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Review Paper

Detection of Salmonella in Eggs

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Abstract: The purpose of this review paper is to describe improved test methods for estimating the extent of *Salmonella* contamination on an egg, in order to assist in the control and elimination of this food infection hazard. *Salmonella* a member of the bacterial family *Enterobacteriaceae* may be recovered from foods and processing facilities. *Salmonella* has been recognized as an important zoonotic pathogen of economic significance in animals and humans. The egg's contents are an ideal growth medium for microorganisms that are hazardous to humans. A variety of methods has been developed in order to expedite the detection of *Salmonella* in eggs, including Gene Quence DNA hybridization, PCR analysis, and enzyme-linked immunosorbent assay. In addition, brilliant green agar found to support more growth of all the types of studied *Salmonella*, while bismuth sulphite act as inhibitory to *S. typhimurium, S. anatis, S. Worthington and S. oranienburg.* In each mentioned article of this review, different methods are used for detection of *Salmonella* on an egg.

***Keyword**:* Salmonella, *egg, PCR, DNA microarray, agar*

Introduction

Salmonella – pathogenesis

The genus *Salmonella* are facultative bacteria, gram-negative rods in the family *Enterobacteriaceae*. *Salmonella* are small bacteria in diameter around 0.5 µm with a length 2 to 3 µm; most strains are motile with peritrichous flagella. Typical *Salmonella* is distinguished from other members of the family by lack of fermentation of lactose, fermentation of glucose with production of gas and production of H_2S from thiosulfate. The optimum temperature of *Salmonella* growth is usually 37^{o}C (Cox *et al*., 2000). There are more than 2200 different *Salmonella* serotypes and most of these are human pathogens (Cocolin *et al*., 1998). *Salmonella* has long been recognized as an important zoonotic pathogen of economic significance in animals and humans. The genus *Salmonella* is divided into three species: *Salmonella enterica*, *Salmonella bongori* and *Salmonella subterranea* (Kim *et al*., 2006; Garcia et al., 2011; Gole et al., 2014). *Salmonella is* present everywhere in nature. Foodborne salmonellosis is an important public health problem worldwide, both in developed and developing countries (Camps *et al*., 2005). They are found in contaminated foods, water and the intestines of animals. Salmonellosis is usually caused by consuming inadequately cooked meat or meat products, poultry, dairy products, raw egg and egg products contaminated with the pathogens (Wong et al., 2013). Infection with *Salmonella enterica serovar Enteritidis* causes fever, stomach cramps and diarrhea (Schroeder *et al*., 2005). More than 1.4 million cases of salmonellosis happen in the United States each year, causing more than 300,000 hospitalization events and around 500 deaths. In Hong Kong, *Salmonella* is the second leading cause of food-borne illnesses. Many cases over 3,000 *Salmonella* infection reported the Department of Health within the last several years. Based on studies, antibiotics are not essential for the treatment of most cases of salmonellosis, but they can be protector in invasive infections, which often occur in children and old people. Conventional drugs, including ampicillin, chloramphenicol and tetracycline noticed resistance of *Salmonella*, frequently. Fortunately, the resistant cerates of fluoroquinolones and broad-spectrum cephalosporins, which have been the choices of treatment for multidrug-resistant (MDR) nontyphoidal *Salmonella* infection in adults and children remain extremely low (Wong *et al*., 2013). The microbiological safety of food production is an important concern of regulatory agencies and the food industry. The most important aspect is to avoid potential negative consequences to human health and economic losses, as well as the loss of consumer confidence.

