PREPARATION AND EVALUATION OF CHEMICALLY INACTIVATED *SALMONELLA ENTERITIDIS* VACCINE IN CHICKENS

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ABSTRACT

Objective: *Salmonella enteritidis* ghosts (SEGs) is a non-living empty bacterial cell envelopes which were generated using a different concentration of sodium hydroxide (NaOH) 6.4 mg/mL and evaluated as a vaccine candidate in specific pathogen-free (SPF) chicken. SEGs have been produced by chemical-mediated lysis and evaluated the potential efficacy of chemically induced SEG vaccine and its ability to induce protective immune responses against virulent *S. enteritidis* challenge in SPF chickens.

Methods: SPF chickens were divided into three groups: Group A (non-vaccinated control), Group B (vaccinated with prepared vaccine), and Group C (vaccinated with commercial vaccine).

Results: Vaccination of SPF chicken with SEGs induced higher immune responses before and after virulent challenge. SPF chicken vaccinated with SEGs showed increasing in serum enzyme-linked immunosorbent assay (ELISA) antibodies. During the vaccination period, Groups B and C showed higher serum antibody titer compared to Group A. The minimal inhibitory concentration (MIC) of NaOH was capable of inducing non-living SEGs, and it has successfully generated non-living SEGs by MIC of NaOH.

Conclusion: It is a one-step process which means easy manufacturing and low production cost compared to protein E-mediated lysis method. Chemically induced SEG vaccine is a highly effective method for inducing protective immunity. This study strongly suggests that SEGs will be a permissive vaccine, as the method of inhibition of *S. enteritidis* was safe and cheaper than other methods, and it gave a good protection.

Keywords: *Salmonella enteritidis* ghost, Specific pathogen-free, Vaccine, Enzyme-linked immunosorbent assay, Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

INTRODUCTION

The *Salmonella* consists of a range of very closely related bacteria, some of which cause illness in both animals and people, and some types cause illness in animals but not in people [1]. *Salmonella enteritidis*, a Gram-negative bacterium, is a worldwide leading zoonotic food-borne bacterial pathogen, causing diarrhea and gastroenteritis in humans and animals [2]. *S. enteritidis* is transmitted to progeny through developing embryo and infects generally through contaminated eggs or undercooked specific pathogen-free (SPF) chicken products [3]. Salmonellosis is an important public health problem and causes large economic losses in the SPF chicken industry. Furthermore, *in vivo* and *in vitro* studies suggested that the bacterial virulence ability to invade and survive in epithelial cells is crucial for salmonellosis [4]. The SPF chicken model of salmonellosis has been extensively studied [5]. These studies indicate that an SPF chicken model can be used for salmonellosis.

Progress in science has always been the major driving force for the development of effective agents that are given to healthy individuals to prevent infections [6,7]. Vaccination is an effective tool for the control and prevention of *Salmonella* infections. Previous studies have shown that the generation of live attenuated, killed, and subunit vaccines have been attempted [8], but their efficacy is not always optimal. Whole-cell killed *Salmonella* vaccine has the ability to stimulate beneficial antibody responses. However, it confers incomplete protection and is more reactogenic. Live attenuated vaccine is able to induce mucosal, cellular immune responses in chickens [9,10]. In addition, it protects chickens

against a lethal challenge, but there is a possibility of dangerous reversion [11]. To overcome these drawbacks, there is an urgent need to find other safe and efficacious vaccine approaches to protect against salmonellosis.

Bacterial ghosts (BGs) are novel and non-living empty bacterial cell envelopes raised from Gram-negative bacteria, which represent a new avenue in vaccine technology [12]. In general, the most common method for producing BGs is based on the phage-derived lysis gene E. The expression of protein E-mediated lysis leads to the formation of transmembrane pores, which consequently leads to loss of cytoplasmic contents. The resultant BGs share their functional and antigenic determinants of the envelope and represent an excellent vaccine candidate [12]. BGs from pathogenic bacteria were reported to be controlled and can protect against infectious diseases [13]. The mucosal immunizations with *Escherichia coli* ghosts were reported to induce protective immunity in an SPF chicken model [14]. Furthermore, the oral vaccination of *Edwardsiella tarda* ghosts induced higher protection against infection in mice [15]. Moreover, BGs were able to target-specific organ or tissue, indicating that they are a powerful system for drug and DNA delivery.

