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Original Article

# Evaluation of Analytical Sensitivity of Sdf I based PCR and Sandwich ELISA for Salmonella Enteritidis detection and On-Farm prevalence in Punjab, Pakistan

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## ABSTRACT

*Salmonella* Enteritidis (SE) is a dominant serotype among non-typhoidal *Salmonella* which renders poultry products unsafe for human consumption. Due to frequent reporting of egg associated outbreaks, broiler breeder flocks are understudied although farm environment present supporting conditions for the growth of SE. In this study, two rapid detection techniques for SE were compared in terms of analytical sensitivity and the extent of SE contamination in broiler breeder farm environment was determined. Analytical sensitivity as limit of detection (LOD) was evaluated quantitatively for serotype specific PCR based on amplification of Sdf I gene and a commercially available sandwich ELISA for antigen detection. In triplicate experiments, tenfold serial dilutions of SE were prepared and tested with each technique. Using pure cultures, analytical sensitivity of PCR and ELISA were found to be 18.6 CFU/ml and $2.77\times10^5$ CFU/ml respectively. PCR (LOD, log 1.2) was found to be more sensitive and rapid than ELISA (LOD, log 5.4). Environmental swab samples (n = 260) were collected from 22 hen houses representing 8 broiler breeder farms located in and around Lahore and Sheikhupura districts of Punjab province. From each hen house swab samples were collected from litter, nests, feeders, drinkers, fans, pads, ceiling, walls and walkways. Following selective enrichment, pooled swab samples were subjected to PCR. Results showed that 36.3 % (8/22) hen houses were detected positive for SE. These findings suggest improvement in farm biosecurity measures and advocate implementation of integrated Salmonellosis control programs in broiler breeder houses to minimize carcass contamination.

## INTRODUCTION

*Salmonella enterica subsp. enterica serovar* Enteritidis (*Salmonella* Enteritidis) is a reemerging zoonotic pathogen which causes severe gastroenteritis in human beings, Chai *et al.* (2012); Woolhouse & Gowtage-Sequeria (2005). Human salmonellosis (food poisoning) is contracted mainly due to consumption of contaminated food derived from poultry origin especially layer eggs and broiler meat, Osimani *et al.* (2016). *Salmonella* Enteritidis (SE) is a non-host adapted serotype for avian species with an outcome of persistent subclinical infection in poultry birds, Guard-Petter (2001). *Salmonella* Enteritidis is transmitted between poultry flocks via both vertical (trans-ovarian) as well as horizontal channels, De Reu *et al.* (2006); Singh *et al.* (2010). Poultry houses provide suitable environmental conditions which contribute towards pathogen survivability, persistency of infection and resultant product contamination with SE, Omwandho & Kubota (2010). Survival and persistence of SE in poultry house environment even after thorough cleaning and disinfection procedures has been

reported, Luyckx *et al.* (2016). Zoonotic threats posed by SE can be effectively reduced by eliminating the SE environmental contamination at poultry production facilities, Trampel *et al.* (2014). Effectiveness of *Salmonella* Enteritidis control programs designed for poultry production facilities require monitoring the presence of this organism. Therefore, detection of SE requires a rapid but analytically sensitive technique.

A number of different conventional culture and rapid detection techniques are available for SE confirmation. For the detection of *Salmonella*, Polymerase Chain Reaction (PCR) has been found to be a very rapid and sensitive technique with high sample throughput as compared to conventional culture techniques which are laborious and time consuming, Langkabel *et al.* (2014). *Salmonella* difference fragment I (Sdf I) is a gene fragment exclusively found in *Salmonella* Enteritidis, Amplification of 304 bp fragment of Sdf I region by using primer set (ENTF, ENTR) is confirmatory for SE, Agron *et al.* (2001); Alvarez *et al.* (2004). Immunology based technique, Enzyme linked immunosorbent assay (ELISA) has also demonstrated to be an effective and rapid technique which allows the detection of injured viable but non-culturable bacteria as well, Maciorowski *et al.* (2006). Analytical sensitivity or limit of detection (LOD) is a primary parameter allowing the comparison of detection techniques based on assay's ability to detect the lowest concentration of analyte, Saah & Hoover (1997). Therefore, a cost effective and robust diagnostic technique coupled with high analytical sensitivity is desirable for effective monitoring of SE in poultry farm environment. In this study, analytical sensitivity of Sdf I based PCR and a commercially available sandwich ELISA (SAL 0096S, Solus *Salmonella* ELISA) for *Salmonella* Enteritidis detection has been evaluated.

