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Evaluation of different plating medias and PCR in the detecting of *Salmonella* Enteritidis from eggs laid by experimentally infected hens

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Abstract

Salmonellosis is one of the most important food-borne diseases. Outbreaks of Salmonellosis are mainly related to the consumption of contaminated eggs or egg-products and, less frequently, of poultry meat. In this study fifty 29-week-old broiler breeder hens were randomly divided in two groups of 25 birds. One group of birds inoculated orally with $10\log_{10}$ CFU and other group inoculated intravenously with $6\log_{10}$ CFU of *S.* Enteritidis parent strain bacteria. During 35 days of experimental period, produced eggs were cultured using different medias and *S.*Enteritidis was detected using different cultural and PCR method. SE was isolated during 32.5 % of experimental days and more isolation rate of *Salmonella* was during the first two weeks of post infection period. In contrast, using pre-enrichment media resulted more isolation rate of *Salmonella* cells and SS agar was more sensitive than BG agar. PCR was more sensitive than cultural methods for detection of *Salmonella* Enteritidis from contaminated eggs.

Key words: *Salmonella* Enteritidis, PCR, Culture, hen

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Introduction

Salmonellosis is one of the most important food-borne diseases. The World Health Organization (WHO) has reported 1.3 billion cases per year of acute gastroenteritis due to non-typhoid Salmonellosis with 3 million fatal cases (Gomez et al., 1997). During the 2006 there were 160,645 reported human cases of Salmonellosis in the 25 Member States of the European Union (equivalent to an incidence of 35.4 cases per 100,000 populations) (Lahuerta et al., 2008). At that time *Salmonellosis* was the second most commonly reported gastrointestinal zoonotic infection across the EU. Outbreaks of Salmonellosis are mainly related to the consumption of contaminated eggs or egg-products and, less frequently, of poultry meat (Coyle et al., 1988). The overall European Union prevalence of *Salmonella* in table eggs was 0.8% in 2006 and more than 90% of all egg isolates were *S*. Enteritidis whereas; *S*. Enteritidis is the most common serotype (52.3%) in the laying flock environment (Lahuerta et al., 2008, Coyle et al., 1988). The persistence of this organism in poultry house environments poses a continuing threat of infection for laying hens (Davies et al., 2003). Additionally, there is suggestion that *S.* Enteritidis has some intrinsic characteristics that allow a specific interaction with either the reproductive organs of laying hens or the egg components (Gantois et al., 2009).

In poultry, an important step in *Salmonella* pathogenesis is bacterial entry in the epithelial cells of the intestinal tract, especially the caeca (Desmidt et al., 1996).

Oral infection of hens with *S*. Enteritidis leads to the invasion of a variety of internal organs, including the ovary and oviduct (Gast et al., 1990) and produced sporadic egg contamination for several weeks (Gast et al., 2000). The colonization of reproductive tissues in infected laying hens is a pivotal stage in the production of contaminated eggs that can transmit *S*. Enteritidis infections to off spring and consumers (Okamura et al., 2001). Egg contamination is caused by penetration through the eggshell by *S*. Enteritidis contained in feces after the egg is covered by the shell (DE Reu et al., 2005). The second possible route is by direct contamination of yolk or albumen originating from the infection of reproductive organs with *S*.Enteritidis before the egg is covered by the shell (Keller et al., 1995). The location of *S*. Enteritidis deposition in a developing egg (yolk or albumen) is likely a consequence of which regions of the laying hens reproductive tract are colonized (Bichler et al., 1996).

A wide variety of methods and selective medias have been developed to isolate and identify this microorganism. The use of different isolation techniques has a major impact on the outcome of the experiments. (Gantois et al., 2009). On the other hand, use of several agars in the detection and isolation of *Salmonella* increases the amount of labor and cost (Gast et al., 2000). Rapid detection methods, such as PCR detection methods and nucleic acid hybridization have been developed. In vitro amplification of

DNA by the PCR method is a powerful tool in microbiological diagnostics. Several genes have been used to detect *Salmonella* in natural environmental samples as well as food, egg and feces (Monzur., 2012).

The objective of this study was to evaluate the effectiveness of pre-enrichment broth and two plating agars and PCR procedure for *Salmonella* isolation and identification from experimentally contaminated eggs.