There were several studies demonstrated BG vaccine production by genetically engineered method [16,17]. However, this genetic inactivation method still has some risk in its use as a vaccine. Therefore, in this study, it was demonstrated an alternative method to produce BGs from *S. enteritidis* strain using the minimal inhibitory concentration (MIC) of sodium hydroxide (NaOH).

NaOH has been proven for its ability to loose and create pores in the cell wall and DNA degradation [18]. The specific concentration of NaOH was responsible for the generation of empty cell envelopes. Amara *et al.* [19] demonstrated a method for production of BGs from *E. coli* BL21 and JM109 strains using MIC and minimum growth concentration of NaOH, sodium dodecyl sulfate (SDS), and calcium carbonate.

In the present study, *Salmonella enteritidis* ghosts (SEGs) have been produced by chemical-mediated lysis and evaluated the potential efficacy of chemically induced SEG vaccine and its ability to induce protective immune responses against virulent *S. enteritidis* challenge in SPF chickens.

METHODS

Bacterial strain and culture condition
A strain of *S. enteritidis* virulent previously isolated was used for the BGs production. The strain was freshly grown in Luria–Bertani (LB) broth for 24 hrs at 37°C. The colony-forming unit (CFU) was adjusted to contain 1×10^6 CFU/mL as previously described by Sambrook and Russell [20].

Determination of MIC
NaOH in a concentration of 50 mg/mL was added to the adjusted culture of *S. enteritidis* as a starting concentration and then doubled, fold diluted in phosphate buffer saline according to Tavares *et al.* [21]. All tubes mixed well at room temperature then a loopful of each tube was cultured on LB agar and incubated at 37°C for 24 hrs to check the growth of *S. enteritidis*. The minimum concentration that stopped growth of bacteria was considered as the MIC.

Production of SEGs
Pellet of *S. enteritidis* produced after centrifugation (10,000 ×*g*, for 10 minutes at 4°C) was suspended in phosphate buffered saline (PBS) and adjusted to concentration of 1×10^6 CFU/mL. About 2 mL of this suspension was added to 1 mL of MIC of NaOH and was incubated at 37°C for different time points (15, 30, 45, 60, and 70 minutes). About 25 µL of each incubated time points were cultured on LB agar plates and incubated for 24 hrs at 37°C. The specific time that inhibits bacterial growth was determined as a lysis time that produces SEG. SEGs were harvested by centrifugation (10,000 ×*g* for 10 minutes at 4°C) and washed 3 times with PBS. The final cell pellets were resuspended in ice-cold PBS and stored at 4°C until further use.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) analysis
The SDS-PAGE analysis was performed on 12% polyacrylamide gels as previously described by Laemmli [22]. SEG for SDS-PAGE was taken. The whole cell pellet of SEGs and non-treated *S. enteritidis* was resuspended in 20 µL of PBS and boiled at 100°C for 5 minutes in 5 µL of 5× SDS loading buffer. The SDS-PAGE gels containing samples were electrophoresed at constant 40 V for 2 hrs. Protein bands were visualized after staining with Coomassie brilliant blue.

Isolation and quantification of DNA by polymerase chain reaction (PCR)
To confirm the absence of genetic content in SEGs and non-treated control cells were quantitatively analyzed by PCR. Bacterial genomic DNA was extracted using a commercial kit QIAamp™ DNA Mini Kit (QIAGEN), catalog no. 51304, according to kit manufacturing. After extraction of genomic DNA from non-treated control cells, PCR was performed [23]. Preparation of PCR reaction mix was according to EmeraldAmp GT PCR mastermix (Takara) Code No. RR310A kit mixing.

Oligonucleotide primers sequences
The primers for PCR and sequencing were synthesized by Metabion (Metabion International AG, Lena-Christ-Str. 44/I, 82152 Martinsried, Germany) (Table 1).