In Pakistan, the poultry sector is considered a rapid growing industry which contributed 1.4% in national GDP and produced 1.39 million tons of poultry meat and 18 billion eggs, Ministry of Finance (2019). Intensive poultry farming requires regular monitoring of breeder poultry flocks for vertically transmitted salmonellosis. For this purpose, a mini scale on-farm surveillance of SE in broiler breeder houses has been conducted. Broiler breeder farms located in Lahore and Sheikhupura districts of Punjab province were selected for this study. Environmental swab samples were processed bacteriologically by pre-enrichment and selective enrichment. Hen house representative pooled samples were further tested by PCR to determine on-farm prevalence in selected districts.

## MATERIALS AND METHODS

### Collection and processing of environmental swab samples for breeder farm screening

The study area included eight different broiler breeder farms (A-H) located in and around two districts (Lahore and Sheikhupura) of Punjab province. These farms contained 22 hen houses, which were coded in an alphanumeric way. A total of 260 environmental swab samples were collected from 22 hen houses (Table 03). Samples were collected and initially processed by the technique described by Food and Drug Administration (2008) in agency's prescribed laboratory methods. Sterile swabs were moistened with evaporated skim milk and dragged over nine different hen house environmental surfaces consisting of: litter, nests, feeders, drinkers, fans, pads, ceiling, walls and walkways. For all hen houses, at least one swab sample was collected from each target surface. Each swab was packed in an individual whirl-Pak bag containing 15 ml sterile evaporated skim milk. Each swab sample was pre-enriched in 100 ml of Buffered Peptone Water (BWP) (Oxoid, CM 0509) and incubated at 35 ˚C for 24 h. Enrichment was made by inoculating 100 µl of incubated BWP in 10 ml of Rappaport-Vassiliadis (RV) broth (Solus Scientific, RVS001) which was incubated at 42˚C for 24 h. Incubated RV broth samples from each house were pooled together to form 22 representative samples. DNA was extracted from 1 ml of representative RV broth sample by using boiling method described by Croci *et al.* (2004). Representative samples were centrifuged at 12,000 rpm for 5 minutes. Supernatant was discarded and bacterial pellet was resuspended in 1 ml of Tris Borate EDTA (TBE) buffer. Centrifugation was performed at 12,000 rpm for 5 minutes. After discarding the supernatant, pellet was resuspended in 100 µl of TBE buffer. Samples were boiled at 100°C for 10 minutes and rapidly cooled on ice for 5 minutes. Supernatant (80 µl) was collected in a new micro-centrifuge tube, 2 µl supernatant was used for PCR as template DNA.

### Standardization of PCR for *Salmonella* Enteritidis detection

Polymerase Chain Reaction was standardized by using primer set ENTF (TGTGTTTTATCTGAT GCAAGAGG) and ENTR (TGAACTACGTTCGTTCTTCTGG) as reported by Alvarez *et al.* (2004). Genome DNA was prepared from reference strain; *Salmonella* Enteritidis (ATCC 13076) via Purelink® Genomic DNA Kit (Invitrogen, K182001). The genomic DNA was used as a template DNA for PCR

standardization and as a positive control for the result validation. Reaction mixture was prepared in a total volume of 25 µl by using Dream Taq Green2x PCR master mix (Thermo Scientific , K1081) as 12.5 µl, template DNA 2 µl, each primer 1 µl (10 pmol/µl) and nuclease free water as 8.5 µl. PCR was conducted in thermocycler (Esco, Swift mini) by programming initial denaturation at 95ºC for 10 minutes, 35 cycles comprising denaturation at 94ºC for 1 minute, annealing at 52ºC for 1 minute and extension at 72ºC for 1 minute with one final extension step at 72ºC for 10 minutes. The result was visualized by gel electrophoresis by using 1.3% agarose gel stained with ethidium bromide (0.5 µg/ml). Gel documentation system (AlphaImager EP) was used for image processing.

