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Sampling and bacteriological detection of *Salmonella* in poultry and poultry premises: a review

J.J. Carrique-Mas & R.H. Davies

Department of Food and Environmental Safety, Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom

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Summary

The detection of *Salmonella* in primary poultry production is an issue of great concern in the European Union (EU), since control of this zoonotic disease is in part based on the reduction of the prevalence at the farm level. Success of detection is likely to be highly dependent on the choice of an adequate sampling procedure combined with a sensitive culture method. In poultry farms 'naturally pooled' faeces/litter and dust are the matrices of choice. In floor systems boot swabs are the preferred method for the collection of faeces. A wide range of culture methods is available, but ISO 6579:2002 (Annex D) is currently the standard for poultry environmental samples in the EU. In this review, the authors discuss in detail the range of sampling and culture methodologies for *Salmonella* in poultry farms. The review also covers sampling and testing of poultry hatcheries, live birds and poultry carcasses.

Keywords

Chickens – Enrichment – European Union – Isolation – Media – Poultry – Pre-enrichment – Salmonella – Sampling.

Introduction

The monitoring and control of *Salmonella* in poultry has been a priority over the last few decades in the United Kingdom (UK) and elsewhere in the European Union (EU), since most human cases of *Salmonella* are thought to be linked to the consumption of eggs and poultry products (34, 78). Because the EU works as a single market, the European Commission (EC) has played a major role in harmonising and co-ordinating monitoring and control programmes aiming to reduce the prevalence of *Salmonella* in primary production of poultry across EU Member States. A key piece of EU legislation is Regulation (EC) No. 2160/2003, which requires that Member States put in place national control plans so that reduction targets for the prevalence of *Salmonella* at farm level can be met (2, 41). These control plans are likely to require harmonised sampling of production flocks, in addition to more sensitive monitoring of breeding flocks and hatcheries.

The investigation of *Salmonella* in poultry at the primary production stage may involve:

– the collection of material from the environment in the poultry house (e.g. faeces, litter, dust), or

– the collection of samples from the birds themselves (e.g. blood, cloacal swabs, post-mortem samples of the small intestine and caecum).

Laboratory tests can be broadly classified into those that aim to detect the presence of viable *Salmonella* organisms (culture, immunological detection of antigens following culture), specific components of these organisms (i.e. molecular DNA amplification techniques), or a response of the host to infection (i.e. serological testing). The type and number of samples is determined by the type of test. The following is a review of published information regarding methods of sample collection and testing for detection of *Salmonella* in primary poultry production (i.e. farms and hatcheries), as well as in live poultry, poultry carcasses and organs, using bacteriological (i.e. culture) methods only. It is aimed at public health and laboratory scientists, veterinarians, poultry health specialists, and policy makers with an interest in the control of non-typhoidal *Salmonella* in poultry.

Sampling of living birds

The investigation of *Salmonella* in poultry is normally driven by public health considerations. In most cases, infection in adult birds is asymptomatic (88) and the investigation of the individual bird is relatively unimportant compared with establishing whether a flock is infected. A flock is normally regarded as infected with *Salmonella* if at least one sample from the birds, or a relevant environmental sample, tests positive. In some studies, birds have been sampled using cloacal swabs (5, 6). Cloacal swabs should be taken as aseptically as possible, avoiding cross-contamination of the swab from the integument of the birds. Any previous antibiotic treatment may mask the success of the isolation. Cloacal swabs are relatively insensitive due to, among other reasons, a typically low prevalence of infection in individual birds, combined with intermittent shedding and the relatively low number of organisms excreted by infected birds in many cases. Moreover, since cloacal swabs only obtain a small amount of faeces and *Salmonella* may be present in low numbers or be non-uniformly distributed in the faeces, this method is likely to be relatively insensitive compared with the culture of more voluminous faecal material (44, 62). The sensitivity of detection may be further reduced when swabs from a large number of birds are pooled and processed as a small number of cultures. Unfortunately, this has been the preferred method in many *Salmonella* monitoring schemes in poultry. The peak and level of excretion in the birds is likely to depend on the timing of entry of the infection into the flocks, the type of *Salmonella*, the level of challenge and the species, age and stage of production of the flock. All of these variables may greatly affect shedding of *Salmonella* and the chance of detection. In broiler flocks, for example, samples taken at the age of three weeks were more successful in detecting infection than sampling later in the production cycle (46), whereas commercial egg laying flocks sampled later are more likely to test positive (94). Also, the authors have seen a lower prevalence of infection in adult turkey breeders compared with turkeys in the brooding or meat finishing stage (data not shown).

