Molecular and Virulence Characterization of Recently Isolated *Salmonella* in Comparison with Standard Strains Used in Vaccine Evaluation

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**Abstract:** This study aimed to conduct molecular, phylogenetic and virulence characterization of recently isolated *Salmonella* Enteritidis strains in comparison with standard strains used in evaluation of live *S.* Enteritidis vaccines potency. A total of (75) fecal swab samples including 20 apparently healthy chickens and 55 diseased chicken were collected as representative from 20 broiler and layers chicken farms reporting 7 farms from Giza, 9 farms from Kalioubia and 4 farms from Sharkia governorates during the period of January 2016 to August 2016. According to morphological, biochemical and serotyping results, only 5 isolated *Salmonella* samples (5/75, 6.7%) were belonging to *S.* Enteritidis and were confirmed by molecular characterization where the PCR amplification of these *Salmonella* isolates produced a product of approximate molecular size 310 bp DNA fragments of the *Salmonella sefA* gene. Fimbrial *sefA* virulence gene was found in all 5 detected field isolates and also standard reference *S.* Enteritidis strain (100%) and can be used as a potential target for identification of *S.* Enteritidis. Salmonella enterotoxin virulence gene (*stn*) was detected in standard reference *S.* Enteritidis strain and field isolate No. (1) which produced 617 bp DNA fragment product. Phylogenetic analysis and homology of *stn* genes of *S.* Enteritidis field isolate No. (1) and standard reference strain at nucleotide level: The NCBI search of the *stn* gene showed maximum homology (98.7%) of *S.* Enteritidis field isolate No. (1) with standard reference strain and (100%) with *Salmonella enterica* subsp. Enteritidis strains EC20120200, EC20121177, SA19970769, SA19980677, SA19981522, SA19981857, SA19992322, SA20094352, SA20095440, EC20100325. *S.* Enteritidis field isolate No. (1) gave protection rate in vaccinated group (1) 84% and had the ability to produce mortality rate was 100% in control group with high pathogenic effect and typical homologous with standard reference strain gave (88% and 100%) protection rate and mortality rate respectively with high reliability and efficacy in evaluation and quality assurance of live *S.* Enteritidis vaccines.

**Key words:** *S.* Enteritidis · PCR · *sefA* gene · *stn* gene · Sequencing · Evaluation · Challenge test

**INTRODUCTION**

*Salmonella* infection is one of the most serious problems that affects poultry industry causing high economic losses not only due to high mortality in young chickens but also for the debilitating effect which predisposes for many other diseases. The genus *Salmonella* is of particular clinical relevance in both developed and developing countries, where this pathogen is one of the most common causes of food-borne illness and a major cause of diarrheal diseases and several outbreaks attributed to different *Salmonella* serovars are reported each year, highlighting the frequency of *S.* enterica Enteritidis among the most common causal agents [1-5]. *Salmonella enterica* serovar Typhimurium and *S.* enterica serovar Enteritidis are the most frequent isolated serovars worldwide [6]. In Egypt *S.* Enteritidis was isolated from broiler chicken, chicken meat and food poisoning patients. The clinical illness is characterized by fever, nausea and diarrhea,
vomiting and abdominal pain after an incubation period of 12 to 72 hrs [7]. *Salmonella* enterica subspecies serovar Enteritidis is the world leading cause of salmonellosis and is often implicated in over 60% of cases of human salmonellosis in Europe [8].

Traditionally, detection and quantification of salmonellae have been largely based on the use of selective culture and standard biochemical tests. This requires confirmatory test of all typical and atypical colonies and can be very cumbersome and time consuming [9]. The use of DNA based methods is becoming increasingly important in epidemiological survey and differentiation of *Salmonella* species. A lot of attention is being paid to a rapid detection of *Salmonella* using molecular techniques like polymerase chain reaction (PCR) as it is highly sensitive, specific and reproducible [10]. Several genes have been used to detect *Salmonella* in natural environmental samples as well as food and fecal samples. Virulence chromosomal genes—including *sefA* and *stn* are target genes for PCR amplification of *Salmonella* species [11]. *SefA* is one of nine genes set associated with the virulence in 100% of *S. Enteritidis* isolates [12]. The Salmonella enterotoxin (*stn*) gene encodes stn protein, causing acute gastroenteritis. It has shown an enterotoxic activity in a murine ileal loop model with symptoms that include nausea, vomiting, abdominal pain, fever and diarrhea [13-15]. *Stn* is an important virulent gene which code for *Salmonella* toxin, increases the level of C-AMP in the host and ultimately results into diarrhoea and vomiting [16]. Therefore, *stn* is a *Salmonella* virulence factor and is responsible for the enterotoxicity of *Salmonella*. It is possible that *stn* will play a pivotal role in special functions of *Salmonella*. To explore more information about *stn* protein in the present study *stn* gene of S. Enteritidis was cloned, sequenced and phylogenetic analysis was done by using bioinformatics approach. The general aim of this work was molecular, phylogenetic and virulence characterization of recently isolated *S. Enteritidis* in comparison with standard reference *S. Enteritidis* strain used in evaluation of live *S. Enteritidis* vaccines potency and specific aim was to achieve a clear vision in difference between these field recently isolated and standard strains to improve and studying the likelihood of presence or innovate newly field isolates used in vaccination-challenge tests for qualifications and evaluation of *Salmonella* vaccines in poultry.