### Evaluation of Analytical Sensitivity of PCR

Analytical sensitivity was evaluated by preparing bacterial suspensions with known bacterial load as described by Paião *et al.* (2013). For this purpose, three different experiments were conducted. Each experiment was initiated by inoculating 10 ml of Rappaport Vassiliadis (RV) broth (Solus Scientific, RVS001) with 0.1 ml of *Salmonella* Enteritidis (ATCC 13076) culture, preserved in broth form. RV broth was incubated at 37 ºC for 22 h, 42 ºC for 24 h and 37ºC for 15 h in experiment 01, 02 and 03 respectively. Following incubation, 1ml of RV broth was used to make 10 fold serial dilutions in sterile phosphate buffered saline (PBS) as shown in table 01. From each dilution, an aliquot of 100 µl was spread on XLD agar plates (Oxoid, CM 0469). The plates were incubated at 37 ºC for 24 h and used to determine bacterial load as colony forming units (CFU) per ml in each respective dilution. Following thorough vortex mixing, 1ml aliquot from each dilution was transferred to a micro-centrifuge tube and processed for DNA extraction by using genomic DNA Kit (Invitrogen, K182001). Genomic DNA (2 µl) from each dilution was used for PCR.

### Evaluation of Analytical Sensitivity of Antigen Detection ELISA

In triplicate experiments, bacterial dilutions from the reference strain *Salmonella* Enteritidis (ATCC 13076) with known bacterial concentrations were prepared and analyzed by using sandwich ELISA kit (SAL 0096S, Solus *Salmonella* ELISA) for the detection of *Salmonella* Enteritidis antigen (Table 01). For each experiment, an aliquot of 1 ml from each dilution with known bacterial concentration was processed by heating at 100˚C for 18 minutes followed by cooling to 25 ºC. ELISA was performed by following manufacturer's instructions. Optical density (O.D) values were determined at a wavelength of 450 nm by using microplate photometer (Thermo Scientific, Multiskan® EX).

## RESULTS

### Analytical Sensitivity of PCR

For each of the triplicate experiments, 10 fold serial dilutions of reference SE strain varied in bacterial quantity which was confirmed by plate count (Table 01). For each standardized PCR, 1ml of dilution was processed for DNA extraction and 2 µl of extracted DNA was used per reaction. Bacterial concentrations were gradually decreased in tested dilutions and gradually decreasing band intensities were noted as shown in fig 1 & 2. The highest dilution detected positive in experiments I, II and III contained 17.2, 14.6 and 24.2 CFU/ml bacterial load. In experiments I and II, the dilutions containing <10 CFU/ml were negative for DNA amplification. In PCR Experiment III, the dilutions tested ranged from $2.42\times10^{10}$ to 24.2 CFU/ml, while the highest dilution in this experiment, 10-10 (24.2 CFU/ml) was found to be positive. It was observed that the exact numerical value for LOD was dependent upon the CFU/ml of the original bacterial suspension from which the 10-fold serial dilution was prepared. Therefore, the mean value of LOD 18.6 CFU/ml derived from LOD values of 17.2, 14.6 and 24.2 CFU/ml found in triplicate experiments.

### Analytical Sensitivity of Sandwich ELISA

SE dilutions tested via ELISA are described in table 01. Dilutions with $O.D_{450}\geq 0.200$ were considered positive. Table 02 represents the optical density values recorded for each dilution. LOD values in these experiments were found to be $2.70 \times 10^5$, $2.40 \times 10^5$ and $3.22 \times 10^5$ CFU/ml respectively. Therefore, mean LOD for ELISA was found to be $2.77 \times 10^5$ CFU/ml.