Environmental sampling of poultry houses

The presence of a large number of competing bacteria is a major limiting factor in the isolation of *Salmonella* from faeces and other environmental samples. However, environmental sampling of the poultry house is regarded as more cost-effective (1) and sensitive than sampling a limited number of individual birds, provided that appropriate samples are collected and tested (31). The most common samples collected from poultry houses are faeces, litter or dust. Faeces (especially if fresh) provide an indication of current infection, whereas *Salmonella* positive dust may also indicate previous infection. It is normally easier to isolate *Salmonella* from dust than from faeces. This is likely to be due to the comparative advantage of *Salmonella* in this type of matrix compared to other Enterobacteriaceae, which do not tend to survive as well in dry conditions (50). *Salmonella* has been reported to survive in poultry houses for at least 53 weeks in dust (31) and up to 26 months in thin layers of litter, dried faeces and feed (29) following depopulation of a flock. In laying houses, for example, sampling faeces alone may fail to detect infected flocks that have passed the peak of infection but which are still producing contaminated eggs (84). Because of this, sampling of both faeces/litter and dust is recommended in *Salmonella* monitoring programmes. It must be acknowledged that in most cases it will not be economically possible to employ a sampling method which has 100% detection sensitivity, but a possible option would be the use of the sampling method on repeated occasions during the life of a flock. It is at least theoretically possible that positive environmental samples may not reflect established infection in the flock. This may be the case with transient infections or when infected rodent droppings or contaminated feed are included with the sample. In most cases however *Salmonella* found in faeces and/or dust is concurrent with infection in the flock.

Collection of faeces

Faeces can either be carefully collected as individual droppings, and later combined into a smaller number of composite samples. However, for detection purposes, it is more practical (and sensitive) to take pinches of 'naturally pooled' faeces from the areas where manure gets deposited in cage laying houses, or to use boot swabs in floor housing systems. During the life of an infected flock *Salmonella* is

normally shed at any time by a small proportion of birds, often intermittently (35). This may partly explain the non-uniform distribution of *Salmonella* often found in poultry houses (49, 84, 85). The collection of naturally pooled faecal material (i.e. originating from a large number of birds) is preferable to the collection of individual droppings or cloacal swabs. The former method increases the chance of inclusion of faecal material from infected birds which may contain high numbers of organisms, hence compensating for the typically low bird-level prevalence of infection (3). The maximum number of individual droppings that could be combined for culture without compromising the sensitivity of detection is still not well established for poultry faeces, but it is likely to be highly variable, depending on both the within-flock prevalence of *Salmonella* and the type and number of competitor organisms present. In most cases, the sampling programme is determined by a combination of sensitivity and economic considerations. In situations of high prevalence fewer samples would be necessary, but it is clear that a standardised protocol (with minor variations depending on the house type) is preferable in order to allow meaningful prevalence comparisons so that epidemiological analyses can be carried out. Representative collection of faeces presents a particular challenge when flocks are in cage houses because of limited access. The specifications for commercial layers contained in the 2004/2005 *Salmonella* 'baseline survey' for laying flocks (one of three surveys carried out between 2004 and 2007 to ascertain the prevalence of *Salmonella* among poultry in the EU) required the collection of five naturally pooled samples of faeces/litter from each laying house (38, 92). For cage houses, each faeces sample consisted of 200 g to 300 g made up of 20 to 40 pinches from representative locations, which varied according to the housing type:

– if the manure collection system involved belts or scrapers, faeces were to be collected from the scrapers or bars at the discharge ends of the cage rows

– in 'A-frame' (step-cage) houses, faeces were to be collected from separate locations from the accumulated manure in the deep pit (this can sometimes be a challenging and unpleasant task since deep pits may not always be accessible).