Electrophoresis was performed on PCR product of SEG with modification using electrophoresis [24]. Grade agarose (1 g) was prepared in 100 mL

Table 1: Oligonucleotide primers sequences

Primer	Sequence	Amplified product	Reference
sefA gene	AGGTTCAGGCAGCGGTTACT GGGACATTTAGCGTTTCTTG	312 bp	[23]

tris-borate-ethylenediaminetetraacetic acid (TBE) buffer in a sterile flask. It was heated in microwave to dissolve all granules with agitation, and allowed to cool at 70°C then 0.5 µg/mL ethedium bromide was added and mixed thoroughly. The warm agarose was poured directly in gel casting apparatus with desired comb in apposition and left at room temperature for polymerization. The comb was then removed, and the electrophoresis tank was filled with TBE buffer. About 2 µL of each PCR product samples, negative and positive control were loaded to the gel. The power supply was 1-5 V/cm of the tank length. The run was stopped after about 30 minutes and the gel was transferred to ultraviolet cabinet. The gel was photographed by a gel documentation system, and the data were analyzed through computer software.

Examination of SEGs and *S. enteritidis* by transmission electron microscopy (TEM)
Morphological features of chemically induced SEGs and native *S. enteritidis* were analyzed by TEM as previously described by Zhu *et al.* [17]. Cells were fixed in 4% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.3) for 4 hrs after two rinses in the same buffer (for 20 minutes) followed by post-fixation in osmium tetraoxide OsO_4 (for 2 hrs). The fixed cells are washed three times for 30 minutes in the same buffer. The samples were then dehydrated in ascending grades of ethanol; from 10% to 100%, 10 minutes in each one except the finely one (100%) for 30 minutes for three changes (each one for 10 minutes). Then, the samples were cleared in propylene oxide for two changes in 10 minutes. The specimens were put in equal volumes of propylene oxide and Epon 812 for 1 hr at room temperature. Half of the propylene oxide - Epon mixture was poured off in a waste bottle and add 2 volume of Epon 812 for 3 hrs at room temperature. The samples were putted in a pure Epon 812 resin overnight. The samples were embedded in Epon 812 resin mixture and polymerized in the oven at 60°C for 24 hrs. Semi-thin (1 pm) sections were cut from these blocks using Ultramicrotome (Ultracut, Reichert-Jung, Vienna, Austria) stained with toluidine blue and examined by the light microscope. Ultrathin sections obtained from selected blocks were mounted on copper, single-hole grids, and double stained with uranyl acetate and lead citrate then examined with transmission electron microscope Zeiss EM 10 (Carl Zeiss, 7082 Oberkochen, West Germany).

SPF chicken
A total of 210 of 3-week-old SPF chicken were obtained from the national project for production of SPF eggs (Nile SPF eggs), Kom Oshim, Fayoum, Egypt. They were reared in separated isolator in the Central Laboratory for Evaluation of Veterinary Biologics (CLVB), Cairo, Egypt.

Vaccination safety and challenge experiment
A group of 120 experimental SPF chicken was equally divided into three Groups (A, B, and C) to evaluate the protective efficacy of chemically induced SEGs as a vaccine candidate. The 40 SPF chicken in Group A were considered the non-vaccinated control group. Group B was vaccinated with 0.2 mL of SEGs as primary vaccination. A booster dose following the primary vaccination was taken with the same dose and route after 3 weeks. Group C SPF chicken received the commercial vaccine of *S. enteritidis* with the same route and dose as group B. Blood samples were collected individually from all chicken after 1 week of the second dose and all the groups were challenged by 1 mL of the virulent *S. enteritidis* strain containing 10^9 CFU and observed for 4 weeks.

Another group of 90 experimental SPF chicken was equally divided into three groups (D, E, F) to evaluate the safety of chemically induced SEGs as a vaccine candidate 30 SPF chicken in Group D (non-vaccinated

control group). Group E was vaccinated with 0.4 mL of SEGs. Group F SPF chicken received the commercial vaccine of *S. enteritidis* with the same route and dose as Group E. All the experiment groups were observed for 3 weeks.

Antibody response assessment

Detection of specific immunoglobulin G antibodies was measured in the sera of SPF chicken by an indirect enzyme-linked immunsorbant assay (ELISA) test kit (Jordan Bio–Industries Center, JOVAC), and the result was calculated according to the following equation.

Calculation of results

ELISA (EU) calculation: Calculate S/P ratio between corrected sample absorbance and corrected positive control absorbance (average).

$$S/P = (Abs_{Test\ Sample} - Abs_{Negative})/(Abs_{Positive} - Abs_{Negative})$$

Calculate ELISA unit (EU):

$$EU_{sample} = (S/P) \times 100$$

Titer calculation

Titers (T) for the tested samples are calculated with the following equations:

$$log_{10}T = 0.9397 \times log_{10}(S/P) + 3.0244$$

$$T = Antilog\ (log_{10}T).$$

Where, S/P - The sample/positive ratio; Abs - Absorbance; T - Antilog $(log_{10}T)$.

