*Rev. sci. tech. Off. int. Epiz.*, 2008, **27** (3), 657-664

# Bacteriological detection of *Salmonella* Enteritidis in eggs: a review

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Submitted for publication: 17 April 2007
Accepted for publication: 22 July 2008

**Summary**
The detection of *Salmonella enterica* serovar Enteritidis (SE) in eggs is hampered by a typically low prevalence of contaminated eggs, the low number of SE organisms in such eggs, and the presence of inhibitory substances in the egg albumin. For these reasons, the analysis of large pools of eggs is normally necessary, which presents logistic and microbiological challenges associated with a low number of target organisms from a large volume of sample matrix. In some studies using artificially inoculated eggs the standard procedure for *Salmonella* culture consisting of pre-enrichment, followed by selective enrichment and plating has been replaced by incubation of the egg pools at 25°C to 37°C followed by direct plating. However, in most cases using pools of naturally contaminated eggs, it may be necessary to enhance the traditional three-step method by addition of antibiotics or iron supplements.

**Keywords**
Albumin – Culture – Eggs – Isolation – Pool – Pre-enrichment – Salmonella Enteritidis – Testing.

## Introduction

Contaminated eggs produced by infected laying hens are thought to be one of the main sources of human infection with *Salmonella enterica* serovar Enteritidis (SE) throughout the world (6, 22, 23, 40). This serovar has a predilection for colonisation of the hen's reproductive tract, which may lead to internal contamination of the eggs prior to being laid (24, 30, 38). Also, when a high level of environmental contamination is present in the hen houses, there is a possibility that SE organisms deposited on the shell due to external soiling, may subsequently penetrate it. This risk is known to increase when eggs are washed (25).

Most modern SE monitoring programmes in laying flocks are primarily concerned with determining whether infection is present in the flock or not. Studies measuring the prevalence of contaminated eggs are normally carried out by public health authorities. Eggs are usually sampled and tested as part of national or small-scale retail surveys, to assess the overall risk to the consumer or in trace-back investigations in human outbreaks. The following is a review of published information regarding bacteriological (i.e. culture) methods that test for SE in whole eggs or its contents. It is aimed at veterinary and public health laboratories and professionals with an interest in the monitoring and control of SE in poultry and poultry products.

## Main challenges

The main challenge concerning the detection of SE in eggs is the typically very low rate of egg contamination, even among eggs originating from SE-infected flocks (29, 30). In addition, only very few SE organisms are deposited within contaminated eggs (30). In many countries flocks of commercial laying hens are vaccinated against SE, which further reduces the rate of contamination in eggs (8). These factors make the detection of SE in raw eggs a challenging

task. In many countries, such as the United Kingdom (UK), SE-infected flocks represent a small proportion of all flocks, which translates into a very low overall prevalence of contaminated eggs at the retail level. For example, a recent survey of retail eggs in the UK indicated a prevalence of infection of 0.34% (none in egg contents) in boxes of six eggs on shells only, which represents an even lower prevalence of infection of individual eggs (11). In such situations of very low prevalence, if a sufficient precision of the prevalence estimate is desired, the required sample size approaches the size of the population, which is clearly uneconomical (37). In most situations, in order to avoid overwhelming laboratory resources, the contents of between 5 and 40 eggs are pooled and cultured together. However, there might be some reduction in the test sensitivity due to a dilution effect derived from pooling eggs (31), although the precise magnitude of this effect is unknown. The culture of large pools of eggs, especially if pre-enrichment is used, constitutes an additional limitation for some laboratories where there may not be sufficient incubator space. Because direct plating would not be able to detect fewer than $10^5$ CFU/ml of *Salmonella* (17), additional steps or enhancements to the culture procedures are necessary. Pools of egg contents are either incubated or pre-enriched (or both), with or without supplementation of additives that promote the growth of *Salmonella*. More recently, molecular techniques have been developed in order to speed up testing times, although most of them still use a pre-enrichment or an incubation step, and fall outside the scope of this review.

## Egg surface washing and disinfection

In some studies eggs are cracked and the contents (albumen and yolk together) are separated from the shell and associated membranes for culture. However, other studies aim to exclude any potential *Salmonella* contamination on the external surface of the shells. In that case, the shells must be cleaned and disinfected prior to cracking. Cleaning of egg shells may be achieved by brushing and using a multipurpose liquid detergent (43). Surface disinfection may be achieved by soaking in a chlorine solution (2), tincture of iodine (13), 70% ethanol (39, 43) or by dipping in boiling water (27).

