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*Original Research Article*

# An Alternative Method to Evaluate the Virulence of *Salmonella* Enteritidis Challenge Strain using Embryonated Chicken Eggs

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**Abstract**

**The chicken pathogenicity test is an old method to determine the bacterial virulence. In the present study, ELA (Embryo Lethality Assay) was designed as an alternative, rapid and easy model to evaluate the S. Enteritidis virulence. Previously confirmed virulent and avirulent strains, using the traditional chicken pathogenicity method, were used to apply the ELA. Based on $ELD_{50}$ (50% embryo lethal dose) calculation, virulent strain has 3.6 $Log_{10}$ $ELD_{50}$ but the avirulent strain has 6 $Log_{10}$ $ELD_{50}$. Strain virulence was determined according to bacterial count in the allantoic cavity (AC) fluid and rate of liver invasion score. The virulent strain count in the allantoic fluid was declined and maintained to $10^7$ CFU/ml on the 5th post inoculation, but the avirulent strain declined to $10^6$ CFU/ml. The bacterial load of embryonic liver of virulent strain was about 10,000 times more than the avirulent one on the 5th post inoculation. In conclusion, these results recommend the application of embryonated chicken eggs assay as an alternative method to evaluate the virulence of *Salmonella* Enteritidis challenge strain.**

**Keywords:** Alternative, ELA, Pathogenicity, *Salmonella* Enteritidis, Virulence

## INTRODUCTION

*Salmonella enteritis*is a well-known food-borne bacterial pathogen which causes annual deaths of 715,000 birds due to diarrhea as mentioned by WHO (World Health Organization) (Besser, 2018). *Salmonellaenteritis* spreads from chicken to human (Knodler and Elfenbein, 2019). The virulence includes intestinal bacterial invasion survival and transmission. Among various SPIs, the role in virulence is well proven for SPI1 and SPI2 and further insight into the complex regulatory network of SPIs can contribute to drug investigation and prevention of infection. Mainly SPI1 and SPI2 are included in salmonella virulence (Sarika and Navneet 2021).

To evaluate salmonella vaccines, chicken models are used for determination of effectiveness of the used vaccines. The chicken pathogenicity test is used as a most common infection model. Recently, animal experimental models were recommended to be avoided for some ethical considerations. Different international laws tried to control any unethical animal uses to maximize the animals' welfare (Doke and Dhawale 2015). Even in case of using animals at the last evaluation step, it is important to find an alternative protocol within the evaluation steps. Additionally, the price of experimental animal is high and requires special facilities and specially trained work team (Jacobsen et al., 2010). To overcome the above mentioned disadvantages and avoiding the unethical methods, embryo lethality assay (ELA) was applied as an alternative procedure (Doke and Dhawale, 2015).

The chicken embryo lethality assay (ELA) could be

applied as an alternative assay for studying the bacterial virulence of different bacteria such as *Escherichia coli*, *Enterococcus, Staphylococcus*, *Yersinia*, *Campylobacter*, *Riemerella*, *Listeria*, *and Clostridium* (Wooley et al., 2000; Nix et al., 2006; Townsend et al., 2008; Polakowska et al., 2012; Alnassan et al., 2013; Seo et al., 2013; Andersson et al., 2015; Blanco et al., 2017). The main advantages of ELA are being rapid, sensitive, cheap, specific and simple method with ethical considerations.

Concerning *Salmonella*, the previous reports by us in the ELALA mainly involved *Salmonella* Pullorum, *Salmonella* Typhimurium and *S*. Gallinarum (Geng et al., 2017, Guo et al., 2017, Lacharme-Lora et al., 2019, Zhang et al., 2020). Therefore, it was supposed that ELA may be applied as an alternative to determine the virulence of *S. enteritidis* for salmonella vaccines evaluation. In this work, chicken embryo model was established to evaluate the virulence of *S. enteritidis* challenge strain in comparison to vaccinal strain.

So, according to the above mentioned points, the goal of this work is to find a rapid, accurate alternative method for evaluation of *Salmonella* Enteritidis virulence and pathogenicity.

## MATERIAL AND METHODS

### Ethical statement

All animal related procedures were applied with relevant guidelines and regulations of Veterinary Cairo University Institutional Animal Care and Use Committee (Vet. CU-IACUC) according to local Egyptian laws. The study was approved ethically by the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Abbasia, Cairo, Egypt.

### Competing interests

The authors declare that they have no competing interests.

### Growth conditions of Bacterial strains

Virulent and avirulent vaccinal strains of *S. enteritidis*used in this study were kindly obtained from Strain Bank of Central Laboratory of Evaluation of Veterinary Biologics (CLEVB), Abbasia, Cairo, Egypt (Thomson et al., 2008).