Recovery of *Salmonella* strains from challenged chickens

On the 4th week post-challenge, samples were collected from the heart blood, liver, spleen, and cecal junction from vaccinated and non-vaccinated challenged chickens for recovery of the organism.

RESULTS AND DISCUSSION

S. enteritidis (1 mL culture) was used and the tested range of concentration of NaOH was 6.0-7.0 mg/L using *Lactobacillus* (LB) medium and incubation for 24 hrs. There was no growth was observed with the concentration of 6.4 mg/mL. Fig. 1 illustrated the MIC of NaOH (6.4 mg/mL).

Table 2: Determination of the suitable time for *S. enteritidis* inactivated by NaOH

Incubation time (minutes)	Colonies on agar
10	+ve
15	−ve
30	−ve
45	−ve
60	−ve
75	−ve

S. enteritidis: Salmonella enteritidis, NaOH: Sodium hydroxide

Determination of the suitable time for *S. enteritidis* inactivated by NaOH was represented in Table 2.

In the current study, the MIC of NaOH treated *S. enteritidis* cells exhibited lysis within 15 minutes (Fig. 2).

It should be noted that the new strategy for NaOH-induced BGs was fast [13]. Moreover, other researchers [17] have shown the lysis efficiency of genetically inactivated BGs after lysis was 99.9%. In addition, collected MIC of NaOH-treated cells and non-treated control cells were subjected to SDS-PAGE analysis. Non-treated control cells showed very clear strong protein bands. These clear protein bands indicate the presence of cytoplasmic and cell wall proteins while MIC of NaOH-treated cells shows weaker protein bands. This is due to lack of cytoplasmic contents in MIC of NaOH-treated cells. Furthermore, agarose gel electrophoresis showed that there was no DNA band of
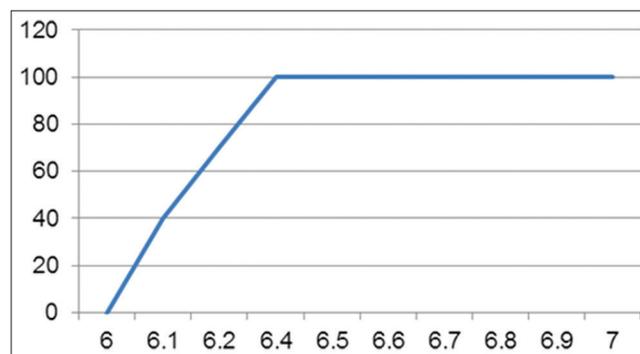

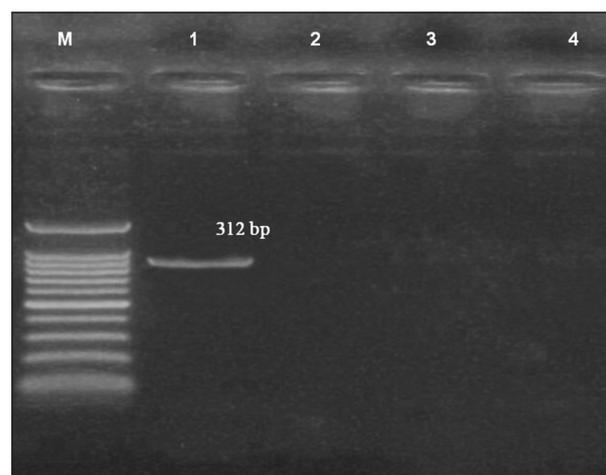**Fig. 1: Determination of minimal inhibitory concentration of sodium hydroxide**

Fig. 2: Agarose gel electrophoretic analysis polymerase chain reaction product extracted from non-treated control cells (Lane 1), minimal inhibitory concentration of sodium hydroxide treated cells (Lanes 2-4), and M is 1 kb marker ladder

Table 3: *S. enteritidis* antibody titer measured by ELISA

Groups	Antibody titer			
	Pre-vaccination	Weeks post-booster		
		1st week	2nd week	3rd week
Group B vaccinated with SEGs	169.0	430.0	1073.8	1710.4
Group C vaccinated with commercial vaccine	169.0	270.0	945.0	1133.8
Group A control	166.0	170.0	171.0	177.0

S. enteritidis: Salmonella enteritidis, SEG: *Salmonella enteritidis* ghosts, ELISA: Enzyme-linked immunosorbent assay

treated cells detected after 20 minutes (Fig. 3 - Lanes 1-5), compared to non-treated control in which clear bands were shown in Fig. 3. Similarly, *Salmonella* virulence gene of *sefA* gene [25,26]-based PCR analysis shows degraded genetic content and complete absence of detectable concentration of genetic content in chemically induced SEGs (Fig. 3). This result indicates that DNA free SEGs are successfully induced by MIC of NaOH.