**MATERIALS AND METHODS**

**Standard Salmonella Enteritidis Strain:** Was kindly obtained from Central Laboratory for Evaluation of Veterinary Biologics (CLEVB) and used in genotypic and virulence comparison with field local isolates and also in vaccination-challenge tests.

**Samples Collection:** A total of (75) fecal swab samples including 20 apparently healthy chickens and 55 diseased chicken were collected from 20 broiler and layers chicken farms representing 2 farms from Giza, 3 farms from Kaliouba and 2 farms from Sharkia governorates during the period of January 2016 to August 2016.

**Isolation of Salmonella Organism:** It was done according to ISO [17]. Fecal samples were weighed and suspended in Buffered peptone water as 1:10 dilution and then incubated at 37°C for 18 h. From the pre-enrichment culture, 0.1 ml of the broth was transferred to a tube containing 10 ml of the Rappaport Vassiliadis broth and then incubated at 41.5°C for 24 h. From the enrichment culture, 10 µL were inoculated onto the surface of Salmonella Shigella agar (SS agar) (Merck) and Xylose Lysine Deoxycholate (XLD, Oxoid), Hektoen Enteric (HE, Oxoid), separately, then incubated at 37°C for 18 h. From the pre-enrichment culture, 0.1 ml of the broth was transferred to a tube containing 10 ml of the Rappaport Vassiliadis broth and then incubated at 41.5°C for 24 h. From the enrichment culture, 10 µL were inoculated onto the surface of Salmonella Shigella agar (SS agar) (Merck) and Xylose Lysine Deoxycholate (XLD, Oxoid), Hektoen Enteric (HE, Oxoid), separately, then incubated at 37°C for 24 h. The plates were then checked for the growth of typical *Salmonella* colonies. The purified cultures were subjected to further investigation as Gram staining and motility test.

**Identification of Salmonella Isolates**

**Microscopical Examination:** Suspected colonies were Gram stained according to Quinn *et al.* [18] and examined microscopically.

**Biochemical Identification:** Biochemical system was applied on isolated organisms according to The Analytical Profile Index 20E (API 20E) catalogue [19, 20]. Serological Identification: Typical Salmonella isolates were serologically identified by slide agglutination tests using somatic and flagellar reference *Salmonella* antisera according to Kauffmann-White serotyping Scheme for determination of somatic (O) and flagellar (H) antigens [21].

**Molecular Identification:** All the isolates and standard reference *S. Enteritidis* strain were examined by PCR for the presence of *S. Enteritidis* fimbrial (*sefA*) virulence
Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers sequences 5'--3'</th>
<th>Amplified segment (bp)</th>
<th>Amplification (35 cycles)</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>sef gene</td>
<td>GCAGCGGTATATATGCGACG</td>
<td>310</td>
<td>94°C 94°C 52°C 72°C 72°C</td>
<td>7 min.</td>
</tr>
<tr>
<td></td>
<td>TGTGACAGGGACATTACCGG</td>
<td></td>
<td>94°C 94°C 59°C 72°C 72°C</td>
<td></td>
</tr>
<tr>
<td>Stn gene</td>
<td>TTG TGT CGC TAT CAC TGG CAA CC ATT CGT AAC CCG CTC TCG TCC</td>
<td>617</td>
<td>5 min. 30 sec. 40 sec. 45 sec. 10 min.</td>
<td></td>
</tr>
</tbody>
</table>

associated gene then detected the presence of Salmonella enterotoxin encoding gene (stn) in standard S. Enteritidis strain and field isolate No. (1). The primers sequences and PCR product sizes are shown in (Table 1).