In floor housing systems, litter/faeces can be easily collected from a number of representative locations. In these systems boot swabs (or 'socks') are preferred, since they can be used in connection with ordinary inspection of the flock and they can be conveniently posted to the laboratory for analysis. Boot swabs are also more likely to be taken in a representative manner than litter. In a Danish study, sampling of a poultry house using two pairs of socks, analysed as one sample, was at least as sensitive as sampling of 60 faeces, analysed as one sample (46). Collection of a pair of gauze socks for each of the five subsections of a chicken house, five pairs in all, was at least as sensitive as the collection of 300 fresh droppings analysed in groups of five (i.e. 60 cultures). This method was superior to other sampling methods (i.e. one pair of boot swabs, one paper sheet in each of five sampling areas of the house) (91). This may be attributable to a typically very low within-flock prevalence, which would give a comparative advantage to boot swabs, which probably collect faecal material from a much larger number of birds. These findings were instrumental in the decision to adopt five pairs of boot swabs (cultured separately) as a sampling method for the EU *Salmonella* baseline surveys of broilers, turkeys and egg laying flocks raised on floor systems (i.e. barns and free-range houses) (38, 39, 40), and for the statutory monitoring of chicken breeding flocks (Commission Regulation [EC]) No. 1003/2005). A very important consideration when using boot swabs is to avoid contamination or contact with disinfectant (i.e. boot dips) prior to use. Before use, boot swabs should be pre-moistened with a suitable diluent, normally potable water, to avoid problems of non-adherence if the litter is dry. There is a wide range of commercial products that can be used as boot swabs, such as socks, surgical shoe covers, mob caps, which may all vary in their absorbency, ease of fitting, and resistance to tearing. A concern with larger types of boot swabs is that they also require larger volumes of pre-enrichment medium (i.e. buffered peptone water [BPW]) (65), which increases the cost of culture, as well as requiring more incubator space. Sampling both turkey rearing and finishing flocks for *Salmonella* using boot swabs (one pair cultured as one sample) was more effective than the culture of pooled litter (unpublished data).

In some countries, drag swabs are used, which in their original form consisted of an assembly of at least three separate moistened 10 cm × 10 cm surgical gauze swabs, attached to a string stapled to a wooden spatula (60, 68). Commercial adaptations of this are based on a small, pre-moistened cellulose sponge, which has limitations due to the small surface area and light weight. The sampling procedure consists of dragging the assembled kit over the manure along the length of the chicken house using the water and feeding line as sectioning guides, or along the pits (when present) in laying houses. This method is currently the favoured one for sampling poultry houses in the United States of America (USA) (16). Variable results were obtained depending on whether drag swabs were used dry or with transport media of different types (75, 85). Other factors affecting performance relate to the water content of the manure sampled (85), exposure times and storage conditions (75). The use of certain transport media was shown to increase the recovery rate if there was a substantial delay in the processing of the samples (10, 75, 85). Refrigeration of the drag swab for up to three days before culturing yielded better results than freezing swabs two weeks prior to culture (75). A disadvantage of the drag swab method is that it may be difficult to apply in houses

with older birds that occupy most of the floor space and refuse to move, in addition to costs associated with assembly preparation (66). Boot and drag swabs become quickly saturated, and may therefore be unable to trap further bacteria, and thus it has been suggested that swabs should be changed based on their level of soiling, rather than the distance travelled (85). Studies in the USA have shown that boot swabs performed similarly to, or better than, drag-swabs when both were evaluated in parallel (11, 66). It has recently been shown that stepping on drag swabs enhances the adherence of faeces and thus the rate of detection is increased compared with the standard sampling methodology. However, the highest overall detection rate was still achieved using boot swabs (7).

Collection of dust

Dust is regarded as a sensitive sample type for detecting *Salmonella* (33) and is a potential vehicle of transmission of *Salmonella* in some types of poultry houses (48). In poultry houses accumulation of dust is more common around fans and extraction vents, although it may be easier to collect it from horizontal surfaces where it deposits, such as partitions, ledges, beams and (in cage houses) underneath the cages (27). In laying houses where automated egg collection is used accumulated dust from the ends of the egg belts and in spillage trays on egg elevators is a convenient sample.

The specifications for cage houses in the EU *Salmonella* baseline survey for laying flocks required the collection of two 250 ml composite samples of dust from beneath the cages, each to be collected from 20 separate locations within the house. For barn and free-range houses two dust samples were to be taken: one consisted of dusty spillage discharged from egg belts (if present), and another one from the rest of the house including exhaust fan baffles, ledges, beams, partitions or pipes (38). However, in some barns or naturally ventilated houses dust is not always available or easy to sample, particularly if the building contains no horizontal surfaces where the dust can settle. Dust is also less copious in houses with young flocks. More recently, aerosol sampling (64) and electrostatic dust sampling (45) devices have been developed. The authors do not yet have data about the performance of these methods in relation to the EU sampling methods.

