Salmonella in Shell Eggs: Mechanisms, Prevention and Detection

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Abstract

Contaminated shell egg is one of the key Salmonella infection routes for human, causing food-borne illnesses. Multiple salmonellosis infections due to egg contamination still occur in developed countries even though preventive methods, such as vaccination or washing followed by rinsing process, are carried out following certified national standards. Recent outbreaks indicated that current strategies for Salmonella control need to be optimized to further minimize contamination of commercial eggs. Therefore, there is a critical need to develop more sensitive and rapid Salmonella detection methods. In this review, we address (i) egg production; (ii) preventive methods that minimize contamination; (iii) mechanisms of Salmonella contamination; (iv) Salmonella detection methods.

Keywords: Shell egg; Salmonella; Vaccine; Rapid detection; Food safety

Introduction

Salmonella, a gram-negative, facultative anaerobic, non-spore forming, rod-shaped bacteria are one of the most dangerous food-borne pathogens [1,2]. The Centers for Disease Control and Prevention (CDC) estimate about one million of events of Salmonella cases annually in the United States [3]. Among the various foods that cause salmonellosis, eggs are regarded as one of the potential sources of Salmonella reservoirs in the human food chain that cause illness when consumed by people [4-8].

Recent outbreaks include a Salmonella enteritidis contamination that led to the recall of more than 500 million eggs from Iowa between May and November in 2010. There were 1,939 infections linked to that outbreak [9]. Outside of the US, egg contamination has also been responsible for 247 cases and 3 deaths in the UK, 130 cases in other European countries in 2014 [10,11], and 353 and 1895 cases in Australia in 2014 and 2015 (Table 1) [12,13].

Eggs have also been used to produce vaccines for more than 70 years [14]. Even though there are alternative methods to produce vaccines (i.e., DNA-based, cell culture based, recombinant/purified protein based methods), egg-based vaccine production is still regarded as the preferred method with respect to productivity and scale by global health organizations and industry [15].

<table>
<thead>
<tr>
<th>Country</th>
<th>Date</th>
<th>Reported Case</th>
<th>Organization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Austria</td>
<td>Apr-14</td>
<td>353</td>
<td>Government of South Australia</td>
</tr>
<tr>
<td>Australia</td>
<td>Mar-15</td>
<td>1895</td>
<td>AIFS</td>
</tr>
<tr>
<td>Austria</td>
<td>Jun-14</td>
<td>61</td>
<td>EFSA</td>
</tr>
<tr>
<td>France</td>
<td>Aug-14</td>
<td>45</td>
<td>EFSA</td>
</tr>
</tbody>
</table>

Table 1: International incidence of Salmonella associated with eggs. AIFS denotes Australian Institute of Food Safety. EFSA denotes European Food Safety Authority.

The alternative methods still have technical obstacles to overcome high production costs, oncogenicity and tumorigenicity risks [16]. The World Health Organization (WHO) has been transferred technologies to build and develop egg-based vaccine production capacities in several countries, including Brazil, India, Mexico, Thailand, Islamic Republic of Iran and Romania [17]. Major safety concerns in the vaccine manufacturing industry are microbial contamination by Salmonella and Campylobacter [16,18]. The development of fast detection methods for Salmonella in eggs is important for quality assurance and risk control not only in the food, but also in the pharmaceutical industry.

Egg production

According to CIWF (Compassion in World Farming) [19], there are more than 6.6 billion laying hens, which produce more than 65 million metric tons eggs in 2011 worldwide. As the largest shell egg producing country, more than 2.5 billion laying hens are bred in China producing 23.9 million metric tons of eggs. In the US, about 338 million laying hens are bred, compared to 363 million in the European Union (EU), with an estimated 5.4 million metric tons of eggs in the US and 7.1 million metric tons of eggs in the EU [19]. As the primary egg exporting country, the Netherlands contributed to more than 30% (0.6 million metric tons) of the global shell egg exports in 2010. Germany is the primary egg importing country and imported 27% (0.5 million metric tons) of the global share for shell eggs in 2010 [19,20]. According to AGMRC (Agricultural Marketing Resource Center)
Preventive methods for minimizing contamination