Materials and Methods

Bacterial strain: *S*. Enteritidis phage type 4, strain NIDO 76Sa88 Nalr (parent strain) was used in this experiment, obtained from Ghent University, Belgium. The nalidixic acid resistant strain is well characterized (Van Immerseel et al., 2002).

Hens

Fifty 29-week-old broiler breeder hens were selected from an Arian Grand Parent farm (north of Iran) that was under strict control for *Salmonella* and other infectious diseases. They were free of any apparent disease throughout the growing and laying periods. Before starting of the experiment, cloacal swabs were taken from all hens and checked for *Salmonella* infection, to confirm that animals were *Salmonella*-free. Hens randomly divided in two groups of 25 birds. One group of birds inoculated by oral route in the crop, using a plastic tube with $10\log_{10}$ colony forming units (CFU) of *S*. Enteritidis 76Sa88 Nalr parent strain in a volume of 1 ml of PBS as reported previously (Barrow et al., 1991), Other group inoculated intravenously (IV) with $6\log_{10}$CFU of *S*. Enteritidis 76Sa88 Nalr parent strain bacteria, using 0.1 ml of PBS.

Bacterial Culture

At days 2, 7, 14, 21 and 35 post-inoculation, 10 eggs from every groups were pooled together into sterile honey jars and contents mixed and homogenized by shaking the jars. Egg pools cultured in a two ways, one part of egg pools directly incubated in 37 ºC without using any pre enrichment medias, second group cultured in Pre-enrichment and enrichment medias was performed in Buffered Peptone Water and selenite cystein broth and then all groups plated onto the antibiotic containing SS or BG agars.

PCR

DNA was extracted from isolated suspected colonies of *Salmonella* from agar medias and was used as a template to detect ST, spv, SefA genes by mono and multiplex PCR. (Table 1). For multiplex PCR,

three sets of primers (Table 1) were selected from different genomic sequences amplifying a 429 bp fragment specific for the genus *Salmonella* within a randomly cloned sequence (ST gene), a 250 bp fragment within a spv gene, and a 310 bp fragment within the sefA gene specific for *Salmonella* Enteritidis (Okamura et al., 2001). PCR reactions were performed in a final volume of 25 µl containing template DNA, PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 1.5 mM MgCl2, 0.25 mM of dNTPs, and 1 U of Taq DNA polymerase, 20 pmole of a specific forward and reverse primers. Amplification was carried out using a Techne TC- 512 thermocycler (Techne, UK), as follows: 35 cycles of 30 s for denaturation at 94 °C, 90 s for annealing at 56 °C, and 30 s for primer extension at 72 °C, followed by a terminal extension at 72 °C for 10 min. The amplification products were electrophoresed on 1.2% agarose gels and 100-bp ladder was used as a molecular weight marker. The gels were stained with ethidium bromide (2 µg mL–1) to visualize fluorescent bands while using UV in the gel document system (BIORAD, UK).

Table 1. Primers used for identification of *Salmonella* Enteritidis by multiplex polymerase chain reaction (Pan and Liu, 2002).

Primer		Sequence	Amplification product [bp]
ST	ST11	5' –GCCAACCATTGCTAAATTGGCGCA- 3'	429 bp
	ST14	5' –GGTAGAAATTCCCAGCGGGTACTGG- 3'	
Spv	S1	5' –GCCGTACACGAGCTTATAGA-3'	250 bp
	S4	5' –ACCTACAGGGGCACAATAAC- 3'	
*Sef*A	*SEF*A2	5' –GCAGCGGTTACTATTGCAGC- 3'	310 bp
	*SEF*A4	5' –TGTACAGGGACATTTAGCG- 3'	

Results

Table 2 describes total days and percentages of *S*. Enteritidis isolation rate from different cultures. As it has shown, SE was isolated from 32.5 % of the examination days (13 days of 40 experimental days) and the mean days of isolation rate were 13 day.

Table 3 describes total results of SE isolation rate from eggs that directly incubated (without using pre-enrichmentmedia) and then cultured in BG or SS agars. Using this method, *Salmonella* was isolated at 26.66% of examination days and mean days of isolation rate was 10.1 days. The isolation rate of SE from eggs cultured in enrichment medias and plated onto BG or SS agars was 30% and mean days of isolation rate was 14.4 days(table4).