## Homogenisation of egg contents

The most frequent site of contamination of eggs is the outside of the vitelline membrane, followed by the albumen, and lastly the yolk (14, 19, 32). However, the yolk is high in iron content, making it an excellent medium for bacterial growth compared with the albumen, which is iron-deficient (34, 43). Homogenisation of egg contents prior to culture in the laboratory therefore increases the chance of detection, since it makes the yolk contents available to the bacteria. Homogenisation can be achieved using stomaching, blending or (manual) stirring. Careful manual homogenisation of artificially inoculated eggs is preferable to electrical blending or stomaching for detection (42). This is thought to be because electric blending allows antimicrobial proteins in the albumen to better mix with the yolk, or because the physical stress caused by the electrical blender leads to death or injury of any SE organisms present.

## Culture methods

Because of the typically low prevalence of contaminated eggs, the low numbers of SE organisms in such eggs, and the bacteriostatic effect of the albumen, multiplication of these relatively few organisms is necessary to reach detectable levels in culture. This can be achieved using traditional three-step *Salmonella* culture methods. These methods proceed as follows:

– incubation of the sample in non-selective pre-enrichment media to resuscitate sub-lethally damaged SE organisms

– incubation in selective enrichment media that contain inhibitory substances to suppress competing organisms

– plating onto selective agar media to allow the differentiation of SE organisms from other Enterobacteria.

Three-step culture methods are thought to be considerably more sensitive than other methods involving fewer steps (45), and have been used in several studies (1, 9, 33, 35), although the pre-enrichment step was not always carried out (10). The main disadvantage of three-step culture methods is that they take more time, and use more media and incubation space. Therefore abbreviated methods and/or modifications have been attempted.

Some studies on artificially inoculated eggs have reported that selective enrichment does not add any advantage to pre-enrichment followed by plating (31) or direct plating only (13). This is dependent on careful inoculation techniques that avoid contamination of the egg contents with other bacteria, and the absence of competing bacteria in the egg content. Also, most artificial inoculation studies have typically involved a higher than normal number of SE organisms in the initial inoculum, which may not reflect a realistic level of 'natural' contamination of eggs in most cases. The addition of novobiocin or cefsulodin to pre-enrichment media (e.g. buffered peptone water [BPW]) has been used to inhibit other bacteria that may interfere with the isolation of SE in a small proportion of eggs (31), resulting in a greater sensitivity of detection. Longer periods of pre-enrichment (48 h rather than 24 h) have been shown to further increase the sensitivity of detection in individual eggs (15) or in pooled egg content (31), as

long as only a few competitive organisms are present. The addition of BPW to egg contents also prevents the egg contents from becoming semisolid during incubation. The volume of pre-enrichment media may be an issue when culturing large pools of eggs, and the use of smaller amounts may be justified. Instead of the standard 1:10 dilution, the authors have found that 1:1 ratio of pooled egg material to BPW is adequate for pools of up to 40 eggs when pre-enrichment is followed by selective enrichment in modified semi-solid Rappaport Vassiliadis (MSRV) media, and plating on to Rambach (unpublished data). A wide variety of methods have been used in studies aiming at investigating SE in naturally-contaminated eggs. These are summarised in Table I. In those studies where different techniques and conditions are compared, only those giving a further advantage are reported. In practice it is very difficult to rank the methodologies across studies, given their complexity. In most cases only partial steps aimed at improving already established culture methods in individual laboratories are evaluated.

## Incubation of pools of eggs

The incubation of individual egg contents or pools of eggs for four days at room temperature prior to plating has been shown to improve the sensitivity of the culture (16). This requires a very careful technique for sterile crushing of eggs to avoid the introduction of any other bacteria, since their multiplication may mask the target organisms (28). Increased detection sensitivity can be achieved by incubating the egg pools at 37ºC, rather than 25ºC, although the number of SE bacteria present would not typically be detectable by direct plating within the first 12 h of incubation (20). An increase of temperature beyond 37ºC may not achieve any further increase in sensitivity (21), but will result in some solidification of the egg pool.

## Use of additives

The albumin of the egg has a strong bacteriostatic effect due to the presence of ovotransferrin, which limits the amount of iron available to the bacteria (3). Iron supplementation can therefore help overcome this effect, enhancing the growth of *Salmonella* and therefore increasing the sensitivity of detection (5, 36). This is especially necessary in situations where abbreviated methods (i.e. incubation followed by direct plating) are used, rather than the three-step culture method using pre-enrichment, followed by selective enrichment (17) and plating, since detection of *Salmonella* in egg pools by direct plating requires the presence of at least $10^5$ CFU/ml (20).