Vaccination of laying hens is considered an effective method in reducing human salmonellosis by shell eggs in European countries [18]. A variety of vaccines (e.g., live attenuated and inactivated whole cell vaccines) are officially approved to reduce *Salmonella* contamination of eggs [29]. According to Organization of the United Nations (FAO), the vaccination for *Salmonella* reduced egg contamination by 75% by reducing the *Salmonella* invasion and colonization inside of the reproductive organs [30-33]. However, the vaccination of laying hens does not completely guarantee the control of internal egg contamination by *Salmonella* in entire chicken flocks. Several reports showed the presence of *Salmonella* inside the reproductive organs from vaccinated laying hens that could enter the internal egg [34,35].

Regulatory agencies for food safety follow different methods in different countries. In the US, vaccination is not required, but eggs must be washed and refrigerated. The entire Grade A shell eggs are carefully washed and microbial load should be minimized on the surface of shell before they go to market. Recently, the U.S. Food and Drug Administration (FDA) [36] stated that more research data are necessary to support regulation of egg safety for mandating vaccination to laying hens for *Salmonella* prevention. The US Department of Agriculture (USDA) grade mark on egg cartons means the plant processed the eggs following USDA's sanitation and good manufacturing processes. Eggs were washed followed by sanitizing rinse stages over the producing lines. Egg producers in Australia, Canada, and Japan have also tried to prevent the invasion of *Salmonella* from outside of the shell instead of vaccine treatment of laying hens [37,38]. In these counties, processing for the Grade A (Class A) shell eggs including washing and rinsing is common and/or is regarded as a safety procedure [39]. Figure 1 shows the flow of the primary processing for shell eggs [40].

Surface washing is not acceptable in the European Union except some Scandinavian countries [41]. The reason is the egg cuticle, which serves as an obstacle against invasion of microorganisms, can deteriorate during the washing and/or rinsing process [42,43]. The major goal of the regulations is to not only promote better feeding environments, with higher hygiene standards and quality eggs, but also to reduce the processing time between egg laying and its packaging for consumers [44,45].

Despite being the world's largest volume egg producer, China's egg processing and safety regulations are behind the times [46]. Since the mid-1990's, artificially produced eggs (fake eggs), made from resin, starch, coagulants, pigments, and sodium alginate, have appeared in Chinese food markets.

![Figure 1: Work flow for primary processing of shell eggs. Adapted and modified from Food Standards Australia New Zealand (40).](image)

This has been a serious food safety issue [47,48], although quite different from the topic addressed in this paper. Less than 10% of the shell eggs are washed and packed, and less than 0.3% of the shell eggs undergo processing for the purpose of use as food supply supplements and ingredients (i.e., dry food products, liquid products) [49]. On the other hand, China's duck egg industry is well developed and about 45% of duck eggs are processed using their traditional methods (i.e., salty eggs, century eggs) [49].

**Contamination mechanism**

Mechanisms of egg contamination by *Salmonella* have been described in numerous studies during the last two decades [50-54]. Both egg whites and yolks can be contaminated. However, *Salmonella* contaminations have been more often observed in egg whites at *in vivo* condition [55-57].

**Causes of *Salmonella* contamination in eggs can be categorized by intrinsic and extrinsic factors** [43,58,59] and the detailed contents are shown in Table 2.

<table>
<thead>
<tr>
<th>Intrinsic factors</th>
<th>Shell porosity</th>
<th>Shell thickness</th>
<th>Distribution of cuticle</th>
<th>Translucency</th>
</tr>
</thead>
</table>

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negatively charged cell surfaces. Ovotransferrin is considered a major antimicrobial ingredient of the egg whites because it chelates iron, lysozyme, which are positively charged and easily interact with other microorganisms because of the distinctive genes related to their cell wall formation and metabolism [64,67,68]. Moreover, using the siderophore, *Salmonella* can effectively uptake iron to survive in egg white [69,70].