### On farm screening

Prior to PCR, initial bacteriological processing including pre-enrichment and selective enrichment was performed to enhance the detection sensitivity and to dilute possible PCR inhibitors present of environmental swab samples. Alphanumeric coding of hen houses is described in table 03. Out of all the tested pooled samples (n= 22), 08 samples were recorded positive (Fig 3 & 4). Therefore, 36.3 % houses were found contaminated with *Salmonella* Enteritidis.

**Table 1 –** *Salmonella* Enteritidis dilutions used in Analytical Sensitivity experiments.

| Dilution No. | PCR Experiments (Bacterial load as CFU/ml) | | | ELISA Experiments (Bacterial load as CFU/ml) | | |
|---|---|---|---|---|---|---|
| | I | II | III | I | II | III |
| $10^{-1}$ | $1.72 \times 10^{8}$ | $1.46 \times 10^{4}$ | $2.42 \times 10^{10}$ | $2.70 \times 10^{7}$ | $2.4 \times 10^{7}$ | $3.22 \times 10^{7}$ |
| $10^{-2}$ | $1.72 \times 10^{7}$ | $1.46 \times 10^{3}$ | $2.42 \times 10^{9}$ | $2.70 \times 10^{6}$ | $2.4 \times 10^{6}$ | $3.22 \times 10^{6}$ |
| $10^{-3}$ | $1.72 \times 10^{6}$ | $1.46 \times 10^{2}$ | $2.42 \times 10^{8}$ | $2.70 \times 10^{5}$ | $2.4 \times 10^{5}$ | $3.22 \times 10^{5}$ |
| $10^{-4}$ | $1.72 \times 10^{5}$ | $1.46 \times 10^{1} = 14.6$ | $2.42 \times 10^{7}$ | $2.70 \times 10^{4}$ | $2.4 \times 10^{4}$ | $3.22 \times 10^{4}$ |
| $10^{-5}$ | $1.72 \times 10^{4}$ | 1.46 | $2.42 \times 10^{6}$ | $2.70 \times 10^{3}$ | $2.4 \times 10^{3}$ | $3.22 \times 10^{3}$ |
| $10^{-6}$ | $1.72 \times 10^{3}$ | 0.146 | $2.42 \times 10^{5}$ | $2.70 \times 10^{2}$ | $2.4 \times 10^{2}$ | $3.22 \times 10^{2} = 322$ |
| $10^{-7}$ | $1.72 \times 10^{2}$ | 0 | $2.42 \times 10^{4}$ | $2.70 \times 10^{1} = 27$ | $2.4 \times 10^{1} = 24$ | $3.22 \times 10^{1} = 32.2$ |
| $10^{-8}$ | $1.72 \times 10^{1} = 17.2$ | 0 | $2.42 \times 10^{3}$ | $2.70 \times 10^{0} = 2.7$ | $2.4 \times 10^{0} = 2.4$ | $3.22 \times 10^{7} = 3.22$ |
| $10^{-9}$ | 1.72 | 0 | $2.42 \times 10^{2}$ | 0.27 | $2.4 \times 10^{7} = 0.24$ | $3.22 \times 10^{7} = 0.322$ |
| $10^{-10}$ | 0.172 | 0 | $2.42 \times 10^{1} = 24.2$ | 0.027 | $2.4 \times 10^{7} = 0.024$ | $3.22 \times 10^{7} = 0.0322$ |

Concentration of dilutions used in each experiment is represented as Colony forming unit (CFU) per milliliter. Highest dilutions detected positive for SE in PCR experiments I, II and III are $10^{-8}$, $10^{-4}$ and $10^{-10}$ respectively. In ELISA experiments highest dilution detected positive is $10^{-3}$consistently in all experiments.

**Table 2 –** ELISA plate optical density readings.

| Dilution No. | *$O.D_{450}$ Values | | |
|---|---|---|---|
| | Experiment I | Experiment II | Experiment II |
| $10^{-1}$ | 1.558 | 1.441 | 1.651 |
| $10^{-2}$ | 1.692 | 1.329 | 1.487 |
| $10^{-3}$ | 0.298 | 0.266 | 0.271 |
| $10^{-4}$ | 0.079 | 0.084 | 0.188 |
| $10^{-5}$ | 0.088 | 0.100 | 0.121 |
| $10^{-6}$ | 0.188 | 0.087 | 0.098 |
| $10^{-7}$ | 0.101 | 0.133 | 0.117 |
| $10^{-8}$ | 0.103 | 0.106 | 0.081 |
| $10^{-9}$ | 0.105 | 0.091 | 0.091 |
| $10^{-10}$ | 0.015 | 0.082 | 0.071 |

*Optical density values at 450 nm wavelength. Positive sample threshold = $O.D_{450} \geq 0.200$).

