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The running title:

Salmonella Enteritidis in Raw Egg Samples

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Research Article

Isolation of *Salmonella* Enteritidis from liquid whole eggs sold in retail outlets in Egypt and Bangladesh

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ABSTRACT

The main objective of this survey was to establish the current prevalence of *Salmonella* enterica subspecies enterica serovar Enteritidis (*S.* Enteritidis) in raw egg samples from the following countries: Japan, Egypt, and Bangladesh.

A total of 151 sample units of eggs were tested over a five-month period from December 2013 to April 2014. Following conventional bacteriological procedures the samples subjected for molecular identification by PCR using specific primers for *Salmonella* Enteritidis sp. All samples were purchased and analyzed within their stated shelf life. *Salmonella* contamination was recorded 17.6% (9/51) of eggs' contents of Egypt samples and recorded in 38% (8/21) of eggs' contents of Bangladesh samples while none of the samples from Japan (0/69) contaminated with *S*. Enteritidis in the eggs' contents.

Detection of *Salmonella* in chicken eggs' contents needs a greater concern for effective control of Salmonellosis in poultry.

Key Words: *Salmonella*, Contamination, Poultry, Eggs contents

INTRODUCTION

The consumption of raw or lightly cooked eggs, eggs containing foods in particular mayonnaise, desserts and sauces that contain raw eggs has been associated with high percentage of many human *S.* Enteritidis outbreaks. Over 99% of human *Salmonella* spp. infections is caused *by S.* enterica subsp. enterica (Bell and Kyriakides 2002; Crum-Cianflone 2008).

The intestinal tract is the primary reservoir of *Salmonella* in poultry birds leading to contamination of chicken eggs in cloacal region through horizontal route. Transovarian contamination is another important route of *S*. Enteritidis egg contamination. Several studies have isolated *S.* Enteritidis from ovaries and oviducts of naturally and experimentally infected hens. The presence of *S.* Enteritidis in the reproductive tract was consistent with the production of *S.* Enteritidis contaminated eggs in the albumen, the yolk or the both (Keller et al 1995; Gast and Beard 1990; Gast 1993; Timoney et al 1989).

The goal of this study was to determine the incidence of *S.* Enteritidis in raw egg samples sold in the following countries: Japan, Egypt, and Bangladesh, using both conventional method, and a specific PCR detection technique.

MATERIALS AND METHODS

Sample Collection:

Eggs were purchased from a retail outlets in a residential area of (Gifu Prefecture; Japan, Kafr Elsheik; Dkhalia; and Ismailia cities; Egypt, Dhaka; Bangladesh) over a period of 5-months (December 2013‑April 2014). A total of 151 eggs' content were analyzed for the presence of *Salmonella.* In order to collect the eggs' contents, eggs' surfaces were sterilized by swabbing with 70% alcohol, drying with the air and then cracked with a sterile knife. Yolks and whites were mixed thoroughly, so that their contents pooled; each pool consisted between 6 and 10 eggs.

According to The World Organization for Animal Health 2008, the conventional culture methods for detection of *Salmonella* in food include non-selective pre-enrichment followed by selective enrichment, plating on selective and differential agars, and biochemical tests.

Non-Selective Pre-Enrichment:

A 25 gram portion of the pooled sample was added to 225 ml BPW and mixed well before incubation at 35° C for 24 hours.

Selective Enrichment:

1ml samples were taken and mixed with 10 ml of Rappaport Vassialidis broth (RV) and incubated at 35° C for 24 hours.

Plating on Selective Agar:

A loopful of each enriched sample was streaked on selective agar medium such as deoxycholate hydrogen sulphide lactose agar (DHL) plate (EIKEN, Tokyo, Japan), using such medium enables testing of the bacterium for lactose fermentation and H_2S production.

Suspect colonies are confirmed by molecular identification.

Molecular identification:

DNA extraction:

The DNA was released by heating at 100° C for 5 min. The cellular debris was pelleted by centrifugation at 8000 rpm for 5 min, and the clear supernatant fluid containing nucleic acids was conserved until it was used in subsequent PCR assays.

Polymerase chain reaction:

The DNA samples (5 µL) were amplified in an optimized 20 µL reaction mixture consisting of 2.5 µL of each primer 0.4 µM /reaction mixture, (10 µL) 2× SYBER Premix Extaq buffer (TAKARA, Japan). The reaction mixture was incubated in a programmable DNA thermal cycler (Thermogen, model QB-0224B). *Salmonella* genus-specific primers 139 (AMR No DCR227, Japan), which were based on the invA gene of *Salmonella*, were used in the PCR assay.

A reagent blank containing all the components of the reaction mixture with the exception of template DNA (which was replaced by sterile distilled water) was included with every PCR assay. Furthermore, positive DNA control was included, which was prepared from *Salmonella* sp., respectively. The cycling parameters used were initially denaturated at 95° C for 1 min followed by 40 cycles of amplification of 10 s at 95° C, and 10 s at 60° C. Then, the PCR tubes were held at 4° C.

Agarose Gel Electrophoresis

The PCR products were analyzed by gel electrophoresis. Five microliters of each sample was loaded onto 2.0% of agarose gel in 1× TAE buffer at 100 V/cm for 30 min. The gel was stained with ethidium bromide and electrophoresed products were visualized with a UV transilluminator (FAS-III, Toyobo, Osaka, Japan). A 100-bp ladder (Toyobo, Japan) was used as a molecular weight marker.