Collection of samples into buffered peptone water

An alternative to the collection of 'dry' faeces, litter or dust, which require further processing in the laboratory, is to take samples using BPW-impregnated hand-held swabs taken directly into 225 ml BPW jars. In occupied laying houses, the collection of faeces/litter (25 g) and dust (15 g) from 10 different points in the house is more sensitive than the EU layer survey method, although on a 'per sample' basis each sample is less sensitive than EU survey samples (unpublished data). By taking more samples from different locations the specific areas of contamination in the houses can be more precisely identified. Also, if each swab was used to sample multiple sites, then the per sample sensitivity would be expected to increase, allowing a reduction in the number of samples taken. However, this has never been investigated in detail. The jars of BPW containing the samples are placed directly into incubation after arrival in the laboratory without the need of further processing. This 'wet swabbing' method is only suitable if samples can be quickly returned to the laboratory so that culture can begin soon after collection, and this usually requires trained staff to take the samples. A delay in the start of the culturing process may result in *Salmonella* being overgrown by competitor organisms in the BPW. This method is particularly useful for assessing the efficacy of cleaning and disinfection (C&D) of poultry houses, since any residual disinfectant that may still be present on the surfaces is diluted by excess BPW. The highest rates of detection in disinfected chicken breeding houses were observed in floor sweepings, nest box floors, slave hoppers, wall fabric junctions, feed troughs and high beams and pipes (25, 31). In cage systems, drinkers, feeders, cage interiors, dropping belts / boards and floors are routinely sampled to assess C&D (95). Recently a semi-quantitative method for estimating residual contamination of cleaned and disinfected houses (95), as well as infection of flocks (96), has been developed, but the extra costs of this are not likely to be justifiable for routine testing.

Sampling of poultry hatcheries

Culture of meconium, dead-in-shell or cull chicks has up to recently formed the basis of statutory monitoring of hatcheries in many countries, but may underestimate contamination, especially for serovars that are not transmitted via the ovary (27). Collection of hatcher fluff samples has also been used for monitoring purposes (19), although broken egg shells and hatcher basket liners are more productive types of sample (20, 27). The latter is now included in recent EU legislation for monitoring of chicken breeding flocks (Commission Regulation [EC] No. 1005/2005). Macerated hatchery basket waste or swabs from the macerator after use are also very sensitive samples for detection of *Salmonella* in the hatchery throughput.

Sampling of poultry carcasses at the abattoir

Microbiological sampling of poultry carcasses may be conducted by whole-carcass rinsing, tissue excision or skin

swabbing. Whole-carcass rinsing involves placing the carcass in a sterile bag with a suitable wash, such as maximum recovery diluent, and performing microbiological tests on the rinse fluid after manual shaking (14, 21). However, results may vary depending on when the sample is taken in the process (i.e. pre-chill, post-chill), as well as differences in rinse and sub-sampling volumes (43). Tissue excision involves removing small areas (<50cm^2) of the carcass skin, typically neck skins. The tissue is then homogenised by blending or stomaching (4) and then cultured. A stomacher is a relatively simple device, which homogenises specimens in a special plastic bag by the vigorous pounding of two paddles. Stomaching is now the preferred method of choice for homogenising foods, including meat samples. Stomaching involves less labour than blending, since bags can be disposed of after samples have been processed or can be used for further culture of the samples (87), and is at least as effective as blending in recovering Gram-negative bacteria from meat (55). Blenders need to be thoroughly cleaned and sterilised to avoid cross-contamination, but can be more convenient for high-throughput testing when serial blenders can be used and sterilised in boiling water. Tissue excision may be destructive (e.g. breast tissue removal) or non destructive (e.g. neck skin removal). The former is not considered acceptable for routine sampling to verify process control because it devalues the carcass. Neck skin samples contain higher numbers of *Salmonella* than vent samples, and sampling 5 g to 10 g is more sensitive than sampling 1 g (63). Skin swabbing is performed using an abrasive sponge or gauze which is used to remove bacteria. The swab is then shaken or stomached in diluent prior to culture. In general, neck skin or carcass rinse sampling is regarded as more sensitive than skin swabbing (89). Studies have shown a higher recovery of *Salmonella* when whole-carcasses (90) or neck skins (58) were cultured with pre-enrichment media compared to just culturing an aliquot of the rinse. Turkeys are larger than broilers and therefore it is more difficult to manually perform whole-carcass rinses on them. For this species a special 'modified rinse' method was devised, in which the carcass was introduced in an autoclave bag with sterile diluent, and was later placed on the seat of a custom-made free-standing swing, which was used to swing the carcass back and forth a set number of times. Both whole-carcass swabbing and the modified rinse method were more effective in detecting *Salmonella* than swabbing in several locations or excision of neck tissue (67).