**Table 3 –** Summary of On-farm screening of SE in broiler breeder farms.

| Serial # | Farm Code | House Codes | Total Houses | *No. Of Positive Houses | No. of Samples processed |
|---|---|---|---|---|---|
| 1 | A | A1, A2 | 2 | 2 (A1, A2) | 22 |
| 2 | B | B1, B2, B3, B4 | 4 | 1 (B1) | 48 |
| 3 | C | C1, C2, C3 | 3 | 1 (C1) | 27 |
| 4 | D | D1, D2 | 2 | 0 | 13 |
| 5 | E | E1, E2 | 2 | 2 (E1, E2) | 34 |
| 6 | F | F1, F2 | 2 | 0 | 22 |
| 7 | G | G1, G2, G3, G4 | 4 | 0 | 64 |
| 8 | H | H1, H2, H3 | 3 | 2 (H2, H3) | 30 |
| Total Samples | | | | | 260 |

*PCR result shown in fig. 3 & fig. 4.

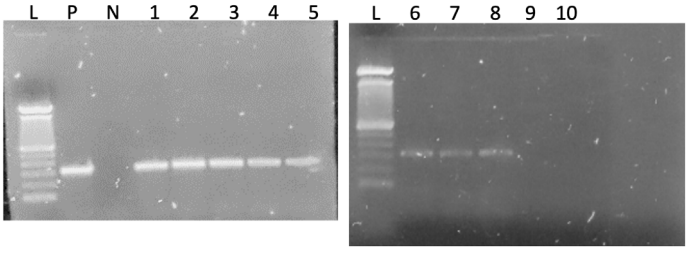


**Figure 1** – PCR Analytical Sensitivity, Experiment I.

Amplification shows 304 bp PCR product. (SdfI gene specific for SE)

L: Ladder, P: Positive Control, N: Negative Control, Lane 1-10 contain test dilutions containing $1.72 \times 10^{8}$ to 0.172 CFU/ml. Lane 8 contains 17.2 CFU/ml (Highest dilution detected positive).

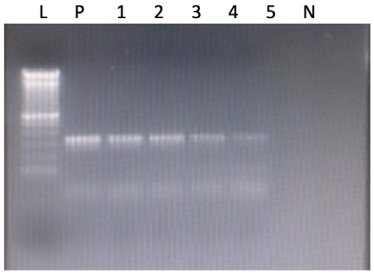


**Figure 2** – PCR Analytical Sensitivity, Experiment II.

L: Ladder, P: Positive Control, N: Negative Control, Lane 1-5 contain test dilutions containing $1.46 \times 10^{4}$ to 1.46 CFU/ml. Lane 4 contains 14.6 CFU/ml (Highest dilution detected positive).

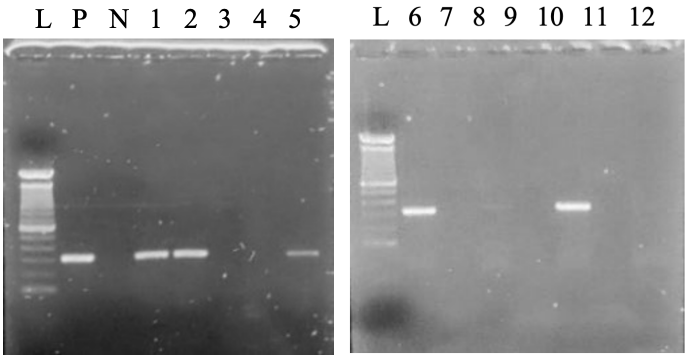


**Figure 3** – On-farm SE Detection I.

L: Ladder, P: Positive Control, N: Negative Control, Lane 1-12 contain representative sample from hen houses 1:A1, 2:A2,3:D1, 4:D2, 5:E1, 6:B1, 7:B2, 8:B3, 9:B4, 10:C1, 11:C2, 12:C3.