Iron salts such as ferrous sulphate have also been shown to promote the isolation of SE from egg contents (5, 7), and may replace the pre-enrichment step of the culture when added to the egg pool prior to incubation (4). However, iron salts may also stimulate the growth of competitive bacteria. Ferrioxamines, on the other hand, act by supplying SE with useable iron, rather than saturating ovotransferrin, and therefore do not promote the growth of *E. coli* and the *Proteus-Providencia-Morganella* group,

**Table I**
**Published pre-enrichment/culture methods of detecting *Salmonella* Enteritidis**
(reported in different peer-reviewed studies and surveys of naturally contaminated eggs)

| Study | Matrix | No. of eggs pooled | Pre-enrichment | Enrichment | Agar plates | Country | Reference |
|---|---|---|---|---|---|---|---|
| 1 | Homogenised egg contents | 5 | BPW (1:9) | MSRV TBG | BGS BSA | Canada | (39) |
| 2 | Homogenised egg contents | Single egg, 4 | BPW or BPW+NC (1:3) | RV | XLD | UK | (31) |
| 4 | Homogenised egg contents from an egg-breaking plant | 10 ml liquid egg | No | TT | BGN | USA | (10) |
| 5 | Homogenised egg contents | 6 | 180 ml BPW | No | XLD+BSA, BGA, BGMA | UK | (1) |
| 6 | Homogenised egg contents | 6 | 250 ml BPW | RVS | XLD, BGA | UK | (46) |
| 7 | Egg yolk | Single egg | 225 ml TSB | TT | XLD | Korea | (2) |
| 8 | Liquid eggs and shell eggs | Single egg, 25 ml liquid egg | 225 ml BPW | TT | XLT4, XLD, BGM, SMID, ES-II, CHROM | Japan | (26) |

BGA: Brilliant green agar
BGMA: Brilliant green MacConkey agar
BGM: Brilliant green agar modified
BGN: Brilliant green plus novobiocin
BGS: Brilliant green sulpha agar
BPW: Buffered peptone water
BPW+NC: BPW plus cefsulodin and novobiocin
BSA: Bismuth sulphite agar
CHROM: CHROMagar
ES II: ES *Salmonella* II agar
MSRV: Modified semisolid Rappaport-Vassiliadis
RV: Rappaport-Vassiliadis broth
RVS: Rappaport-Vassiliadis soya peptone broth
SMID: *Salmonella* detection and identification medium
TBG: Tethrathionate brilliant green
TSB: Tryptone soy broth
TT: Tetrathyonate
XLD: Xylose lysine desoxycholate agar
XLT4: Xylose lactose thiosulphate tetradecylsulfate agar
UK: United Kingdom
USA: United Status of America

**Table II**
**Summary of additives and the concentrations used in conventional microbiological culture of eggs and egg contents**

| Type and amount of additive | Additive added to | Matrix of investigation | Technique steps | Reference |
|---|---|---|---|---|
| Ferrioxamine G (1 µl/ml) | BPW | Egg albumen pool | Selective enrichment, plating | (41) |
| Ferrous sulphate (35 mg/l) | TSB (pre-enrichment) | Homogenised egg contents | Incubation of egg contents, pre-enrichment, selective enrichment, plating | (12) (45) |
| Concentrated (×10) TSB or (×10) RV | Homogenised egg contents | Homogenised egg contents | Incubation of egg contents and direct plating | (18) |
| Ferrous sulphate (0.5 mg/g egg content) | 50 g pooled egg contents | Pooled egg contents | Incubation of egg contents and direct plating | (4) |
| Cysteine (0.2 mg/l) | BPW | Eggs contents and liquid egg | Pre-enrichment, selective enrichment, plating | (26) |
| Ferrous sulphate solution (60 mg/ml) or concentrated (×5) TSB | Homogenised egg contents | Homogenised egg contents | Incubation of egg contents and direct plating | (20) |
| Ferrioxamine E (500 µl/ml) | Egg albumin pool | Egg albumin | Direct plating | (44) |

BPW: Buffered peptone water
RV: Rappapport-Vassiliadis broth
TSB: Tryptone soy broth

provided that they are not supplied at high concentration (<50 µg/ml) (44). The addition of ferrioxamine G to pre-enrichment media increases the growth rate of bacteria such that they reach detectable numbers within 6 h, thus potentially accelerating the detection of SE from artificially contaminated eggs (41).