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Salmonella on Eggs

Eggs are considered as the main source of infection of *Salmonella Enteritidis* for humans (Carrique-Mas *et al*., 2009). The egg contents are an ideal growth medium for microorganisms that are hazardous to humans. Gram-positive bacteria dominate the microflora of the eggshell, while gram negative bacteria are best equipped to overcome the antimicrobial defenses of the egg content (Chousalkar et al., 2012). There are two pathways for eggs to become internally contaminated with *Salmonella*. Direct contamination occurs during the formation of an egg in the reproductive tract of hens (including the ovary and oviduct); while, indirect contamination occurs after an egg has been laid. These pathways for contamination can be affected by the egg production process, food preparation, storage and handling (Whiley et al., 2015). The surface of the eggs may be contaminated with *Salmonella* present in feces, as well in the other matters such as yolk, fluff, dust and other debris present in domestic fowl (Poppe et al., 1998). The contact between fecal material and the eggshell is often unavoidable and could strength entrance of microorganisms into the egg. Small defects and abnormalities in eggshells (thin shells, increased shell pore numbers, translucency) may potentiate the entry of food-borne pathogens into the egg contents. Shell contamination depends on either intestinal or oviduct infection; nevertheless, the process responsible for eggshell contamination by Salmonella-infected birds is not clear. Bacterial contamination of shells and egg contents is more common in eggs from older hens than from younger hens (Chousalkar *et al*., 2012). Egg washing can reduce the microbial load on the eggshell surface and thus may lower the rate of penetration of Salmonella across the eggshell and decrease the incidence of food poisoning. Egg washing uses to reduce eggshell contamination in many countries such as the United States, Australia and Japan. However, some researchers claim that egg-washing chemicals can damage the cuticle layer of the eggshell, which may result in moisture loss, and deterioration of the internal quality of the egg. Furthermore, egg washing may favor the transmission of *Salmonella* across the eggshell particularly, when the post-washing storage and drying conditions are below required standard (Gole *et al*., 2014). Therefore, the contamination and persistence of *Salmonella enterica serovar Enteritidis* in chicken eggs represent a unique epidemiological characteristic of this bacterium that is essential for its eventual transmission to humans. Little is known about the bacterial factors that allow *Salmonella enterica serovar Enteritidis* to survive in eggs and contribute to its epidemiological association with chicken eggs. *Salmonella enterica serovar Enteritidis* can be deposited into both albumen and yolk. It is more frequently deposit into the albumen, especially in naturally contaminated eggs (Clavijo *et al*., 2006). The deposition of *Salmonella Enteritidis* inside the contents of eggs is a consequence of bacterial spreading to reproductive tissues (ovary and oviduct) in systemically infected hens (Gast *et al*., 2014). Freshly laid eggs usually contain no more than a few hundred *Salmonella* cell; immediate refrigeration can prevent extensive bacterial multiplication during storage, which could increase the threat of egg borne transmission of illness to consumers. Accordingly, components of many risk reduction plans and state or federal regulations have specified that eggs must be stored at 7.2°C or lower within 1 or 2 d after being laid. The efficacy of egg refrigeration for preventing the expansion of small populations of pathogens such as *Salmonella Enteritidis* are reached depends on the initial level and location of contamination, the potential for movement of bacteria or nutrients within eggs during storage, and the rate at which growth-restricting temperatures (Gast *et al*., 2010).

Methods for the Detection of *Salmonella* on Eggs

The isolation and identification of members of the *Salmonella* group from food products present many difficulties. Various methods and modifications have been proposed throughout the years (Byrne et al., 1955).

Culture media for *Salmonella* detection

Culture based methods are still the most widely used detection techniques and stay the gold standard for the detection of *Salmonella* due to their selectivity and sensitivity. To decrease the risk of obtaining the negative results, a nonselective pre-enrichment media and selective enrichment media are performed in isolation of Salmonella from animal feces and food (Odumeru *et al*., 2012):

- Pre-enrichment in non-selective medium (buffered peptone water).

- Selective enrichment in Tetrathionate broth (Müller-Kauffmann) and Rappaport Vassiliadis soy peptone (RVS) broth.
- Subcultivation on Xylose Lysine Desoxycholate (XLD) agar and on Brilliant Green agar (BGA) (or another selective agar media).

Some of the enrichment media tend to inhibit the growth of certain types of *Salmonella*. It has also been found that some of the routinely used selective enrichment broths are inhibitory towards *Salmonella enterica serovar Enteritidis*. It was found that tetrathionate broth was definitely inhibitory to *Salmonella paratyphi* (Wells *et al.*, 1957). Also brilliant green agar was found to support more growth of all the types of *Salmonella* studied, while bismuth sulfite was found to be inhibitory to *S. typhimurium, S. anatis, S. Worthington and S. oranienburg*. Bismuth sulphite agar is used because it is more inhibitor than brilliant green agar to organisms other than *Salmonella* (Wells *et al*., 1957). Detection and isolation of *Salmonella Enteritidis* from eggs is essential for the surveillance and control of *Salmonella Enteritidis* in eggs and egg products implemented of FDA egg rule 21 CFR Parts 16 and 118 (Food and Drug Administration, 2009). Preenrichment broths, tryptic soy broth (TSB) + ferrous sulfate (TSB + Fe), buffered peptone water (BPW), and nutrient broth (NB) or BPW are used by FDA, USDA, and Health Canada, respectively (Food & Drug Administration, 2011; USDA, 2011; Health Canada, 2009; Zhang *et al*., 2013). The improved test methods are used for estimating the extent of *Salmonella* contamination in foods in order to assist in the control and elimination of this food infection hazard (Byrne *et al*., 1955). Using brilliant green agar plates have been compared with those obtained using bismuth sulphite agar plates. It is obvious from the data shown that the bismuth sulfite agar is more effective than brilliant green agar for the isolation of *Salmonella* from liquid eggs, especially from the liquid before pasteurization (Byrne AF et al., 1955). In another article (Chousalkar K et al, 2012) nutrient broth was used in the prevalence of *Salmonella spp* on the eggshell surface, eggshell membranes or pores and in egg internal contents. Clean eggs (n=1,560 from 26 flocks) were collected from commercial caged layer farms in Australia. *Salmonella* spp was not isolated from any eggshell crush or egg internal contents. It leads to understand that the occurrence of Salmonella in the Australian egg industry is low (Chousalkar *et al*, 2012).