### Table 2: *Salmonella* contamination factors for shell egg.

<table>
<thead>
<tr>
<th>Extrinsic factors</th>
<th>Health condition of laying hen</th>
<th>Type of <em>Salmonella</em></th>
<th>Level of initial inoculum</th>
<th>Humidity</th>
<th>Temperature</th>
<th>Storage condition</th>
<th>Washing condition</th>
</tr>
</thead>
</table>

The processes of contamination can be divided into two main parts: vertical transmission (primary contamination) and the horizontal transmission (secondary contamination) [54]. The vertical transmission occurs when *Salmonella* cells migrate to the egg inside of the hen before the egg shell is formed. *Salmonella* cells move into the reproductive track and then enter into the albumen and/or yolk prior to the egg shell formation [53].

Horizontal transmission happens after the egg shell is formed. *Salmonella* cells migrate to the egg albumen from the outside of the shell after the egg has been made. Consequently, the initial contamination by the horizontal transmission occurs in the albumen. This horizontal transmission is considered the most common route for egg contamination by *Salmonella* [60]. Grijseebert et al. [52] reported that it took 58 hours for *Salmonella* to proliferate from one initial cell to about 508 cells in albumen.

Egg whites are reported to include ingredients that have bacteriostatic and bactricidal properties, such as ovotransferrin and lysozyme, which are positively charged and easily interact with negatively charged cell surfaces. Ovotransferrin is considered a major antimicrobial ingredient of the egg whites because it chelates iron, which is a critical growth factor for microorganisms such as *Salmonella* [61-63]. Ovotransferrin and lysozyme are known to generate pores on the surface of gram-negative bacteria resulting in membrane permeabilization [64,65]. However, there are various studies showing that *Salmonella* cells survive effectively inside of egg whites using their defense system. For example, Lu et al. [66] reported that damaged DNA by egg whites can be restored by yafD, xthA and rfbH genes of *Salmonella*. Other researchers have shown that *Salmonella* is more adept at thriving in egg albumen compared with other microorganisms because of the distinctive genes related to their cell wall formation and metabolism [64,67,68]. Moreover, using the siderophore, *Salmonella* can effectively uptake iron to survive in egg white [69,70].

### Salmonella detection

Rapid detection of *Salmonella* is important to assure food safety by *Salmonella* monitoring and risk management in the food industry. There are a variety of methods to detect *Salmonella* and they usually depend on cultural enrichment to enhance total cell concentration and to restore injured microorganisms [71]. In the food industry, *Salmonella*, if present, is often found at low concentrations and as little as 15-20 bacilli of *Salmonella* constitute an infectious dose for human [72].

Food samples are typically enriched for the detection of low concentrations of *Salmonella* using non selective broth (e.g., buffered peptone water, trypticase soy broth, and lactose broth) to enhance microbial activities from target foods [73-76]. Traditional culture-based assays, which are regarded as the "gold standards" are widely used by many organizations, especially by regulatory food safety agencies and food companies, because these are accepted in global regulation for trade and enable detection of low levels of pathogens [77]. However, standardized ways of detecting foodborne pathogenic bacteria by regulatory organizations (i.e., ISO, WHO, FDA) are often labore intensive and time consuming because of the sequence of the following processes: (i) pre-enrichment in non-selective broth (e.g., buffered peptone water, trypticase soy broth); (ii) selective bacterial enrichment using selective broth (e.g., Rappaport-Vassiliadis Soy [RVS] broth, Muller-Kaufmann tetrahionate [MKtt] broth); (iii) plating on selective agar plates (e.g., xylose lysine deoxycholate [XLD] agar, Brilliant Green [BG] agar, bismuth sulfite [BS] agar; hektoen enteric [HE] agar); (iv) biochemical and/or serological confirmation [73,75-77] (Table 3).

#### Table 3: Work flow for *Salmonella* detection from foods using ISO standard method.

This entire process usually requires 5 to 7 days to complete. About 2 to 3 days are needed for limited examination of food samples for the presence or absence of *Salmonella* cells through selective plating methods (See the step 3 in Table 3) [78].