In our laboratory, the addition of ferrioxamines to BPW does not result in any significant improvement in the recovery of *Salmonella* from pools of 40 naturally contaminated eggs when a thee-step method using BPW/MSRV/Rambach is used (unpublished data). Other studies have demonstrated increased sensitivity of the standard culture by pre-enriching the egg content pool in BPW with cysteine (26) or with a concentrate of Rappaport-Vassiliadis or tryptone soy broths (18). Supplementing pools of eggs with ferrous sulphate or tryptone soya broth and incubating the sample has been shown to potentially yield concentrations of $10^5$ SE cells within 12 h (20). The types of additives added and concentrations reported in the literature are shown in Table II. In those studies comparing techniques and conditions, only those reporting an advantage compared with no supplementation are presented.

# Conclusions

A wide range of methodologies for the bacteriological detection of SE in eggs is available. In most cases, because of the low prevalence of contamination in single eggs and economic considerations, the analysis of large pools of eggs is necessary. In some studies that use artificially inoculated eggs, the standard procedure for *Salmonella* culture (pre-enrichment, followed by selective enrichment and plating) has been replaced by incubation of the egg pool followed by direct plating, or suppression of the pre-enrichment/selective enrichment step. However, the examination of pools of naturally infected eggs is likely to require an increase in the sensitivity of detection, so refinements of the existing methods in order to enhance detection are desirable. These include:

– incubation of egg contents at 25°C to 37°C prior to culture

– addition of antibiotics to pre-enrichment media

– longer pre-enrichment incubation time

– addition of additives to egg pools or pre-enrichment media.

There is limited information as to how these methodologies compare. In the future, traditional culture-based methods will be challenged by new molecular-based technologies, particularly those capable of capturing low numbers of target organisms from a large volume of sample matrix. The adoption of these methodologies for routine use must be based on a compromise between improved sensitivity and speed, ease of use and economic considerations.

# Acknowledgements

We are grateful to Ian McLaren and Sarah Evans (Veterinary Laboratories Agency, Addlestone, Surrey, United Kingdom) for comments on the manuscript. This work has been funded by the Department for Environment, Food and Rural Affairs of the United Kingdom through Projects OZ0322 and OZ0325.

# Étude sur les détections bactériologiques de *Salmonella* Enteritidis dans les œufs

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**Résumé**
La détection effective de *Salmonella enterica* sérovar Enteritidis dans les œufs est rendue difficile par la prévalence généralement faible des œufs contaminés, par le nombre limité de *S.* Enteritidis présentes dans les œufs contaminés ainsi que par la présence de substances inhibitrices dans l'albumine. Pour toutes ces raisons, il est souvent nécessaire de procéder à l'analyse de vastes échantillons composites d'œufs, ce qui représente un défi logistique et microbiologique considérable au vu du petit nombre de micro-organismes à détecter à partir d'une telle matrice d'échantillons. Dans certaines études réalisées à partir d'œufs inoculés artificiellement, la procédure standard de culture de *Salmonella*, à savoir le pré-enrichissement suivi de l'enrichissement en milieu sélectif et l'isolement, a été remplacée par l'incubation d'échantillons composites d'œufs à une température comprise entre 25 °C et 37 °C suivie de l'isolement direct. Toutefois, dans la plupart des cas où des échantillons composites d'œufs naturellement contaminés sont utilisés, il peut s'avérer utile de renforcer la méthode traditionnelle en trois étapes par un ajout d'antibiotiques et de sels de fer.

**Mots-clés**
Albumine – Culture – Échantillon composite – Épreuve de détection – Isolement – Œuf – Pré-enrichissement – Salmonella Enteritidis.

# Repaso de los métodos bacteriológicos para la detección de *Salmonella* Enteritidis en los huevos

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**Resumen**
A la hora de detectar eficazmente *Salmonella enterica* serovar Enteritidis (SE) en los huevos existen varias dificultades: el hecho de que los valores de prevalencia de huevos contaminados sean en general bajos; el escaso número de microorganismos SE en ellos; y la presencia de sustancias inhibidoras en la albúmina del huevo. Por estas razones suele ser necesario analizar mezclas de un gran número de huevos, cosa que plantea problemas de orden logístico y microbiológico, asociados con el escaso número de microorganismos presentes en la matriz de una muestra de gran volumen. En algunos estudios realizados con huevos artificialmente inoculados se ha sustituido el método habitual de cultivo de *Salmonella*, consistente en un enriquecimiento previo seguido de enriquecimiento y siembra selectivos, por la incubación de las mezclas de huevos a una temperatura de 25°C a 37°C, seguida de una siembra

directa. Sin embargo, en la mayoría de los casos en que se utilicen mezclas de huevos contaminados de forma natural puede ser necesario mejorar el método tradicional en tres etapas mediante la adición de antibióticos o suplementos férricos.

**Palabras clave**
Aislamiento – Albúmina – Análisis – Cultivo – Enriquecimiento previo – Huevos – Mezcla – Salmonella Enteritidis.

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