**DNA Extraction:** DNA extraction was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer’s recommendations. Briefly, 200 μl of the sample suspension was incubated with 10 μl of proteinase K and 200 μl of lysis buffer at 56°C for 10 min. After incubation, 200 μl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer’s recommendations. Nucleic acid was eluted with 100 μl of elution buffer.

**Oligonucleotide Primer:** Primers used were supplied from Metabion (Germany) are listed in (Table 1).

**PCR Design and Amplification:** Primers were utilized in a 25-μl reaction containing 12.5 μl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 μl of each primer of 20 pmol concentration, 4.5 μl of water and 6 μl of DNA template. The reaction was performed in an Appliedbiosystem 2720 thermal cycler.

**Analysis of the PCR Products:** The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 μl of the products was loaded in each gel slot. A 100 bp and 100 bp plus DNA Ladders (Qiagen, Germany, GmbH) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

**Sequences Similarity and Phylogenetic Analysis:** It was done in Elimbiopharmaceuticals, Germany. PCR products were purified using QIAquick PCR Product extraction kit (Qiagen, Valencia). Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used for the sequence reaction and then it was purified using Centrisep spin column. DNA sequences were obtained by Applied Biosystems 3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis (Basic Local Alignment Search Tool) [22] was initially performed to establish sequence identity to Gen Bank accessions. The phylogenetic tree was created by the MegAlign module of Laser gene DNA Star [23] and Phylogenetic analyses was done using maximum likelihood, neighbour joining and maximum parsimony in MEGA6 [24].

**Vaccination and Challenge Test:**

**Vaccine:** Live freeze dried Salmonella Enteritidis vaccine.

**Standard Salmonella Strain:** Standard strain of Salmonella Enteritisidis (2.7 x 10^6 CFU/ml) was kindly supplied by Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Abassia, Cairo. This strain was used for challenge of vaccinated chickens.

**Experimental Vaccination Design:** A total number of 450, one day old SPF chicks were divided into 6 vaccinated groups and 6 control groups as following: Groups (from 1 to 6): 300, one day old SPF chicks were divided into 6 groups as 50 chicks in each group, all 6 groups were vaccinated with living attenuated Salmonella Enteritidis vaccine orally with 0.1 ml containing 6 x 10^4 CFU per bird after 36 hours from hatching then boosted after 2 weeks orally with the same dose. Control groups from (7 to 12): 150, one day old SPF chicks were divided into 6 groups as 25 chicks in each group unvaccinated left as control.

**Challenge Test:** Vaccinated groups (From 1 to 5) and control groups from (7 to 11) were challenged at 6 weeks of age S/C injected with 0.1 ml of broth culture of each of 5 virulent field local isolates (2.7 x 10^6 CFU/ml), while group (6) and control group (12) were challenged at 6...
weeks of age S/C injected with 0.1 ml of broth culture of virulent standard S. Enteritidis strain containing \(2.7 \times 10^9\) CFU/ml.

**RESULTS**

**Bacterial Isolation:** Out of a total number of 75 fecal swab samples, five locally fields isolates were detected and confirmed phenotypically by culturing, Gram staining, biochemical and serologically to be S. Enteritidis in prevalence of (6.7%).

**Biochemical Identification:** Using Analytical profile index 20E (API 20E) isolated strains revealed that all were identified as Salmonella species as shown in Table (2).

**Serological Identification:** The Serotyping of the 5 isolates revealed that they were identified as S. Enteritidis as antigenic formula as somatic (O) antigen: 1, 9, 12 and flagellar (H) antigen: phase 1(g, m) & phase 2 (1, 7).

**Molecular Identification:** The PCR amplification with sefA specific primers was conducted with genomic DNA, which resulted in a product of approximate size 310bp. Fimbrial sefA virulence gene was found in all 5 detected field isolates and also standard S. Enteritidis strain (100%) (Table 3) and (Fig. 1). As well as the presence of Salmonella enterotoxin virulence gene (stn) in standard S. Enteritidis strain and field isolate (No. 1) which produced 617 bp DNA fragment product (Table 3) and (Fig. 2).