Investigation of *Salmonella* in tissues/organs of birds

In chickens which had been experimentally infected with medium to high doses of *S. Enteritidis*, the caeca, crop, the proventriculus and the bursa of Fabricius were the organs from which *Salmonella* could be isolated most frequently, and the caeca contained the highest numbers of *Salmonella* bacteria (35). Certain phage types of *S. Enteritidis* are successful in colonising the reproductive tissue of hens for long periods (52). In some cases it has been possible to isolate *S. Enteritidis* from the ovary and oviduct in the absence of intestinal colonisation (9, 71), although this may be a reflection of the additional difficulty in recovering *Salmonella* from faeces where a high level of competing flora is present. In chicks dying from septicaemia due to *S. Enteritidis* or *S. Typhimurium* the bacteria may sometimes be isolated directly from liver, gall bladder or yolk sac (57). In some countries, livers taken from poultry on the slaughter line have been routinely sampled and cultured without surface searing to assess infection (1), but in most cases this will reflect cross-contamination in the abattoir and risk of contamination of meat products, rather than infection of the individual bird. This type of monitoring is therefore most suitable for situations in which a low flock-prevalence is expected. Internal tissues and organs at the point of slaughter are virtually sterile, so extra precautions need to be taken to avoid cross-contamination from other materials in the laboratory when culturing these for *Salmonella*.

Bacteriological testing

Sample holding and preparation

There are very few published studies on how different methods of holding, transporting and preparing the sample affect the recovery of *Salmonella* from poultry faeces. A study using pig's faeces reported no significant differences in *Salmonella* isolation rates between culturing the sample on the same day of collection or after storage at 4ºC for six days (73). Results obtained from the recent EU baseline survey for laying flocks suggested an increase in the sensitivity of detection when the samples were analysed three days after refrigeration (42). In some studies using naturally pooled and individual pig faeces, large sample weights increased the likelihood of detection (17, 44), although recent work at the Veterinary Laboratories Agency (VLA) in the United Kingdom has shown better results culturing 10 g, compared with 25 g, of naturally pooled pig faeces samples, probably because lower sample weights reduce the chance of including certain inhibitory factors which may be present in some individual faeces (3). The authors do not currently have reliable data regarding the optimal sample weight necessary to culture poultry faeces, but this is likely to depend largely on the strain and numbers of *Salmonella* organisms present, as well as the nature of the competing flora and the presence of other inhibitory factors. The latter

include bacteriocines, phages, copro-antibodies, and miscellaneous inhibitory substances. However, inhibitory substances may also develop during culture (e.g. due to the multiplication of antimicrobial-producing moulds or alcohol-producing yeasts).

Some researchers have found that homogenising rather than just mixing the pooled faeces sample before sub-sampling, results in a greater sensitivity, since the bacteria are usually found in clusters in faeces (12), but the authors have not observed this effect in their laboratory (unpublished data). In order to reduce the effect of particulates and other competitive factors that may interfere with isolation of *Salmonella*, it may be beneficial to culture an additional 1:10 dilution of the initial sample, or to contain the particulates within a membrane (32). In the EU layer survey faeces samples from caged houses were homogenised by mixing 200 g of each sample with an equal volume of BPW before adding 50 g of this mixture to 200 ml of BPW to make up the final pre-enrichment mixture.