PCR for *Salmonella* determination

Standard conventional culture method for the detection of Salmonella requires five working days to generate and confirm positive results. Conventional methods for detection of *Salmonella* in foods are labor-intensive, time-consuming, and expensive. Rapid methods based on principles including membrane technology, latex agglutination, Gene Quence DNA hybridization, PCR analysis, and enzyme-linked immunosorbent assay have been developed lastly. Methods employing PCR in combination with pre-enrichment broths, immunomagnetic separation or centrifugation are currently being developed (Yoshimasu *et al*., 2001).

Recently, more rapid and specific PCR methods based on the DNA sequence of Salmonella genes have been developed to identify or characterize pure culture strains and to detect the pathogen in clinical, environmental and food samples. PCR technology represents a rapid procedure with high sensitivity and high specificity to detect *Salmonella* in a wide variety of food. PCR is a technique that is used to amplify a single or a few copies of a piece of nucleic acid, to generate thousands to millions copies of a particular nucleic acid. It allows much easier characterization and comparisons of genetic material from different individuals and organisms. Simply stated, it is a "copying machine for DNA molecules" (Cheung *et al.,* 2004). Real-time PCR (RT-PCR) is a promising new method currently used for detection of a wide variety of bacterial pathogens in food matrices (Day *et al*., 2009). The real-time PCR assay is based on an increase in fluorescence from a dsDNA-specific dye or hybridization probe that is monitored during the amplification of a target gene (Hyeon JY et al., 2010). Several PCR assays have been developed by targeting various *Salmonella* genes, such as 16S rRNA, agfA, and viaB, and virulence-associated plasmids. In addition, invA gene is one of the most often used to detect *Salmonella spp.* in a variety of food. A standardized PCR-based method for the detection of food-borne pathogens should optimally fulfill various criteria such as analytical and diagnostic accuracy, high detection probability, high robustness, low carryover contamination, and acceptance by easily accessible and user-friendly protocols for its application and interpretation (Malorny *et al*., 2004).

Many articles have been published in related to the detection of *Salmonella* in eggs by using PCR technique. Comparison of egg contamination in commercial production from different housing system with *Salmonella* spp. on egg shell and egg content was performed by authors (Wiriya *et al*., 2010). Conventional microbiology and PCR technique using *inva* a gene was used for detection of *Salmonella*. The results showed that none of the conventional methods detected any positive samples, while analysis of the PCR products from direct boiling of the enriched cultures showed that 2 cultures were found positive of *Salmonella* spp. (Wiriya et al., 2010). In another article (Soria MA et al., 2012) detection of *Salmonella* was performed by three methods: Tetrathionate broth (TT), Muller-Kauffmann tetrathionate – novobiocin broth (MKTTn) and PCR method. However, no one method has superiority over another and the sensitivity and specificity of the method depends on the sample type as well as the isolation conditions. Comparison of two culture methods and a PCR assay led on learning of their ability to detect low levels of motile and nonmotile *Salmonella* strains in artificially contaminated egg content. Furthermore, it was investigated the accuracy (**Ac**), sensitivity (**Se**), specificity (**Sp**), positive predictive value (**PPV**), and negative predictive value (**NPV**) of each method and the agreement among methods. From results, there was no any significant difference between two cultures methods. The TT and MKTTn methods had a high value of Sp, Ac, Se, PPV, and NPV for motile, like *S. Enteritidis* and nonmotile *Salmonella* strains in their study. It was found the results using PCR were in perfect agreement with the results of the standard culture methods. However, the PCR assay is extremely quick, and results can be obtained within 4 h of testing of enrichment broths (Soria et al., 2012). The same procedure as previously was used for detection of *Salmonella* with PCR by authors (Moosavy *et al*., 2015). The detection level of motile and nonmotile strains was 5 to 54 cfu per 25 mL for both culture methods, but some strains could not be detected by the PCR methods (Moosavy et al., 2015).