Table 2: Biochemical identification of salmonellae using API 20E system

<table>
<thead>
<tr>
<th>Salmonellae</th>
<th>Biochemical reactions</th>
<th>ONPG</th>
<th>ADH</th>
<th>LDC</th>
<th>ODC</th>
<th>CIT</th>
<th>H2S</th>
<th>URE</th>
<th>TDA</th>
<th>VP</th>
<th>GEL</th>
<th>GLU</th>
<th>MAN</th>
<th>INO</th>
<th>SOR</th>
<th>RHA</th>
<th>SAC</th>
<th>MAL</th>
<th>AMY</th>
<th>ARA</th>
<th>OXY</th>
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</thead>
<tbody>
<tr>
<td>API 20E</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>


| Field local isolate No. 1 | + | + | + |
| Field local isolate No. 2 | + | + |
| Field local isolate No. 3 | + | + |
| Field local isolate No. 4 | + | + |
| Field local isolate No. 5 | + | + |
| Standard reference strain | + | + |

Table 3: Results of all field local isolate strains (From No. 1 to 5) and Standard Reference S. Enteritidis strain

<table>
<thead>
<tr>
<th>Samples</th>
<th>S. Enteritidis</th>
<th>sefA</th>
<th>Stn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field local isolate No. 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Field local isolate No. 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Field local isolate No. 3</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Field local isolate No. 4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Field local isolate No. 5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Standard reference strain</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Fig. 1: Agarose gel showing polymerase chain reaction amplified products of sefA gene (310bp) for Salmonella Enteritidis, lanes 1- 5: Analyzed samples of Salmonella Enteritidis showing positive 310 bp DNA fragment of sefA fimbrial virulence gene, Lane Standard Strain: standard Salmonella Enteritidis strain, lane (pos.): positive control, lane (Neg.): Negative control, lane (L) Molecular weight 100 bp ladder (DNA marker).
Fig. 2: Agarose gel showing PCR amplified product of 617 bp of enterotoxin virulence (stn) gene for *Salmonella Enteritidis*, lane (1): sample positive for *stn* gene, lane (Standard strain): Standard *Salmonella Enteritidis* strain, lane (pos.): positive control, lane (Neg.): Negative control, Lane (L): 100bp ladder (DNA marker).

Table 4: Protection percentage in vaccinated chicken groups after challenge with 5 field local isolates and standard reference *S. Enteritidis* strain

<table>
<thead>
<tr>
<th>Birds</th>
<th>Total No. of birds</th>
<th>1st week</th>
<th>2nd week</th>
<th>3rd week</th>
<th>4th week</th>
<th>No. of dead/Total No.</th>
<th>Mortality rate</th>
<th>Protection %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gp 1</td>
<td>50</td>
<td>3</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>8/50</td>
<td>16%</td>
<td>84%</td>
</tr>
<tr>
<td>GP7</td>
<td>25</td>
<td>13</td>
<td>9</td>
<td>3</td>
<td>0</td>
<td>25/25</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>Gp 2</td>
<td>50</td>
<td>4</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>10/50</td>
<td>20%</td>
<td>80%</td>
</tr>
<tr>
<td>GP8</td>
<td>25</td>
<td>10</td>
<td>7</td>
<td>2</td>
<td>0</td>
<td>19/25</td>
<td>76%</td>
<td>24%</td>
</tr>
<tr>
<td>Gp 3</td>
<td>50</td>
<td>4</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>9/50</td>
<td>18%</td>
<td>82%</td>
</tr>
<tr>
<td>GP9</td>
<td>25</td>
<td>10</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>18/25</td>
<td>72%</td>
<td>28%</td>
</tr>
<tr>
<td>Gp 4</td>
<td>50</td>
<td>6</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>14/50</td>
<td>28%</td>
<td>72%</td>
</tr>
<tr>
<td>GP10</td>
<td>25</td>
<td>9</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>17/25</td>
<td>68%</td>
<td>32%</td>
</tr>
<tr>
<td>GP5</td>
<td>50</td>
<td>4</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>12/50</td>
<td>24%</td>
<td>76%</td>
</tr>
<tr>
<td>GP11</td>
<td>25</td>
<td>11</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>19/25</td>
<td>76%</td>
<td>24%</td>
</tr>
<tr>
<td>Gp 6</td>
<td>50</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>6/50</td>
<td>12%</td>
<td>88%</td>
</tr>
<tr>
<td>GP12</td>
<td>25</td>
<td>12</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>25/25</td>
<td>100%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Group 1: vacc. with living attenuated *S. Enteritidis vaccine* and challenged with local field *S. Enteritidis* strain No. (1).
Group 2: vacc. with living attenuated *S. Enteritidis vaccine* and challenged with local field *S. Enteritidis* strain No. (2)
Group 3: vacc. with living attenuated *S. Enteritidis vaccine* and challenged with local field *S. Enteritidis* strain No. (3)
Group 4: vacc. with living attenuated *S. Enteritidis vaccine* and challenged with local field *S. Enteritidis* strain No. (4)
Group 5: vacc. with living attenuated *S. Enteritidis vaccine* and challenged with local field *S. Enteritidis* strain No. (5)
Group 6: vacc. with living attenuated *S. Enteritidis vaccine* and challenged with virulent standard *S. Enteritidis* strain
Control groups from (7) to (12): unvaccinated control groups and challenged with local field *S. Enteritidis* strains from No. (1) to (5) and virulent standard *S. Enteritidis* strain, respectively.