Pre-enrichment

Buffered peptone water is the usual pre-enrichment medium for recovering *Salmonella* from pigs and poultry environmental samples across the EU. Analysis of results of international ring trials coordinated by the *Salmonella* Community Reference Laboratory in Bilthoven in the Netherlands, established the optimal incubation time of pre-enrichment for poultry environmental samples as 16 h to 20 h, with shorter times resulting in reduced sensitivity of detection. The addition of ferrioxamines (59) to pre-enrichment media is contra-indicated in nutrient-rich samples such as poultry faeces or freshly contaminated environmental samples (28), but may be beneficial for iron-limited samples such as whole egg, egg albumen (83) or water (82). Addition of novobiocin to BPW may increase the level of *Salmonella* cultured in subsequent steps. However, it may also suppress growth of some serovars (56). The presence of bacteriophages in the sample may also alter the results of pre-enrichment, leading to false negative results (70). In practice, it is necessary to optimise the pre-enrichment step so as not to inhibit the recovery of damaged or slow-growing *Salmonella* whilst avoiding overgrowth by competitor organisms. This balance is difficult to achieve and for maximum detection it may be necessary to combine non-selective and direct selective enrichment with direct plating, although this will not normally be feasible for routine use. Recovery of antimicrobial resistant *Salmonella* can be dramatically enhanced by adding relevant antibiotics to pre-enrichment and selective enrichment media.

Selective enrichment

Success in isolating *Salmonella* is usually enhanced by the inoculation of incubated pre-enrichment broth into selective enrichment media. The introduction of semi-solid selective enrichment media such as the modified semi-solid Rappaport-Vassiliadis (MSRV) medium represented an improvement over previously used conventional selective enrichment broths, such as Rappaport-Vassiliadis (soya base) (RVS). There may be, however, differences in the performance of different brands of MSRV. Semi-solid selective enrichment media are easy to use, and negative results can often be obtained by direct visualisation of the plates, leading to a more efficient isolation process (26, 80). This advantage was demonstrated with faeces samples (22, 30, 93). Studies on poultry faeces showed that MSRV was at least as sensitive as the simultaneous use of both RVS and Müller-Kauffman tetrathionate novobiocin (MKTTn) as selective enrichment media. The latter two selective enrichment media are used in the ISO 6579:2002 method (54).

A recent Belgian study investigating a range of poultry and hatchery environmental samples reported greater sensitivity with Diasalm (93.9%) compared with MSRV (79.2%) (51). The authors also found similar results in their laboratory (24), but recent changes in the production of Diasalm media have resulted in a less marked difference, and a greater tendency for overgrowth of non-*Salmonella* organisms in Diasalm.

Samples with a high level of competitive flora such as intestinal and other environmental samples may need to be incubated at higher temperatures (41.5°C with MSRV), in order to provide *Salmonella* with an advantage over most competitive organisms. However, incubation at 35°C to 37°C may be more appropriate for detection of sensitive serovars such as *S.* Pullorum and *S.* Gallinarum in internal organs and tissues. Incubation above 43°C may be lethal for some *Salmonella* serovars (8) such as *S.* Dublin. Selective enrichment media are normally stored cold, so they should be allowed to warm up to at least room temperature before use to avoid reducing the temperature within the incubator, a potential problem where large numbers of plates are incubated together (97).

Growth on MSRV selective enrichment media is plated after 24 h and 48 h onto solid plating media. Although a small number of additional isolates may occasionally be detected beyond 48 h, further plating is not considered cost-effective (23). Non-motile *Salmonella*e, including *S.* Pullorum and *S.* Gallinarum, and the *S.* Enteritidis vaccine strain present in Avipro *Salmonella* Vac E (Lohmann Animal Health) do not grow in MSRV, so they must be identified using different media. For *S.* Pullorum and *S.* Gallinarum the best results were obtained using direct enrichment in selenite cysteine and RVS (77)

followed by direct plating on McConkey agar (in the case of tissues), since these strains are more sensitive and do not endure the pre-enrichment stage. Selenite-based direct enrichment broths are widely used for faeces from human clinical cases in which higher numbers of *Salmonella* organisms are normally present, but are usually inadequate for faeces samples from asymptomatic birds (97). Direct enrichment in selenite cysteine is also the method of choice for the detection of host-adapted serovars (e.g. *S.* Gallinarum, *S.* Pullorum) and some attenuated live vaccines (i.e. Nobilis SG 9R) in faeces. However, these serovars are sensitive and detection in faeces is always difficult, and therefore culture from post-mortem tissues is preferred.

Delayed secondary enrichment involves holding the original selective-enrichment media at room temperature for 5 to 7 days after the initial 24 h incubation and subsequent inoculation onto fresh selective enrichment broth (97). This technique gave increased isolation rates of *Salmonella* from poultry environmental and diagnostic samples compared to incubation of selective enrichment media for 24 h and 48 h (98), and is currently used for environmental monitoring in the California Egg Quality Assurance Program (18). The downside of this method is the large amount of media required. Moreover, it is not clear that this method represents any improvement over culture using BPW-MSRV when growth from the latter is plated out after 24 h and 48 h.