Development of rapid and sensitive bacterial isolation and detection methods from foods has become increasingly important to prevent food poisoning outbreaks from consumers, to meet regulations of safety, and to control processing for the industry. Over the last several decades, there have been significant developments in rapid *Salmonella* detection methods using multiple novel approaches reducing pre-enrichment or detection time (e.g., *In-situ* immuno-gold nanoparticle network ELISA biosensors, immunomagnetic separation, automated microfiltration system, pathogen enrichment device, laser optical sensor, modified media, isothermal amplification) [79-89].

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pre-enrichment in buffered peptone water: non-selective enrichment</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>Enrichment in RVS and MKtt Broth: selective enrichment</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td><em>Salmonella</em> detection using selective agar plates</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>Streaking on nutrient agar</td>
<td>24</td>
</tr>
<tr>
<td>5</td>
<td>Biochemical confirmation</td>
<td>24</td>
</tr>
</tbody>
</table>
Recently, multiple rapid methods have been developed to reduce time for *Salmonella* detection from eggs based on immunology or molecular biology methods. The use of these assays led to equivalent results obtained from methods that have been officially approved by FDA [90]. These include: (i) *Reveal Salmonella* Test System of Neogen Corporation; (ii) SDIX® RapidCheck SELECT™ for *Salmonella*; (iii) BAX® System Polymerase Chain Reaction (PCR); (iv) TaqMan® *Salmonella* Detection Kit from Applied Biosystems™ by Life Technologies™. However, these rapid *Salmonella* detection technologies still require more than one day enrichment process to get a positive signal of *Salmonella* contamination when a low level of cells is present inside of target sample (Figure 2). The accuracy, specificity, and sensitivity of commercially available kits are shown in Table 4 [91-98].

<table>
<thead>
<tr>
<th>Method</th>
<th>Accuracy</th>
<th>Specificity</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RapidCheck</td>
<td>99.4</td>
<td>99</td>
<td>96.6</td>
</tr>
<tr>
<td>Muldoon et al. [97]</td>
<td>100</td>
<td>100</td>
<td>137</td>
</tr>
<tr>
<td>Muldoon et al. [98]</td>
<td>113.3</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Muldoon et al. [98]</td>
<td>325</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Muldoon et al. [98]</td>
<td>84.6</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Reveal [99]</td>
<td>99.4</td>
<td>99</td>
<td>96.6</td>
</tr>
<tr>
<td>Zhang et al. [100]</td>
<td>92</td>
<td>94</td>
<td>83</td>
</tr>
</tbody>
</table>

Table 4: Accuracy, specificity, and sensitivity of rapid *Salmonella* detection methods approved by FDA. *AFNOR* denotes Association French Normalization Organization Regulation.

Figure 2: Comparison of microbial detection processes: The FDA approved molecular biology based methods was compared with classical cultural enrichment method (ISO-6579:2002).

One major recent development has been the reports of the use of an enzyme based approach coupled to a short enrichment and microfiltration steps for reducing time for sample preparation. This allowed *Salmonella* to be detected at very low levels (≤ 1 CFU/g) in different contaminated foods in 8 hours or less than one work shift [82-84]. The major concept is shown in Figure 3.

**Conclusion**

*Salmonella* infection caused by egg contamination is not only an ongoing global food safety issue, but it also has implications in the pharmaceutical industry for vaccine production. Although there are a variety of management practices that may be utilized during egg production, including vaccination and washing processes, salmonellosis caused by egg contamination remains the most common foodborne disease in the world. Therefore, an in-depth study of *Salmonella* and detection tools for eggs is still necessary to ensure food control and quality assurance. While a variety of detection methods have been developed with improved sensitivity and reduced time to result, 2 to 3 days may still be needed sample enrichment to detect low levels of pathogens. Recent reports show that is possible to combine a short enrichment step with enzyme assisted microfiltration in order to reduce the time for *Salmonella* detection to 8 hours or less.
**Figure 3:** Comparison of cell detection processes. FDA approved rapid PCR assay (a) and the microfiltration process (b) for isolation and detection of *Salmonella* from food samples were compared.

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