Both strains were inoculated on S.S agar at 37˚C overnight. One single colony was inoculated in tryptose soya broth to be incubated overnight at 37˚C to be counted and adjusted to obtain $10^9$ CFU/ml through preparing ten-fold serial dilutions for each strain to be used for the assessment.

### Inoculation of specific pathogen free embryonated chicken egg (SPF-ECE)

A total of 210, 13-day-old, SPF eggs was inoculated via intra-allantoic cavity (AC) by 0.1 ml of each strain dilutions as 10 eggs for each dilution and 10 eggs were left as control. Eggs were incubated at 37˚C in 60% humidity.

Eggs vitality was examined daily for 5 days for deaths recording. 24 hr deaths were considered non-specific. $ELD_{50}$ was determined (Reed and Muench., 1938). Dead and survived embryos were chilled at 4˚C for 4 hr for necropsy. Embryonic body and liver gross lesions were scored as shown in Table (1). The allantoic fluid and liver were collected for bacterial re-isolation.

### ECE growth curve

Twenty, 13-day-old, eggs were inoculated by $10^4$ CFU/ml of each strain by the AC. Five eggs were inoculated by phosphate buffer saline (PBS) as control. Three eggs were sacrificed at time intervals 3 h, 6 h, 18 h, 24 h, 48 h, 72 h and so on for 5 days. On the $5^{th}$ day post inoculation, the allantoic fluid was aseptically collected to count the viable bacteria and compare the *in-ovo* growth curves of both strains.

### Assessment of strain virulence

Fifty, 13-day-old, SPF eggs were inoculated into the AC by ten-fold 10 serial dilutions of each strain (5 eggs for each dilution) to determine the $ELD_{50}$ value of each strain.

### Pathogenicity test in chickens

Two groups of 20 SPF 7-day-old chickens were challenged orally by $10^8$ colony forming units (CFU) (Berchieri et al., 2001) of each strain (one strain for each group) to assure strains' virulence *in-vivo*. Chickens were monitored for 14 days. Clinical signs were scored as shown in Table (2). At 14 days post inoculation (dpi), live chickens were humanly euthanized for bacterial re-isolation (Alves et al., 2018).

## RESULTS

### Inoculation of embryonated chicken eggs

Thirteen day/AC SPF egg inoculation could be success-

**Table 1.** Emberyonic body and liver gross lesions scoring system

| Score | Embryonic body | Embryonic liver |
|---|---|---|
| 1 | Normal | Normal |
| 2 | Medium body size | Swelling |
| 3 | Small body size | Few necrotic foci |
| 4 | Small body size plus hemorrhage | Multiple necrotic foci |

**Table 2.** Clinical signs scoring system

| Score | 0 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Clinical signs | Normal | Depression - Ruffled feathers | Previous signs- respiratory distress | Previous signs - anorexia, emaciation – green yellowish diarrhea | Death |

**Table 3.** Pathogenicity test in chickens

| Strain | Chicken mortality | Re-isolation (n = 10) | $Log_{10}$ $ELD_{50}$ |
|---|---|---|---|
| Virulent | 10/10 (100%) | 10/10 | 3.6 |
| Avirulent | 0/10 (0%) | 10/10 | 6.0 |

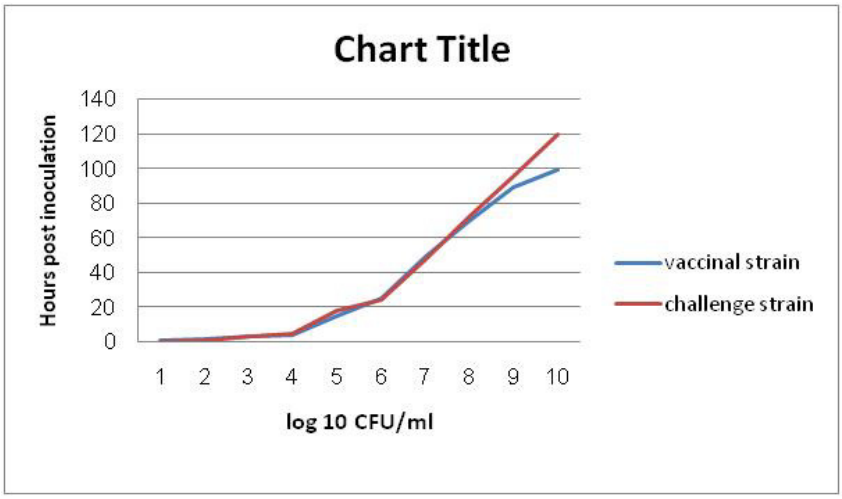


**Figure 1.** Relation between virulence and *in-ovo* growth

fully used to differentiate the virulent and avirulent (vaccinal) *S*. Enteritidis strains. Table 3

Experimentally infected 4-day-old SPF chicks recorded 100% mortality along 14 days post infection by the virulent *Salmonella* Enteritidis virulent strain (Table 3). No Clinical signs or mortality (0%) were observed on group infected by the avirulent strain. Bacterial re-isolation revealed *Salmonella* positive for both strains.