Table2: Total results of SE contamination of egg contents

Groups	Positive (days)	Negative (days)
VS	2, 35	7, 14, 21
VB	2	7, 14, 21, 35
VPS	2, 14, 35	7, 21
VPB	2, 14	7, 21, 35
OS	7, 14	2, 21, 35
OB	7, 21	2, 14, 35
OPS	-	2, 7, 21, 35
OPB	14	2, 7, 14, 21, 35
Total days number	13 days	27 days
Isolation rate	32.5%	67.5%

VS: Eggs produced by intravenous inoculated hens, directly incubated and cultured on SS agar; VB: Eggs produced by intravenous inoculated hens, directly incubated and cultured on BG agar, VPS: Eggs produced by intravenous inoculated hens, incubated in Pre-enrichment and enrichment media and cultured on SS agar; VPB: Eggs produced by intravenous inoculated hens, incubated in pre-enrichment and enrichment media and cultured on BG agar; OS: Eggs produced by orally inoculated hens, directly incubated and cultured on SS agar; OB: Eggs produced by orally inoculated hens, directly incubated and cultured on BG agar; OPS: Eggs produced by orally inoculated hens, incubated in Pre-enrichment and enrichment media and cultured on SS agar; OPB: Eggs produced by orally inoculated hens, incubated in pre-enrichment and enrichment media and cultured on BG agar

Table 3: Total results of incubated eggs without pre-enrichment broth and plated onto BG or SS agars

Group	Positive (days)	Negative (days)
VS	2, 35	7, 14, 21
VB	2	7, 14, 21, 35
OS	7, 14	2, 21, 35
OB	7, 21	2, 14, 35
Total days	8 days	22 days
Isolation rate	26.66%	73.33%

Table 4: Total results of incubated eggs in pre-enrichment broth and plated onto BG or SS agars

Group	Positive (days)	Negative (days)
VPS	2, 14, 35	7, 21
VB	2, 14	7, 21, 35
OPS	-	2, 7, 14, 21, 35
OPB	14	2, 7 , 21, 35
Total days	9 days	21 days
Isolation rate	30%	70%

Table 5 and 6 describes results of SE isolation rates in BG and SS agar mediums. As it is shown the isolation percentage of SE in BG agar was 30%, whereas in SS agar was 35%. The mean days of isolation rate in BG and SS agars were 10 and 15.57 days consequently.

Table 5. Total results of SE cultures in BG agar medium

Groups	Positive (days)	Negative (days)
VB	2	7, 14, 21, 35
VPB	2, 14	7, 21, 35
OB	7, 21	2, 14, 35
OPB	14	2, 7, 21, 35
Total days	6 days	14 days
Isolation rate	30%	70%

Table 6: Total results of SE cultures in SS agar medium

Groups	Positive (days)	Negative (days)
VS	2, 35	7, 14, 21
VPS	2, 14, 35	7, 21
OS	7, 14	2, 21, 35
OPS	-	2, 7, 14, 21, 35
	7 days	13 days
Isolation rate	35%	65%

PCR detection of *S.* Enteritidis

Figures 1 showing results of PCR that carried on DNAs extracted from isolated colonies of *Salmonella*. Three genes for *S.* Enteritidis identification were clearly detected in the isolated colonies. Baced on PCR results, SE detected in 40% of experimantal days (table 7).

Table 7: PCR detection of *S.* Enteritidis suspected colonies in experimental days

PCR	Positive (days)	Negative (days)
IV	2,14	7,21, 35
Or	7,14	2, 21, 35
Results	4 days	6 days
Isolation rate	40%	%60

IV: intravenous inoculated hens, Or: Orally inoculated hens

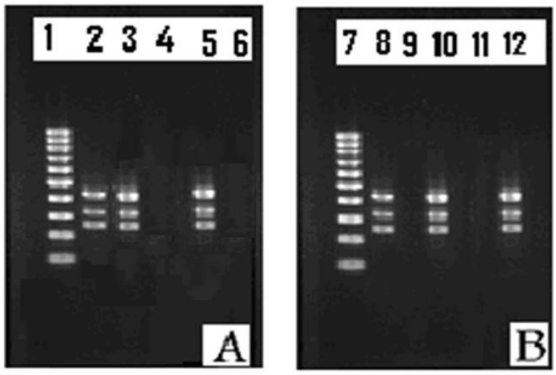

Figure 1. Multiplex polymerase chain reaction for detection of *S.* Enteritidis in eggs of Intravenous [A] and Orally [B] inoculated hens: 1,7= Gene Ruler; 2,8= Control [+]; 3,5= 2& 14dpi; 10, 12= 7 & 14dpi