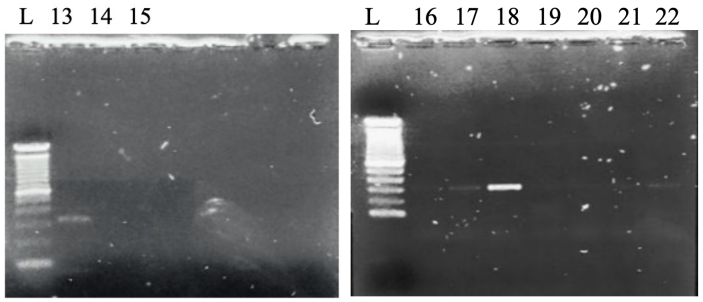


**Figure 4** – On-farm SE Detection II.

L: Ladder, Lane 13-22 contain representative sample from hen houses 13:E2, 14:F1, 15:F2, 16:H1, 17:H2, 18:H3, 19:G1, 20:G2, 21:G3, 22:G4.

## DISCUSSION

*Salmonella* Enteritidis has emerged as a lead cause of human Salmonellosis associated with the consumption of contaminated poultry eggs and meat products, Foley *et al.* (2011). Conventional culture techniques for isolation of *Salmonella* require minimally 4 to 15 days in order to declare a sample as negative or confirmed positive, Gallegos-Robles, *et al.* (2009). Due to this reason rapid detection techniques such as PCR and ELISA are required for devising effective outbreak response and infection control strategy. *Salmonella* difference fragment I (Sdf I) is a chromosome origin gene related to invasiveness of SE in poultry. The amplification of Sdf I gene fragment is confirmatory for SE, Agron *et al.* (2001); Batista *et al.* (2013). In this study, for Sdf I specific primer pair (ENTF and ENTR) we have described PCR optimization at a relatively low annealing temperature (52°C) by using a commercially available master mix, while in previous studies, higher annealing temperature (57°C) was reported where multiplex PCR was developed for epidemiological typing of human stool samples, Alvarez *et al.* (2004) and for poultry meat samples, de Freitas *et al.* (2010). Low annealing temperature enhances PCR sensitivity and improves amplification output, Shen *et al.* (2007). PCR efficiency can be boosted up by decreasing PCR bias which in turn reduces by lowering the primer annealing temperature, Ishii & Fukui (2001). Thus, the Optimization protocol described allows high sensitivity while maintaining specificity. PCR has been demonstrated to be an effective tool for SE confirmation in poultry, food and environment origin specimens. It is a rapid, sensitive and relatively economical technique which provides an additional benefit of detecting non-viable cells as well, Khan *et al.* (2007). Analytical sensitivity is the lowest amount of a substance measured precisely in a sample, Armbruster & Pry (2008). In the present study, we evaluated analytical sensitivity of PCR designed for Sdf Igene. In all three experiments varying incubation parameters were used to ensure the variety of bacterial count ranges. Due to variation in bacterial counts of tested dilutions, the limit of detection was not constant but it was found to be > 10 CFU/ml. An average analytical sensitivity of 18.6 CFU/ml (log 1.26 CFU) was recorded in these experiments. For Sdf Igene, this is the first report of detection limit in pure culture of *Salmonella* Enteritidis. PCR analytical sensitivity is a multifactorial attribute depending upon, but not limited to target gene, primer specificity, sample preparation technique, DNA extraction methodology, sample matrix and PCR inhibitory substances, Aznar & Alarcón (2003). The detection limit of 1 CFU/ml by amplification of a 488 bp fragment of Prot6e gene specific to SE was reported, Li *et al.* (2017). Oliveira *et al.* (2002) found significant variation in PCR analytical sensitivity as 8 CFU and 1.2 × 103 CFU/ml by targeting invA and sefA genes respectively. The analytical sensitivity was reported as 1.2 × 102 CFU/ml based on spv gene (Lampel *et al.* 1996), 102 CFU/ml based on IE 1 gene (Paião *et al.* 2013) and <103 CFU/ml based on sefA gene, De Medici *et al.* (2003).