RESULTS

Growth of *Salmonella* was detected on DHL agar plates as shown in Fig. 1 whitish colonies (non-lactose fermenter) with H_2S production indicated by a blackening of the colonies due to a formation of iron sulfide.

Molecular characterization using PCR produced positive amplification of 139 bp fragments of invA genes (100%) specific for all members of *Salmonella* Enteritidis.

From the previous results, it could be concluded that *Salmonella*-specific PCR test in conjunction with traditional isolation methods could be effective in providing a more accurate profile of the prevalence of *Salmonella* in poultry products.

The results of *Salmonella* incidence in eggs are shown in Table 1. *Salmonella* contamination was recorded 17.6% (9/51) of eggs contents of Egypt samples and recorded in 38% (8/21) of eggs contents of Bangladesh samples while none of the samples from japan (0/69) contains *Salmonella* contamination in the egg's contents.

DISCUSSION

Since *Salmonella* is an important zoonotic bacterium with poultry as largest single reservoir of *Salmonella* (Gupta, Ray, & Sharma, 1999), the assessment of the contamination level and site of contamination are the utmost importance in deciding the control strategies against *Salmonella* in chickens.

Moreover, the use of a three step protocol (pre-enrichment, selective enrichment and selective plating) as specified in the FDA's Bacteriological Analytical Manual (US Food Drug Administration, 2002) was found satisfactory for the recovery of *Salmonella* spp.

The present study demonstrated widespread contamination by *Salmonella* Enteritidis in eggs retailed in Egypt, similar average of contamination of poultry and or poultry products showed previously by (Ibrahim et al 2013, Ibrahim et al in press)

Our results reinforce previous investigations, suggesting that *S.* Enteritidis one of the predominant serovars circulating in poultry in Bangladesh (Barua et al., 2012,2013) and plays a role as a source of human infections (Barua et al 2014), and consistent with the previous studies indicating the very low internal eggs' contamination in Japan (Suzuki H, Yamamoto S. 2009).

It is well known that traditional culturing methods are not able to specify the serovar of *Salmonella* isolates. Molecular identification method could be used in such cases to find out the serovar.

This study emphasized the need to implement proactive measures of hygienic practices, surveillance programs for laying hen flocks should be optimized, and the application of Hazard Analysis and Critical Control Point (HACCP) in the preparation and processing of foods in order to reduce *Salmonella* contamination of eggs in retail and to reduce the risk of human infection.

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Table 1: The prevalence of *Salmonella* isolated from eggs content

Country of origin	No. of eggs samples	No. of positive samples	positive samples %
Japan	79	0	0
Egypt	51	9	17.6
Bangladesh	21	8	38
Total	151	17	11.2

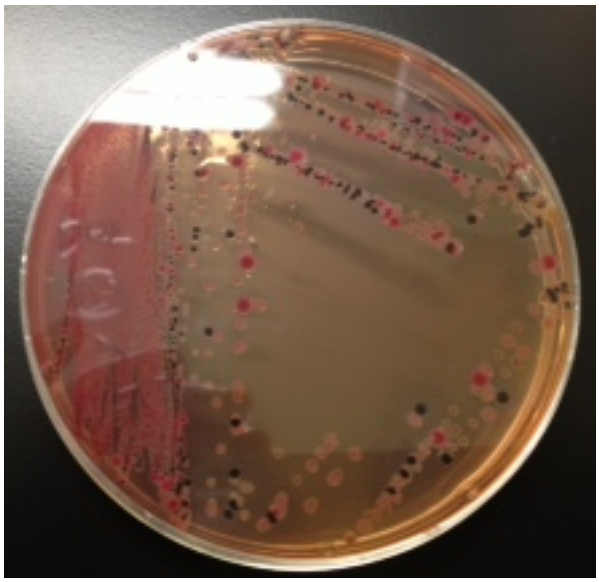
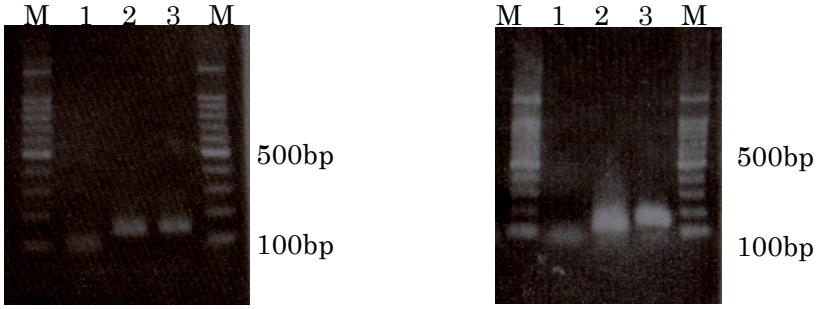


Figure 1: Shows the growth of *Salmonella* on DHL plates with its whitish (non- lactose fermenter) colonies, and blackish colouration because of H_2S production.



A B

Figure 2: A) PCR products of 139 bp specific to invA gene of *Salmonella* Enteritidis in ethidium bromide stained 2% agarose TAE gel electrophoresis. M: 100bp; L1: control negative; L2: control positive; L3: PCR product of the sample from Bangladesh eggs.

B) PCR products of 139 bp specific to invA gene of *Salmonella* Enteritidis in ethidium bromide stained 2% agarose TAE gel electrophoresis. M: 100bp; L1: control negative; L2: control positive; L3: PCR product of the sample from Egypt eggs.