## Determination of strains virulence by ELA (embryo lethality assay)

*S*. Enteritidis virulence in embryonated chicken eggs was distinguished according to the gross lesion scoring the embryonic size and liver. The calculated $Log_{10}$ $ELD_{50}$ of the virulent *Salmonella* strain was 3.6, whereas the value of the avirulent one was 6.0 according to the mentioned scoring method post inoculation by $10^2$ CFU/ml. concerning the titers $10^4$ CFU/ml and $10^6$ CFU/ml, there was no detectable differences between both strains.

The previous results matched with the embryonic liver gross lesion scores. Both $10^4$ and $10^6$ CFU/ml revealed no difference. However $10^2$ CFU/ml resulted in obvious difference between virulent and avirulent strain (Figure 1).

Both allantoic fluid and embryonic livers were used to determine the relationship between bacterial invasion for replication and bacterial virulence (Figure 2). The bacterial count of the allantoic fluid of eggs inoculated by

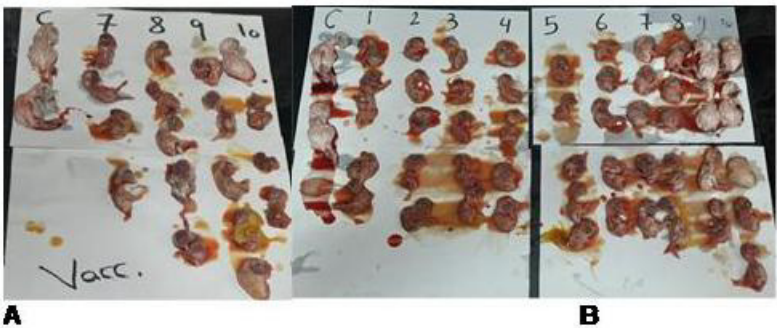


**Figure 2.** Gross lesions scores of embryonic body, (A) Embryonic scores after inoculation by $10^7$, $10^8$, $10^9$ and $10^{10}$ CFU of vaccinal strain. (B) Embryonic scores after inoculation by $10^1$, $10^2$, $10^3$,$10^4$, $10^5$, $10^6$,$10^7$, $10^8$, $10^9$ and $10^{10}$ CFU of vaccinal strain. (9, 10) score 1 for a normal size.(6, 7, 8) score 2 for a medium size. (4,5) score 3 for a small size. (1, 2, 3) score of 4 for a small size with hemorrhage. C (A, B) Scoring is standard for the control of chicken embryo.

**Table 4.** The death pattern of vaccinal and virulent strains

| Dilution | Virulent strain | | Vaccinal strain | |
|---|---|---|---|---|
| | Dead | Total | Dead | Total |
| **1** | 5 | 5 | 5 | 5 |
| **2** | 5 | 5 | 5 | 5 |
| **3** | 5 | 5 | 5 | 5 |
| **4** | 5 | 5 | 5 | 5 |
| **5** | 5 | 5 | 5 | 5 |
| **6** | 5 | 5 | 5 | 5 |
| **7** | 2 | 5 | 0 | 5 |
| **8** | 2 | 5 | 0 | 5 |
| **9** | 1 | 5 | 0 | 5 |
| **10** | 1 | 5 | 0 | 5 |

**Table 5.** The death pattern of vaccinal and virulent strains

| Dilution | 1(Normal) | 2(Medium) | 3(Small) | 4(Small with hemorrhage) |
|---|---|---|---|---|
| **1** | 0 | 0 | 0 | 5/5 |
| **2** | 0 | 0 | 0 | 5/5 |
| **3** | 0 | 0 | 0 | 5/5 |
| **4** | 0 | 0 | 2/5 | 3/5 |
| **5** | 0 | 0 | 3/5 | 2/5 |
| **6** | 0 | 5/5 | 0 | 0 |
| **7** | 0 | 5/5 | 0 | 0 |
| **8** | 0 | 5/5 | 0 | 0 |
| **9** | 3/4 +1 non-specific | 1/4+1 non-specific | 0 | 0 |
| **10** | 3/5 | 2/5 | 0 | 0 |

the virulent strain reached $10^9$ CFU/ml within the 18 hours then declined slightly to $10^7$ CFU/ml on the 5$^{th}$ post inoculation. Whenever, the bacterial load of the avirulent*Salmonella* Enteritidis declined on the 2$^{nd}$ post inoculation to $10^6$ CFU/ml up to the 5$^{th}$ day. Concerning the liver, the bacterial load was clearly different at the 5$^{th}$ day as the virulent strain was count was about 100 times than the avirulent strain Table 4, 5.