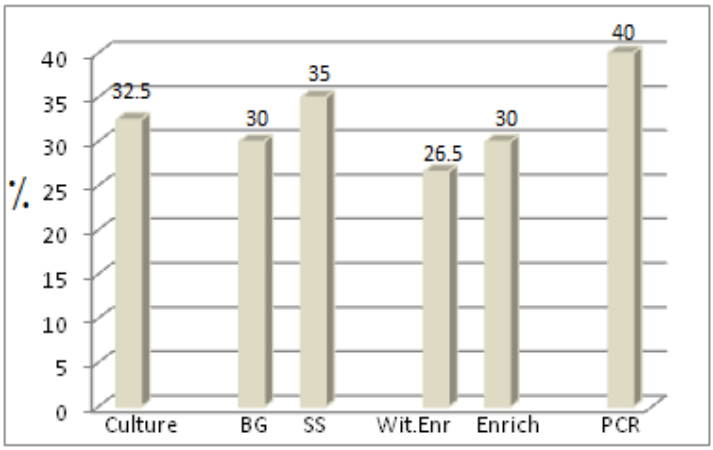

Figure 2. Comparative isolation rate of *Salmonella* Enteritidis in different methods

Discussion

In this study, *Salmonella* was isolated from 32.5 % of experimental days. Mean days of isolation rate were 13 days; it means that *Salmonella* was isolated more during the first two weeks of post infection period (table 2).

Bichler et al. (1996) inoculated 10log10 cfu S.E in laying hens orally; the hens produced SE-positive eggs at high frequencies in the first week postinfection. Contamination rate of egg white and yolk were 43

and 41 % respectively. In another study, De Buck (2003) inoculated 7log10 cfu S.E intravenously in laying hens and indicated that egg yolk and white contaminated 20.3 and 4.3 % consequently during the first week postinfection period. Kinde et al. (2000) inoculated 9 log10 cfu and 6log10 cfu doses of *S*.EPT4 in laying hens by oral and intravenous routs consequently and observed that 2.6% of total eggs contaminated by *S*.E. Gast and Beard (1990), utilizing inoculums of 9 log10 and 6 log10 cfu SE, respectively, showed that the majority of SE-positive eggs were produced within first 2 weeks of inoculated period.

Salmonella inoculated birds can quickly mount an antibody response that peaks within 1-2 weeks. The majority of SE-positive eggs are produced during this time. Once the antibody response has been established, fecal shedding of SE and production of SE-positive eggs decrease (Bichler ET AL., 1996).

Egg contents provide excellent nutrients for SE growth; direct incubation of egg contents without using the enrichment broth is a convenient, rapid and inexpensive method in *Salmonella* diagnosis procedure. In this study by or without using of enrichment broth, *Salmonella* was detected in 30 % and 26.66% of experimental days with mean days of 14.4 and 10.1 days respectively. The use of different isolation techniques has a major impact on the outcome of the experiments (Gantoies et al., 2009). It appears that the pre enrichment method provided greater sensitivity than did the other method. This finding is in agreement with RK Gast (1990) who declared that direct plating provide a relatively rapid and inexpensive method for detecting SE in egg pools, although greater sensitivity is attainable with more intensive methods.

The use of different isolation techniques has a major impact on the outcome of the Experiments (Gantoies et al, 2000). As it is shown in tables 4 and 5, SS agar was more sensitive than BG agar in detecting of *Salmonella* colonies. This result is in agreement with those reported by Vera Lúcia et All (2005) who detected *Salmonella* by SS agar 27.6% and BGA 13.8% of positive samples. Vera Lúcia

declared that his findings were similar to those reported by Rhodes and Quesnel (1986) and Moringo et al. (1989).

PCR was more sensitive than cultural methods and in this study, by using this method, more *Salmonella* cells were detected and identified. This finding is in agreement with Burkhalter et al. (1995) who detected *Salmonella* spp. in eggs by using DNA analyses, culture techniques, and serology techniques. Monzur (2012) Detected *Salmonella* sp. and *Salmonella* Typhimurium in chicken Egg Samples by using Multiplex-PCR and cultural methods. This researcher reported that multiplex-PCR technique requires further optimization for getting higher response for detection of *Salmonella* sp. and *Salmonella* Typhimurium from egg samples.

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