Plating media

Successful detection of *Salmonella* at further plating stages depends on the ability to grow *Salmonella* to levels above 10^4 cells/ml in the enrichment medium. This is because only 1µl of enrichment is typically streaked onto plating media. Most conventional plating media (i.e. brilliant green agar [BGA]) are relatively non-specific, yielding a large number of false positives (e.g. *Citrobacter, Proteus*). This creates an unnecessary workload for examination of presumptive colonies, as well as an increase in the cost associated with the test. Sucrose-fermenting strains of *S.* Mbandaka, which have been reported in Northern Ireland, are not recognised by such media (81). Lactose-fermenting *Salmonella*, such as some rare strains associated with veal production, or some *S. arizonae* and *S. diarizonae* strains may also be missed. False negatives may also occur if *Salmonella* is masked by growth of other bacteria.

Over recent years new plating media which are based on the use of a combination of chromogenic substrates and conventional biochemical reactions have been developed. These allow *Salmonella* to produce distinctive colonies, making the identification easier and faster. Rambach (79) is an example of such media, in which typical *Salmonella* colonies appear as crimson red, and is the medium routinely used in our laboratory. However, in this medium some strains of *S.* Enteritidis (including Merial's Gallivac SE vaccine strain) appear pale orange or colourless. A further disadvantage of Rambach is that *S.* Typhi or *S.* Paratyphi do not appear as typical colonies in this medium, and serovars such as *S.* Moscow and *S.* Wassenar (47, 69) as well as lactose-fermenting strains of *S.* Virchow, *S.* Montevideo, *S. enterica* subsp. *arizonae* and *S. enterica* subsp. *diarizonae* (86), may be missed.

Other recently developed chromogenic media include *Salmonella* chromogenic medium (15), alpha-beta chromogenic medium (74, 76), and BBL™ CHROMagar™ *Salmonella* medium (36). Further work is needed to test the performance of these media on naturally contaminated poultry samples. The use of two selective media with different selective and differentiating properties is likely to help detect atypical strains, but their use may not be justified for surveillance purposes if the aim is to maximise detection of major zoonotic *Salmonella* strains.

Internationally-approved isolation methods

The two EU approved methods for *Salmonella* detection in food and animal feedstuffs are ISO 6579:2002 (54) and NMKL 71 (Nordic Committee on Food Analysis) (72). ISO 6579:2002 is sensitive, but complex and expensive. It consists of pre-enrichment of the sample in BPW followed by selective enrichment in MKTTn and RVS. From each enrichment medium, plating onto two agar media plates (one of which is Xylose-Lysine Desoxycholate [XLD] agar) is carried out after 24 h and 48 h of incubation. Up to five colonies per plate have to be confirmed, which may potentially involve the confirmation of up to 40 presumptive colonies (54). Further research on veterinary faecal samples showed the superiority of MSRV to RVS and MKTTn, and this modification was incorporated into ISO 6579:2002 as Annex D (53). This method has been adopted in the EU *Salmonella* baseline surveys on poultry flocks and pig herds. Using spiked cattle, pigs and poultry samples, the ISO 6579:2002 (Annex D) method has been shown to perform better than commercial polymerase chain reaction (PCR), the BAX method, and three commercial systems based on enzyme-linked immunosorbent assay (37). A simplified version of ISO 6579:2002 (Annex D) using one culture plate (Rambach) is currently used at the VLA for research purposes (13, 95). The authors have adopted this method based on a comparison of the performance of Rambach versus BGA and XLD on samples from the EU *Salmonella* baseline surveys for layers and broilers. A single Rambach plate performed as well as the combination of XLD and BGA, as long as pale orange colonies on Rambach were investigated (unpublished data). Confirmation of *Salmonella* with this method can be obtained within 72 h, although sometimes it has been possible to confirm and isolate within 24 h if

incubation times are abbreviated and tests are carried out directly from selective media (30). The NMKL 71 method (72) was originally developed for the isolation of *Salmonella* in foods and feedstuffs. It is relatively simple and affordable, although it has shown limited sensitivity with veterinary faecal samples compared to other methods (61). It includes a pre-enrichment stage in BPW followed by enrichment in RVS and plating on to XLD plus a second medium of choice. Plating after 48 h of enrichment is optional, and may increase its sensitivity considerably, although in practice this is rarely done.