## DISCUSSION

The traditional chicken pathogenicity test is a routine method used for evaluation the *S. enteritidis* virulence.

This traditional method has many disadvantages as time consuming, ethical considerations, requiring special facilities and cost. Hence, using of embryonated chicken eggs (ECE) became a more suitable alternative model to study bacterial and viral virulence (Andersson et al., 2015). However, ECE has been used as a model for studying the virulence of S. Pullorum, S. Gallinarum, and *S. typhimurium* strains (Zhang et al., 2020). Previous works improved that for S. pullorum, S. gallinarum, and S. typhimurium, the higher the strain virulence by the chicken pathogenicity test, the higher the ECE mortality rate by the inoculated strain. These findings suggested that application of ELA (embryo lethality assay) test could be a successful method to determine the virulence of different *Salmonella* species virulence. So, in the present work, evaluation of *S.enteritidis* virulence was assessed using chicken embryos and compared to the results of the traditional pathogenicity test.

According to previously published works, the susceptible chicken embryo age is 13 days to evaluate virulent and avirulent bacterial strains (Jacobsen et al., 2010, Jacobsen et al., 2011, Seo et al., 2013).

According to a work done by Zhang et al. (2020), AC route was chosen for inoculation as it was proven to be the best route to differentiate between the virulent and avirulent *Salmonella* strains.

The results of growth curve revealed that there is no difference between both virulent and avirulent strains because the difference in virulence is not related to the bacterial proliferation in the allantoic fluid as the allantoic fluid supports good bacterial proliferation rates and growth within the first 24 hours post-inoculation to reach $10^8$ CFU/ml. The bacterial count of the avirulent strain decreased after a few days to be $10^6$ CFU/ml (Zhang et al., 2020).

However, from the colonization rate in embryonic liver, it was observed that the virulent strain showed stronger liver invasiveness than the avirulent one. Concerning the bacterial count of liver samples, the virulent strain also showed more colonization than the avirulent strain. On the $5^{th}$ day post inoculation, the bacterial count of embryonic liver inoculated by the virulent strain was $10^4$ times more than that of the vaccinal avirulent one. On the other hand, mortality rate was 90% of for embryos inoculated by the virulent strain on the $5^{th}$ day post inoculation. In contrast, embryos inoculated by the avirulent strain showed no mortalities.

This finding can be explained due to the ability of the virulent strain to invade embryonic liver, resulting in proliferation of huge bacterial load, which is considered as the main cause of embryonic death. $Log_{10}$ $ELD_{50}$ of the avirulent strain was 6 but the virulent one was 3.6.

Concerning the chicken pathogenicity test, virulent strain showed 100% mortality but the avirulent showed 0% mortality. The present work confirmed the correlation between the embryo mortality and inoculation dose.

This finding represents an alternative method to distinguish the virulent and avirulent strain according to $ELD_{50}$. It was recorded that there was 6-log between the avirulent ($ELD_{50}$= $3.3\times10^8$) and virulent ($ELD_{50}$ $2.2\times10^2$) *Campylobacter jejuni*. Additionally in other work on *Neisseria meningitidis*, it was observed that the avirulent strain $ELD_{50}$ was $10^3$ CFU but virulent strain was less than $10^1$ (Berchieri et al., 2001).

In the present study, there was 3.5-log $ELD_{50}$ difference between the virulent and avirulent *Salmonella* Enteritidis indicating that the *Salmonella* Enteritidis virulence of can be clearly distinguished by the value of $ELD_{50}$. Moreover, there was a correspondence between the ELA and chicken pathogenicity test. *Salmonella* Enteritidis virulence in chickens was also reflected in ELA.

## CONCLUSION

From the obtained results of this study, it can be concluded that the ELA test can be applied as a successful alternative method to assess *Salmonella* Enteritidis virulence and also a successful easy method for isolation and identification of *Salmonella enteritidis*. Moreover, it can be used to differentiate between naturally infected and vaccinated samples.

### Author Contributions

Conceptualization: Mounir Elsafty, Hala Mahmoud. Data curation: Marwa Fathy, Reem Soliman. Formal analysis: Mounir Elsafty, Hala Ahmad. Investigation: Reem Soliman, Marwa Fathy. Methodology: Mounir Elsafty, Hala Mahmoud, Marwa Fathy, Reem Soliman, Hala Ahmad. Supervision: Mounir Elsafty. Validation: Hala Ahmad. Visualization: Marwa Fathy, Reem Soliman. Writing – original draft: Hala Mahmoud. Writing-review and editing: Mounir Elsafty, Hala Mahmoud, Marwa Fathy, Reem Soliman, Hala Ahmad.

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