Indian Journal of Animal Sciences **83** (5): 481–483, May 2013/Article

Detection and quantification of *Salmonella* on chicken egg shell by real time PCR

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Received: 25 April 2012; Accepted: 8 November 2012

ABSTRACT

Quantification of *Salmonella* on chicken eggs collected from selected poultry farms and marketing channels located in and around Bareilly was carried out using RT-PCR targeting *invA* gene of *Salmonella*. RT-PCR conditions were standardised optimizing reaction efficiency and R^2 value and the specificity of the desired amplified product was assessed using melting curve analysis which was found to be 82°C for *Salmonella*. Out of 200 chicken eggs, 8 (4%) were positive for *Salmonella*. Out of 100 eggs which were collected from poultry farms, 3 (3%) eggs were positive for the *Salmonella* while out of 100 eggs from marketing channels, 5 (5%) eggs were found positive for the *Salmonella*. The level of *Salmonella* cells per egg was higher (2.186×10^4 to 3.039×10^6 CFU/egg) in positive eggs collected from marketing channels than eggs collected from poultry farms (2.08×10^3 to 3.62×10^3 CFU/egg). The results indicated RT-PCR technique as a quick and sensitive method of quantification of *salmonella* on chicken egg shell surface.

Key words: Chicken eggs, *invA* gene, Real-time PCR (RT-PCR), *Salmonella*

Salmonella infection causes significant health and economic burden worldwide (Kubota *et al*. 2008). Food-associated salmonellosis is most often associated with consumption of undercooked beef, poultry meat and eggs (DuPont 2007). Among the serovars of *Salmonella*, S. Typhimurium and S. Enteritidis are of great concern from egg-borne human salmonellosis (Messens *et al*. 2007). The traditional methods used to detect *Salmonella* in food are laborious, time consuming and less sensitive. Rapid isolation and identification of *Salmonella* in food will increase the chances of preventing diseases caused by this pathogen. Various rapid methods were developed for this purpose. Real-time PCR technique is highly specific and sensitive. The most widely used gene to detect *Salmonella* spp. to date is *invA* (invasion A) (Malorny *et al*. 2003). The present study was designed for detection and quantification of *Salmonella* on chicken egg shell using SYBR green RT-PCR targeting the *invA* gene of *Salmonella*.

MATERIAL AND METHODS

Revival of standard strain of Salmonella

Standard strain of *Salmonella* Enteritidis was obtained from National *Salmonella* Center, IVRI, Izatnagar, revived and maintained at 4°C on nutrient agar slant to use throughout the experiment.

Standard curve setup

Standard *Salmonella* culture was used for standardization of real time PCR condition and standard curve preparation. For standard curve preparation, a loopful *Salmonella* culture was grown in 10 ml BHI at 37°C for 24 h, after which when turbidity was obtained, 100 µl was transferred into 50 ml BHI broth and incubated again at 37°C for 24 h. The grown culture was transferred into centrifuge tube which was then centrifuged at 6 000 rpm for 10 min. After centrifugation, supernatant was discarded and 2 to 3 washings were given with normal saline solution (NSS). After proper washing the pellet was dissolved in 4 ml NSS and optical density was measured at 540 nm. Subsequently 10-fold serial dilution was made up to 10^{10} from above dissolved pellet and 100 µl from 10^7, 10^8, 10^9, 10^{10} serially diluted standard bacterial culture spread on sterilized triptic soy agar (TSA) plate and then incubated at 37°C for 24 h. After 24 h colonies on TSA were counted.

Number of colonies of cells/ml=No. of colony × dilution factor × 10

Average number of colony which was obtained in 10^8 dilution = 1

Number of colonies or cells/ml = $1 \times 10^8 \times 10 = 10^9$

100µl DNA, which was extracted from 1 ml (standard sample from which serial dilution was made) on the day of

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Table 1. Primers sequence

Primers	Lengths	Primer sequence (5' to 3')	Reference	Amplification product (bp)
Forward primer	26	GTGAAATTATCGCCACGTTCGGGCAA	Galan *et al*. (1992)	284
Reverse primer	22	TCATCGCACCGTCAAAGGAACC		

spreading represents 10^9 bacterial cells.

So 100 µl DNA= 10^9 bacterial cells

1 µl DNA = 10^7 bacterial cells

Starting from10^7, 10-fold serial dilution up to 10^2 was made and triplicate reaction from each dilution was made for standard curve setup. Quantitative PCR was performed with PCR kit and real-time system operated by CFX manager software. A master mix of following components was prepared: 7 µl nuclease free water, 1 µl forward primer (0.25 µM), 1 µl reverse primer (0.25 µM) (Table 1) and 10 µl SYBR mix. The master mix (19 µl) was added to the strip tubes and 1 µl of template DNA (from each serially diluted tube) was added. Following RTPCR protocol was employed throughout the experiment. The PCR condition was as follows, initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min for 40 cycles and last cycle at 94°C for 1 min, at 65°C for 10 sec and gradual increment from 65°C to 95°C @ 1°C /10 sec and continuous fluorescent measurement and a final cooling down to 4°C. Amplification as well as dissociation curve for 10-fold serially diluted standard samples was obtained after the run ended. Standard curve data were obtained as stated in Table 2. By running a standard curve, efficiency of reaction as well as R^2 value was determined for detection and quantification of *Salmonella invA* gene assay prior to processing experimental samples.