**Phylogenetic Analysis and Homology of Stn Genes of S. Enteritidis Field Isolate No. (1) and Standard Reference Strain at Nucleotide Level:** The NCBI search of the *stn* gene showed maximum homology (98.7%) of *S. Enteritidis* field isolate No. (1) with standard reference strain and (100%) with *Salmonella enterica* subsp. *Enterica* serovar *Enteritidis* strains EC20120200, EC20121177, SA19970769, SA19980677, SA19981522, SA19981857, SA19992322, SA20094352, SA20095440, EC201003325, as detected in Figs. (3 and 4).
Fig. 3: Dendrogram for Phylogenetic analysis at nucleotide level of stn gene marked with red circle of *Salmonella* field isolate No. (1) and *Salmonella* reference strain with other closely related sequences.

Fig. 4: Phylogenetic analysis and Homology of (stn) enterotoxin gene of *Salmonella* field isolate No. (1), Standard Reference *Salmonella* strain and different serotypes of salmonellae

**Vaccination and Challenge Test:** Table (4) showed that the isolate No. (1) gave protection rate in vaccinated group (1) 84% and mortality rate was 100% in control group (7) while the isolate No. (2) gave (80% and 76%), the isolate No. (3) gave (82% and 72%), the isolate No.(4) gave (72 and 68%), the isolate No. (5) gave (76% and 76%) and standard Reference *Salmonella Enteritidis* strain gave (88% and 100%) protection rate and mortality rate respectively.


**DISCUSSION**

Regarding to bacterial isolation, out of a total number of 75 fecal swab samples, five locally fields isolates (6.7%) were detected. This finding agrees with Nawar and Khedr [25]. Regarding to molecular identification by PCR, Fimbrial *Salmonella* virulence gene (*sefA*) was present in all salmonellae (100%). The high frequency of *sefA* is consistent with previous findings [26-29] and it can be considered a target gene to identify the serovar *S. Enteritidis* by PCR [28].

*Stn* is an important virulent gene which code for Salmonella toxin, increases the level of c-AMP in the host and ultimately results into diarrhea and vomiting. The *Salmonella* enterotoxin encoding gene (*stn*) was detected in standard reference *S. Enteritidis* strain and field isolate No. (1) by using PCR. It has been shown that the *stn* gene is specifically distributed only in *Salmonella* spp. irrespective of their serotypes [14, 30]. Presence of *stn* gene in all isolates shows that they are the specific targets for *Salmonella* identification and are capable of producing gastroenteric illness to humans where *S. Enteritidis* serovars can be able to produce systemic infection [26, 31]. Regarding to phylogetic analysis and homology of *stn* genes of *S. Enteritidis* field isolate No. (1) and standard reference strain at nucleotide level: revealed that *S. Enteritidis* field isolate No. (1) exhibited higher degree of homogeneity (98.7%) with *S. Enteritidis* standard reference strain and (100%) with *Salmonella* enterica subsp. Enterica serovar *Enteritidis* strains EC20120200, EC20121177, SA19970769, SA19980677, SA19981522, SA19981857, SA19992322, SA20094352, SA20095440, EC20100325 and it *S. Enteritidis* field isolate No. (1) had ability to produce mortalities 100% of SPF control group with high pathogenic effect with typical homologous with standard reference strain with high reliability and efficacy in evaluation and quality assurance of live *S. Enteretidis* vaccines.

**CONCLUSIONS**

It can be considered that *sefA* virulence gene is a target gene to identify the serovar *S. Enteritidis* by PCR. At the nucleotide of *stn* sequence level of *S. Enteritis* field isolate No. (1) exhibited higher degree of homogeneity (98.7%) with *S. Enteritidis* standard reference strain and (100%) with *Salmonella* enterica subsp. Enterica serovar *Enteritidis* strains EC20120200, EC20121177, SA19970769, SA19980677, SA19981522, SA19981857, SA19992322, SA20094352, SA20095440, EC20100325 and it *S. Enteritidis* field isolate No. (1) had ability to produce mortalities 100% of SPF control group with high pathogenic effect with typical homologous with standard reference strain with high reliability and efficacy in evaluation and quality assurance of live *S. Enteretidis* vaccines.

**REFERENCES**