Colonies lysis rate of *S. enteritidis* cells at various time points the lysis efficiency was calculated using the following formula: lysis rate = (1 − (CFU of treated cells/CFU of non-treated control cells)) × 100%. Lysis rate was carried out in triplicate. Table 3 showed the antibody titer of *S. enteritidis* measured by ELISA. The ELISA antibody responses remained elevated during the vaccination in all vaccinated groups.

The classification of titer groups was showed in Table 4. The maximum antibody responses were observed on week 3 post-vaccination period in Group B and C when compared to group A. It is well-documented that the SEG vaccines induce protective immunity [15]. Another study [27] demonstrated that the outer membrane proteins are important to induce protective immunity against *Salmonella* infections. These data indicate most importantly, SEG maintains their original cell envelope and preserve immune stimulating compounds that have adjuvant properties which induce immune responses. On the other hand, groups which double dose of both vaccines showed neither mortality nor signs as the SEGs were safe. Our results suggest that the SEGs-induced immune responses provided protection against *S. enteritidis* infections. This immune response is an important consideration for salmonellosis [28].

In addition, Kwon *et al.* [29] also showed similar results that the genomic DNA was completely degraded. We investigated the formation of the chemically induced SEGs as well as non-treated *S. enteritidis* cells by SEM (Fig. 4).

Electron micrograph images clearly demonstrate the presence of transmembrane lysis tunnel structure in the SEGs (Fig. 4a - arrowheads) compared to unlysed *S. enteritidis* cells (Fig. 4b). During lysis event, specific concentration of NaOH creates sufficient transmembrane tunnel structure in the cell surface. Through this transmembrane tunnel structure, cytoplasmic contents were expelled and turned into an empty cell envelope. Except for these pores, SEGs exhibited cellular morphology including all cell surface structures which were unaffected by the lysis process.

The results of challenge test (Table 5 and Fig. 5) to Groups B and C (vaccinated with SEGs and *S. enteritidis* commercial vaccine) was the same as it gave protection rate of 82.5% compared to Group A (control which showed 20% protection only).

In this study, the protective immunity achieved using SEGs against experimental challenge with *S. enteritidis* in chicken. The results obtained demonstrate that the candidate vaccine confers a suitable protective effect [30].

Concerning the protection efficacy of *Salmonella* vaccine, the protection rate was satisfactory potent in vaccinated chickens when challenged with virulent SE while the control unvaccinated group was unable to withstand the experimental infection with virulent SE strains confirming that the vaccine was effectively potent, and hence able to protect chickens against infection. In our study, the use of SEGs *Salmonella* vaccine for the protection of poultry against infection, in

agreement with previous report [31]. On the other hand, the *S. enteritidis* could be reisolated from vaccinated challenged chickens with SEGs by a
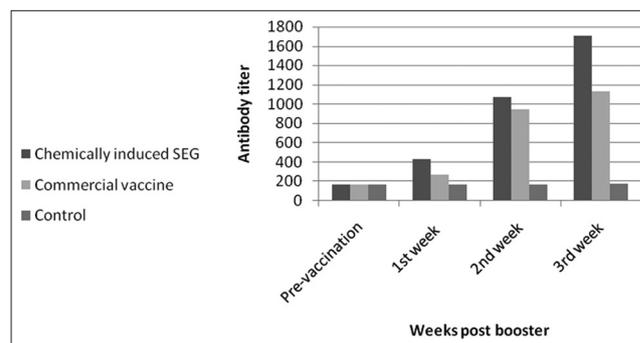

