# Evaluation of the duration of immunity induced by a vaccination program against *Salmonella* Enteritidis 7A in laying hens in Australia (Stage 2).

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# Foreword

This project was conducted to assess the long-term protection of a live *Salmonella* Typhimurium + autogenous *Salmonella* Enteritidis vaccination program in the reduction of *Salmonella* Enteritidis isolate 7A faecal shedding, and colonisation of caeca, air sacs and ovarian tissue.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

This report is an addition to Australian Eggs Limited's range of peer reviewed research publications and an output of our R&D program, which aims to support improved efficiency, sustainability, product quality, education and technology transfer in the Australian egg industry.

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# About the Authors

Dr Peter Scott has more than 30 years' experience in the Australian poultry industry as a veterinary pathologist, veterinarian, researcher, and member of egg industry R&D committees. He has successfully completed many projects for Australian Eggs and other industry organisations and is Managing Director of Scolexia.

Drs Tim Wilson and Jose Quinteros also have extensive experience in vaccine and *Salmonella* studies.

Dr Amir Noormohammadi is the Professor of Avian Medicine at the University of Melbourne and leads the team of researchers and microbiologists involved in assisting with the project. Together with Dr Pollob Shil, the group has also been involved in multiple poultry vaccine studies, both viral protozoal and bacterial, including *Salmonella* vaccine efficacy studies in the past, many of which have been part of the development of successful commercial poultry vaccines in Australia and abroad.

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# Abbreviations

| | |
|---|---|
| ANOVA | Analysis of variance |
| APCAH | Asia-Pacific Centre for Animal Health |
| APVMA | Australian Pesticides and Veterinary Medicines Authority |
| DAE | Days after exposure |
| ELISA | Enzyme linked immunosorbent assay |
| IM | Intramuscular |
| NC | Negative control |
| NSW | New South Wales |
| PC | Positive control |
| PCR | Polymerase chain reaction |
| PI | Protective index |
| SE | *Salmonella enterica* serovar Enteritidis (*Salmonella* Enteritidis) |
| SE 7A | Australian isolate *Salmonella* Enteritidis 7A |
| ST | *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium) |
| TBE | Tris/Borate/EDTA |
| WOA | Weeks of age |

# Executive Summary

During the first stage of this *Salmonella* vaccine study, the protection conferred by different vaccination programs against a *Salmonella enterica* serovar Enteritidis (SE) strain 7A (SE 7A) was assessed. Results demonstrated that the best protection was achieved by the program that included two vaccinations with *Salmonella enterica* serovar Typhimurium (ST) live vaccine at hatch and at 4 weeks of age, followed by two vaccinations with SE 7A autogenous vaccine at 8 and 12 weeks of age. However, this protection was assessed only 5 weeks after the second vaccination with SE autogenous vaccine. This study was undertaken to assess the duration of the immunity observed in the earlier study.

During the present study (stage 2), siblings of the hens included in the stage 1 of the study[1] were challenged at 47 weeks of age, 35 weeks after the second vaccination with SE autogenous vaccine.

SE antibody levels remained above the cut-off threshold (an ELISA value of 654) in the vaccinated hens. The mean antibody level decreased gradually over time. A greater degree of intrinsic resistance of adult hens to *Salmonella* Enteritidis compared to younger hens was observed. A decline in bacterial shedding occurred in both vaccinated and unvaccinated challenged groups, and this decline occurred earlier compared with the previous experiment, where hens were infected at 17 weeks of age. A similar result was observed with the samples taken during the post-mortems at the end of the present experiment. Caecal colonisation in both vaccinated and unvaccinated groups ranged between 56% and 69%, and the colonisation of both air sac and surface of the ovary was almost absent in both groups. As in the initial study no ovarian follicles were infected in the vaccinated group. In the positive control group, one sample collected from the surface of the ovary was positive, while no sample was positive when collected from the inner part of the follicle. This suggests that vertical transmission of the bacteria occurs directly from the peritoneal cavity to the ovarian surface as distinct from a systemic contamination of the germinal tissue of the ovary. However, this concept needs further confirmation.

In conclusion, the vaccination program was capable of inducing a humoral immune response that remained at levels above the cut-off 35 weeks following the last booster and the immunity induced by the vaccination program continued to prevent follicular infection, as noted in the initial study. The infection rate, colonisation and shedding of the bacteria substantially decreased over time compared with infections at the beginning of the laying period. However, no differences in faecal shedding and colonisation were observed in the vaccinated group compared to the positive control group. It is possible that older hens got naturally resistant to Salmonella, explaining why levels where not statistically different between the PC and the vaccinated groups.

# 1 Introduction

*Salmonella enterica* serovars Typhimurium (ST) and Enteritidis (SE) cause disease in the human population. Recent outbreaks in Australia have highlighted the importance of finding a successful method to aid control of SE in poultry placed in previously infected farms.

During stage 1 of the present study which assessed the protection conferred by three different vaccination programs against a challenge with SE 7A, one group of hens was vaccinated with live attenuated ST vaccine at the hatchery by coarse-spray and at 4 weeks of age (WOA) through drinking water (ST group). Another group of hens was vaccinated with the SE autogenous killed vaccine through the parenteral route at 8 and 12 WOA (SE group). A third group was vaccinated with a combination of the two programs (ST+SE group). Results from stage 1 of the study demonstrated a significant protection level conferred by the program including two vaccination with ST live vaccine and two additional vaccinations using the killed SE autogenous vaccine, which was higher than the protection induced by the other vaccination programs. During that study, significant protection was observed in terms of caecal and ovarian SE colonisation when compared with the unvaccinated and challenged group. The initial study demonstrated protection conferred by the vaccination program, as the hens were challenged at 17 WOA, 5 weeks after the last vaccination. However, it is necessary to demonstrate protection over a much longer time frame. If the protection offered is only short term then it may be necessary to handle the birds for vaccination while in lay, which is both expensive and disruptive.