Enzyme linked immunosorbent assay (ELISA) is a serological technique which can be employed for rapid detection of SE. In this study, we have evaluated the analytical sensitivity of a commercially available ELISA kit (SAL 0096S, Solus *Salmonella* ELISA) to determine the limit of detection for SE. Average limit of detection was found to be $2.77\times 10^5$ CFU/ml (log 5.44 CFU). This finding is in agreement with the manufacturer's claim of LOD range $10^5$-$10^6$ CFU/ml in enrichment broth. The analytical sensitivity of antigen detection ELISA varies with the nature of the antigen. Brooks *et al.* (2012) also reported development of an antigen capture monoclonal antibody based ELISA assay for detection of lipopolysaccharide O-antigen of SE, where LOD

was found to be $5\times10^5$ - $5\times10^6$ CFU/ml. ELISA based on recombinant flagellin of SE was developed and reported to have LOD value as $10^3$ CFU/ml, Mirhosseini *et al.* (2017).

Analytical sensitivity of a non-conventional ELISA, using bacteriophages as an alternative to capture antibody for the detection of intact *Salmonella* enterica was found to be $10^6$ CFU/ml, Galikowska *et al.* (2011). However, gold nanoparticles labelled modified sandwich assay format was more sensitive and allowed the detection of $10^3$ CFU/ml, Wu *et al.* (2014). The complexity of the sample matrix reduces the analytical sensitivity of ELISA, as Wang *et al.* (2015) found ten fold decrease in analytical sensitivity of ELISA when the sample medium was tween phosphate buffer saline ($10^4$ CFU/ml) as compared to milk ($10^5$ CFU/ml). In our findings, tween phosphate analytical sensitivity of SE detection for PCR (log 1.26 CFU/ml) was markedly higher than sandwich ELISA (log 5.44 CFU/ml), this is in agreement with the findings of Kumar *et al.* (2008). However, the variation in analytical sensitivity of ELISA is significantly lower than PCR.

Contaminated poultry products especially eggs and meat are implicated for most of the cases of human Salmonellosis. Hen house provides suitable environmental conditions for the survival and propagation of SE. Vertical transmission of this non-host adapted serotype, contributes towards the enhanced vulnerability of commercial broiler flocks through sub-clinically infected parent (breeder) poultry flocks, thus paving the way for human infection, Guard-Petter (2001). For the reduction of commercial broiler carcass contamination with SE at all levels of production, processing, marketing and surveillance of broiler breeder houses play a pivotal role. In this study, we have screened environmental swab samples taken from 8 different broiler breeder farms comprising 22 hen houses. The samples were tested by employing a sensitivity enhanced PCR technique, in which the swabs samples were initially processed via bacteriological technique comprising pre-enrichment in buffered peptone water followed by enrichment in selective broth (RV), and finally PCR was performed. Soumet *et al.* (1999) reported that RV-PCR coupled technique showed comparable results to bacteriological technique by using primer set (S1, S4). The initial bacteriological processing of the environmental samples enhances PCR sensitivity by reducing the inhibitory substances in specimen matrix, Hsu *et al.*(2011). In the present study, we have found 36.3 % of tested hen houses positive for SE contamination of at least 1 out of 9 targeted environmental surfaces. Similar on-farm prevalence of 39.6 %, Berghaus *et al.* (2011) and 38.8 %, Alali *et al.*(2010) were reported and attributed to the absence of any SE control program. While Li *et al.* (2017) found only 1 farm positive for SE in China when control programs were in place.

In conclusion, the analytical sensitivity of PCR has been found clearly higher than ELISA, and the sensitivity enhanced PCR assay can be used as an effective tool for screening hen house environment samples. Cleaning and disinfection alone are not effective at reducing SE contamination at farm level. An integrated farm management approach focusing biosecurity, vector control, feed control and improved chicken immunity is necessary at both breeder and commercial broiler production levels.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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