Fig. 3: Specific immunoglobulin G antibody responses in sera from Group A (non-vaccinated control), Group B (vaccinated with prepared *Salmonella enteritidis* ghosts vaccine), and Group C (vaccinated with commercial vaccine) specific pathogen-free chicken were determined by indirect enzyme-linked immunosorbent assay
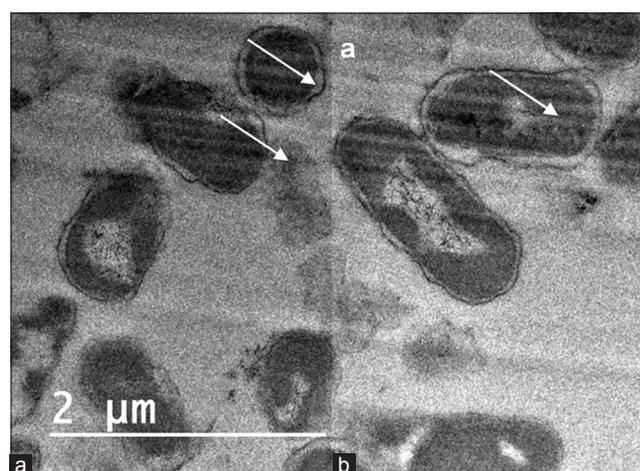

Fig. 4: Transmission electron micrographs of chemically induced Salmonella enteritidis ghosts (Panel A). The small arrows indicate the transmembrane lysis tunnels. (a) Treated cells. (b) Control cells (untreated cells)

Table 4: Classification of titer groups

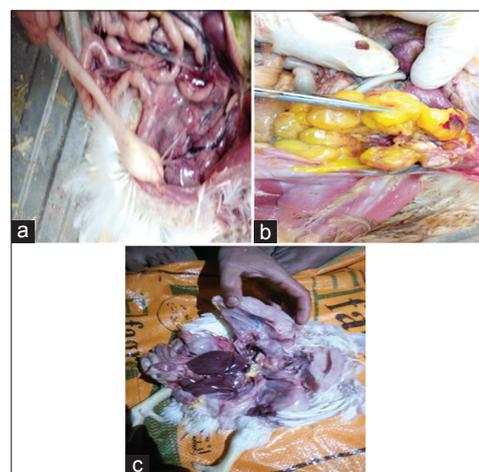| Titer levels	Interpretation
0-233	Negative
234-395	Suspect
>395	Positive

Fig. 5: (a) Fluid accumulation with cecal cores of challenged chicken affected with *Salmonella*. (b) Irregular and misshaped ova with *Salmonella* affected challenged control chicken. And (c) slightly enlarged, pale, and shows focal necrosis

Table 5: Protection rates of vaccinated groups either vaccinated with SEGs or *S. enteritidis* vaccine

Groups	Number of chicken	Challenge dose	Mortality	Protection rate %
Group B (vaccinated with SEGs)	40	10^9	7	82.5
Group C (vaccinated with commercial vaccine)	40	10^9	7	82.5
Group A (control)	40	10^9	32	20

S. enteritidis: Salmonella enteritidis, SEG: *Salmonella enteritidis* ghosts

Table 6: Recovery of *Salmonella* strains from challenged chickens

Groups	Type of vaccine	Number of positive samples for *Salmonella* recovery			
		Heart blood (%)	Liver (%)	Spleen (%)	Cecal junction (%)
Vaccinated	Commercial vaccine	7/30 (23.3)	8/30 (26.7)	9/30 (30)	10/30 (33.3)
	SEG	6/30 (20)	7/30 (23.3)	7/30 (23.3)	9/30 (30)
Control	Non-vaccinated	15/20 (75)	16/20 (80)	15/20 (75)	15/20 (75)

SEG: *Salmonella enteritidis* ghosts

lower ratio than from commercial inactivated *Salmonella* vaccine from the heart blood, liver, spleen, and cecal junction on the 4th week post-challenge and control unvaccinated birds (Table 6). This Table 6 records the recovery of the *Salmonella* from the challenged chickens.

CONCLUSION

It has successfully proved that the MIC of NaOH was capable of inducing non-living SEGs. It has successfully generated non-living SEGs by MIC of NaOH. It is a one-step process which means easy manufacturing and low production cost compared to protein E-mediated lysis method. Chemically induced SEG vaccine is a highly effective method for inducing protective immunity. This study strongly suggested that SEGs will be a permissive vaccine as method of inhibition of *S. enteritidis* is safe and cheaper than other methods, and it gave a good protection.

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