Conclusions and future perspectives

A wide range of methodologies for the sampling and bacteriological detection of *Salmonella* in poultry flocks is available. The EU *Salmonella* baseline surveys in poultry and subsequent regulations have provided a starting point for the harmonisation of results across EU countries, but more work is needed to validate the efficiency of *Salmonella* testing in individual laboratories in the EU and beyond. This would ideally include the introduction of adequate quality assurance schemes using realistic control samples. Existing bacteriological methods will be challenged by new molecular-based technologies that may deliver results quicker or more efficiently, but in the case of faecal and environmental samples of poultry these are still in the early stages of development, and there is considerable work to be done before they can be consistently applied. The adoption of new methodologies for routine use must be based on a compromise between improved sensitivity and speed, ease of use and economic considerations, but it is important to remember that the process has to start with a representative, high quality sample. Testing a larger number of samples using a single but effective method is likely to be more effective than testing a small number of samples using complex laboratory protocols. The purpose of the test is also important. Tests which are excessively cumbersome and laborious may not be appropriate for high throughput monitoring laboratories in zoonosis control programmes. It is necessary to improve and optimise methods of sample preparation, which in the future may involve the separation of the target organisms from inhibitory substances in the sample matrix. This will also be an important consideration for the next generation of PCR-based detection tests which will aim to optimise DNA recovery whilst minimising the impact of inhibitory substances and non-specific elements which may give rise to false positives.

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■

Étude sur l'échantillonnage et la détection bactériologique de *Salmonella* chez les volailles et dans les sites d'élevage

J.J. Carrique-Mas & R.H. Davies

Résumé

La détection de *Salmonella* dans le cadre de la production primaire de volailles revêt une grande importance dans l'Union européenne (UE), dans la mesure où la lutte contre cette zoonose repose en grande partie sur l'objectif de réduction de la prévalence au niveau des sites d'élevage. Dans ce contexte, la réussite de la détection bactériologique dépend du choix d'une procédure d'échantillonnage appropriée et d'une méthode de culture suffisamment sensible. Dans les élevages de volailles, des échantillons composites naturels constitués de

matière fécale, de litière et de poussière forment généralement une matrice d'échantillon de choix. Dans les systèmes d'élevage au sol, l'utilisation de pédisacs pour la collecte de matière fécale est la méthode préconisée. Il existe un large éventail de méthodes de culture, mais c'est la norme ISO 6579:2002 (Annexe D) qui est actuellement suivie dans l'UE pour la prise d'échantillons dans l'environnement des volailles. Les auteurs examinent en détail les différentes méthodologies d'échantillonnage et de culture des *Salmonella* pratiquées dans les élevages de volailles. Sont également abordées les méthodes d'échantillonnage et de tests pour les couvoirs, les oiseaux vivants et les carcasses de volailles.

Mots-clés
Échantillonnage – Enrichissement – Isolement – Milieu de culture – Poulet – Pré-enrichissement – Salmonella – Union européenne – Volaille.

Repaso de los métodos de obtención de muestras y detección bacteriológica de *Salmonella* en aves de corral e instalaciones de producción avícola

J.J. Carrique-Mas & R.H. Davies

Resumen
La detección de *Salmonella* en las instalaciones de producción avícola primaria suscita gran interés en la Unión Europea (UE), pues la lucha contra esta enfermedad zoonótica reposa en parte en la reducción de la prevalencia en las propias granjas. El éxito de la detección depende seguramente en gran medida de la elección de un procedimiento adecuado de obtención de muestras, junto con un método de cultivo sensible. En las granjas de producción avícola, las matrices idóneas son los 'acúmulos naturales' de heces, yacija y polvo. En los sistemas de cría en el suelo, el mejor método para obtener muestras de heces consiste en realizar frotis de la suela de las botas. Existen muy diversos métodos de cultivo, aunque ahora mismo la Norma ISO 6579:2002 (Anexo D) constituye la referencia en la UE por lo que respecta a muestras ambientales de aves de corral. Los autores repasan en detalle los distintos métodos de obtención de muestras y de cultivo de *Salmonella* en granjas avícolas. También se detienen en la extracción de muestras y la realización de análisis de incubadoras avícolas y pájaros vivos y muertos.

Palabras clave
Aislamiento – Aves de corral – Enriquecimiento – Enriquecimiento previo – Medios – Obtención de muestras – Pollo – Salmonella – Unión Europea.

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