Quantification of Salmonella *on chicken egg shell*

Collection of eggs: About 200 chicken eggs were collected (100 from selected layer farms located near Bareilly and 100 from marketing channels in and around Bareilly) for quantification of *Salmonella* on chicken egg shell surface. The eggs were brought aseptically to laboratory and processed immediately for detection and quantification of *Salmonella*.

Processing of eggs: For quantification of *Salmonella* from egg shell surface, a sterile cotton swab was wetted in sterilized normal saline solution and used for surface swabbing and it was re-immersed into the same tube having 2 ml NSS. After that 2ml NSS was transferred into centrifuge tube and centrifuged at 10 000 rpm for 5 min. The supernatant was discarded and the DNA was extracted with DNA extraction kit and extracted DNA was used as template for detection and quantification of *Salmonella* by RT-PCR. Similar protocol and conditions as stated in standard curve setup were followed. After the run ended, cycle threshold (Ct) values and melting curve for every unknown sample were obtained from real-time system. The specificity of desired products was documented using analysis of melting temperature, which is product specific and a high resolution gel electrophoresis to verify that amplified product. Log starting quantity for each Ct value of unknown sample was obtained by using the Ct values and log starting quantities of standard curve with prism 4.03 software. Then number of bacterial cells in 1µl was obtained by using log starting quantity of unknown sample with prism 4.03 software. Cells per egg (CFU/egg) were calculated by multiplying obtained bacterial cells with 10 as 1µl out of total 10 µl DNA was

Table 2. Standard curve data obtained from real time PCR machine

Sample name	Threshold cycle (Ct) value	Melting temperature (°C)	Log starting quantity
Standard 1	14.19	82	7
Standard 2	15.23	82	6
Standard 3	19.37	82	5
Standard 4	23.27	82	4
Standard 5	26.42	82	3
Standard 6	30.06	82	2

Table 3. Detection and quantification of *Salmonella* on chicken egg shell surface collected from layer farms

Sample name	Threshold cycle Ct value	Melting temperature (°C)	Obtained log starting quantity	Cells per egg (CFU/egg)
Unknown 1	27.96	82	2.559514	3.62×10^3
Unknown 2	28.08	82	2.526726	3.38×10^3
Unknown 3	28.85	82	2.31945	2.08×10^3

Table 4. Detection and quantification of *Salmonella* on chicken egg shell surface collected from marketing channel

Sample name	Threshold cycle Ct value	Melting temperature (°C)	Obtained log starting quantity	Cells per egg (CFU/egg)
Unknown 1	24.53	82	3.603402	4.012×10^4
Unknown 2	17.15	82	5.482833	3.039×10^6
Unknown 3	18.00	82	5.296076	1.977×10^6
Unknown 4	23.69	82	3.871	7.434×10^4
Unknown 5	25.34	82	3.339839	2.186×10^4

used as template. The number of *Salmonella* on chicken egg shell were then calculated and expressed as CFU/egg.

RESULTS AND DISCUSSION

Out of 200 chicken eggs, 8 (4%) eggs were found positive for *Salmonella* by RT-PCR technique. Out of 100 eggs which were collected from poultry farms, 3 (3%) eggs were positive for the *Salmonella* while out of 100 eggs from marketing channels, 5 (5%) eggs were found positive for the *Salmonella*. The number of *Salmonella* cells per egg ranged from 2.08×10^3 to 3.039×10^6 CFU/egg. The level of *Salmonella* cells per egg was higher in *Salmonella* positive eggs collected from marketing channels than eggs collected from poultry farms as shown in Tables 3 and 4.

In the mid 1980s eggs and egg containing foods were implicated as the vehicle of infection in nearly 80% of *Salmonella* Enteritidis outbreaks with a known food vehicle (Braden 2006). It has been estimated that of the approximately 477 billion consumed as shell eggs, 2.3 million are contaminated with *Salmonella* Enteritidis (Ebel and Schlosser 2000). In the present study, the Ct values generated from the *Salmonella* positive eggs using RT-PCR ranged from (17.15 to 28.85) as shown in Tables 3 and 4. The lower value of the Ct indicated the higher initial level of *Salmonella* on chicken egg shell surface (Tables 3 and 4). The results of melt curve analysis indicated that the amplicons in positive samples dissociated at temperature of 82°C indicating the specific amplification of *Salmonella invA* gene.

In another study carried out on chicken eggs collected from north Indian states by Yadav *et al*. (2008) indicated that the occurrence of *Salmonella* was 2.90% out of 275 eggs with lower occurrence in chicken eggs collected from market as compared to fresh farm eggs which is in agreement with present investigation. Similar occurrence level of 3.84% (out of 260 chicken eggs) was observed by Singh *et al*. (2010). Contrary to the occurrence detected in the present study, Bajaj *et al*. (2003) observed a higher occurrence level (10.8%) on egg shell surface than internal contents. Surveys conducted in England and Wales have also shown egg contamination level of *Salmonella* to be 0.2% to 1.6% (De Louvois 1994) which is lower than the level observed in the present study. The higher level of contamination in marketing channel eggs than farm eggs may be due to contamination of eggs while handling during transportation.

ACKNOWLEDGEMENT

The authors acknowledge Central Avian Research Institute (CARI) and Indian Veterinary Research Institute (IVRI) for carrying out the research work.

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