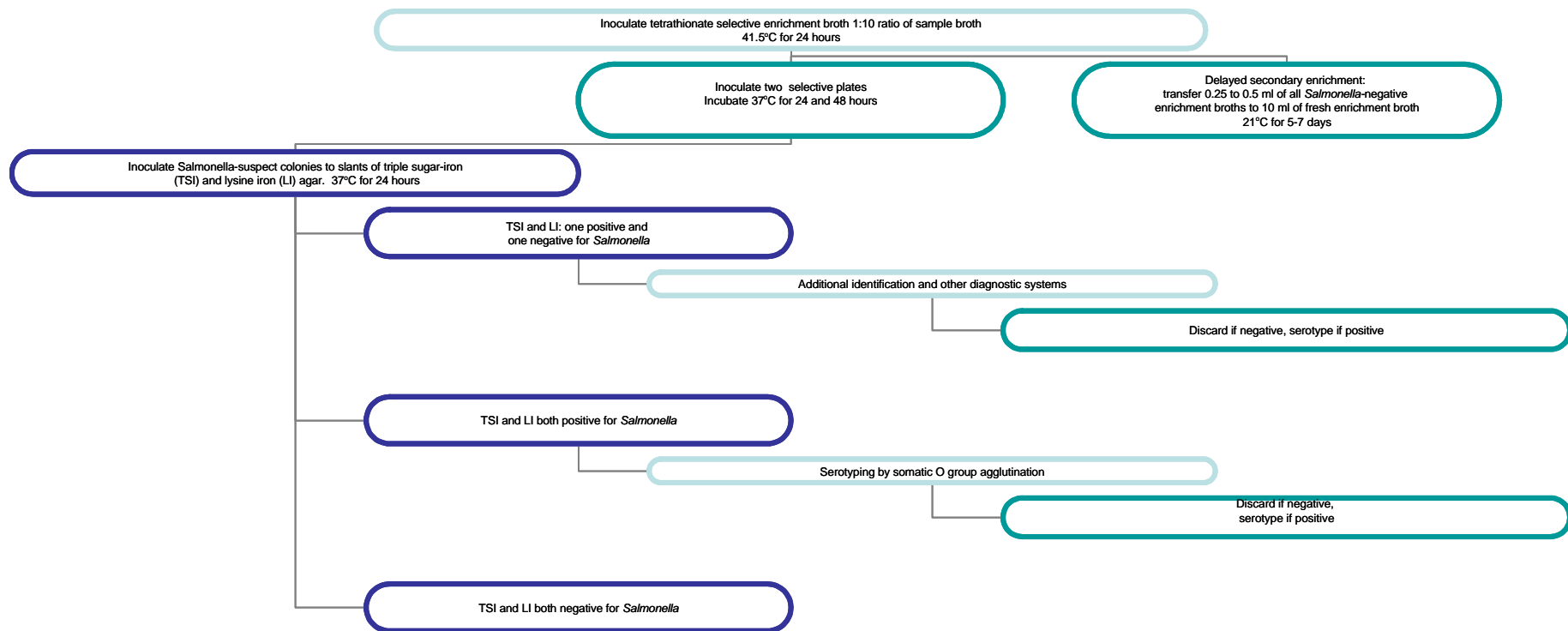


Figure 1: A summary of approved methods for the microbiological analyses of environmental samples collected from poultry housing. Adapted from the USDA-NCIP guidance document (USDA Animal and Plant Inspection Service 1997).

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