Microarrays for *Salmonella* estimation

The potential transfer mechanisms, the characterization of virulence-associated genes and developing an effective detection method in epidemic disease control is important for identification of *Salmonella* pathogenicity. DNA microarrays have a potential for analysis of gene expression, genotyping, pathway analysis, monitoring changes in genomic DNA, and host-pathogen interaction. Microarray techniques have been useful in high-throughput genetic profiling of pathogenic microorganisms. This technology can also detect the presence or absence of thousands of genes simultaneously by a single genomic hybridization step. Spotted DNA microarray method is effective and easy to reproduce in a laboratory setting with basic infrastructure. Furthermore, interpretation of microarray data is easier to automate and standardize than that of gel-based technologies (Zou *et al*., 2011).

Dot Blot Immunoassay

Dot blotting is an important technique used in research and diagnostic laboratories. Dot blotting is a simple technique, which identifies a known protein in a biological sample. It is an ideal diagnostic tool because of simplicity and easy use. The key feature of Dot blotting is the use of immunodetection to identify a specific protein, for example, a protein marker for a disease. Once, the proteins are immobilized on a protein binding membrane, usually nitrocellulose or PVDF (polyvinylidene fluoride), and they can be probed with a primary antibody, an antibody specific for the protein of interest (Yoshimasu *et al*., 2001). For detection of *Salmonella* on egg through dot-blot immunoassay technique, egg homogenate (EH) can be used as the enrichment medium. Detection can be performed by a monoclonal antibody (MAb)-based dot-blot assay. Cholic acid is a detergent that is present in the medium to release the lipopolysaccharide (LPS antigen in gelled egg matrix). Through the addition of sodium chlorate and the application of heat, the LPS antigen of serovar Enteritidis is released from the bacterial membrane. Through diffusional forces, the antigen is able to move through the porous egg sample and onto the solid support for detection. Other detergents have been used for extraction of LPS antigens; however, it was found that a 15% sodium cholate solution is the most efficient. Addition of ferrous sulphate or ferrioxamine E or cholic acid in the enrichment broth has negligible negative effects on the growth of Salmonella. Several media were compared with egg homogenate (EH), trypticase soy broth (TSB) and Lactose broth (LB). *Salmonella enteritidis* grown in TSB showed the greatest visual intensity, shows a positive test when tested by the dot-blot assay. Addition of ferrous

sulphate or ferrioxamine E or cholic acid in the enrichment broth has negligible negative effects on the growth of Salmonella (Jaradat *et al*., 2004).

Other Methods

Because of the complex epidemiology of *Salmonella,* it is necessary to implement control programs based on bacteriological and serological tests, to prevent infection or control the spread of organisms, and to practice preventive hygiene measures. Authors (Rantala *et al.,* 2007) suggested that pretreatment with microbiota isolated in the gastrointestinal tract of adult poultry free of S*almonella* spp. can protect against infection by this species. The use of *Lactobacillus* spp. as a probiotic for hens has been suggested as an interesting option to reduce the infections caused by *Salmonella Enteritidis* (Yamawaki *et al*., 2013). In most conventional cookery, the food remains at high temperatures long enough to yield a product which is safe from pathogenic organisms. However, in the electronic range, the food is at high temperatures for a very short time. *Serratia marcescens, Staphylococcus aureus,* and *Salmonella typhi* were destroyed more completely when baked conventionally for 30 to 40 min. *Bacillus cereus,* a spore former was not completely destroyed by electronic cooking (Baldwin *et al.,* 1968). Unpasteurized liquid egg products are sometimes contaminated with *Salmonella*. When food companies use unpasteurized liquid egg products contaminated with *Salmonella, Salmonella* cells must be inactivated during the heating or cooking process of food production (Sakha MZ et al., 2012).

Conclusions

Salmonella contamination of eggs is a complex issue affected by every stage of the egg production, from farm to the customer. Based on all mentioned articles in this review paper, can be concluded that there are many methods for detection of salmonella on eggs and some ways to prevent this pathogen microorganism to cause the damage to health. However, the current literature does indicate that it is not yet achievable to produce eggs guaranteed to be *Salmonella*-free. This includes post collection, disinfection methods such as washing, pasteurization, and irradiation. There is also the need for further research to optimize storage, temperature and food-handling protocols as currently, the information is highly complex and variable. Given the current shift in consumer's preference and increasing desire for raw food products, there is a need for more informed guidelines regarding the preparation of foods containing raw eggs. Further, research is required to explore different protocols to ensure control of *Salmonella* through temperature and pH of food products. There is also a need to re-educate food handlers and consumers of the risk from raw eggs and cross-contamination of food products and reduce the public health risk.

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