To evaluate the duration of immunity induced by the ST+SE vaccination program, siblings of those hens (which received the same vaccination program) were housed for an additional 30 week-period. The objective of the present study was to evaluate the long-term protection of this vaccination program (at least until mid-lay at 47 WOA) against an oral challenge with SE 7A, using the challenge model used in the earlier study.

# 2 Materials and methods

## 2.1 Animal ethics

This experiment was conducted under the approval of the Animal Ethics Committee, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne (approval ID number 1915043.1).

## 2.2 Production of the SE autogenous vaccine

The SE autogenous vaccine was produced using the strain SE 7A, isolated in Australia. The laboratory reference number for the vaccine was 1914/19 5RXI, and analysis revealed that the strain belonged to the MSLT type 11. The vaccine was produced under APVMA approval, permit number 12576. The vaccine is a whole-cell bacterin, where the bacterium was formalin-inactivated, and contains an aluminium hydroxide and oil in water adjuvant.

## 2.3 Source of hens and treatments

A total of 48 laying hens (Hy-Line Brown) were divided into three groups, each with sixteen birds (Table 1). As displayed in Figure 1, hens from the vaccinated group were coarse-spray vaccinated at the hatchery using a commercial live *Salmonella* Typhimurium (ST) vaccine (Vaxsafe® ST, Bioproperties) and this was repeated in the drinking water again at 4 WOA. At 8 and then at 12 WOA, hens were vaccinated with the killed SE autogenous vaccine. The first vaccination was applied subcutaneously, while the second dose intramuscularly. The dose applied was that recommended by the producing laboratory, 0.5 ml per hen. Hens from both negative and positive control (NC and PC) groups remained unvaccinated. After the first vaccination with the SE autogenous vaccine, all birds were individually identified using leg tags and their cages were identified until their transfer to APCAH facilities.

**Table 1. Distribution of the birds in the different groups included in the SE vaccine trial.**

| Group | Treatment | n | ST vaccine | SE vaccine | Exposure* to SE 7A |
|---|---|---|---|---|---|
| 1 | Negative Control (NC) | 16 | - | - | No |
| 2 | ST+SE (Vacc) | 16 | + | + | Yes |
| 3 | Positive Control (PC) | 16 | - | - | Yes |

** The exposure to SE was, conducted in HEPA filter equipped* isolators *located in the PC2 animal research facilities of The University of Melbourne, FVAS (Werribee Campus). Hens received a vaccination program including both live ST and killed SE autogenous vaccine, or no vaccination at all (controls).*

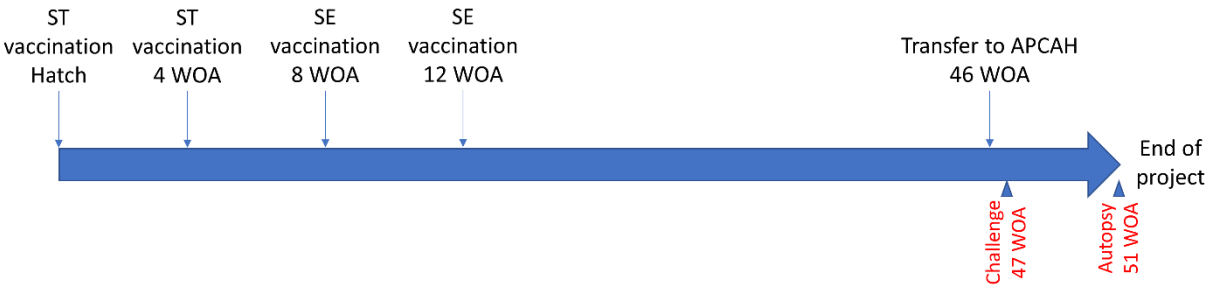


**Figure 1. Experiment timeline.**

Birds were weighed on their arrival to the animal research facilities, and cloacal swabs taken for *Salmonella spp.* isolation (5 days prior to challenge). At 24 and 3 hours before exposure to SE 7A, 0.6 ml of the antibiotic vancomycin at a concentration of 100 mg/ml (approximately 30 mg/kg liveweight) was administered orally to each bird. Application of vancomycin was successfully used during the earlier study[1], and has been successfully used in prior studies with ST by the authors to achieve a more stable infection rate as compared with untreated birds. This challenge model is suitable for the study of anti-*Salmonella* interventions[2], such as vaccination. Also, when mature birds are orally challenged with *Salmonella*, the intestinal flora of the chickens outcompete *Salmonella*[3-5], leading to the potential failure of the challenge model.

The SE 7A challenge inoculum was prepared by ACE Laboratory Services[1] at a concentration of $0.85 \times 10^9$ CFU/ml (Appendix 1), consistent with the dose used in the previous study ($0.81 \times 10^9$ CFU/ml)[1], and also in previous publications[2, 6]. The media used for mock-inoculation of the negative control group was tested to be sterile (Appendix 1). The inoculum was aliquoted into 3 ml syringes containing 1 ml each in a Biohazard cabinet. Two aliquots of 1 ml of the inoculum each were stored at −80°C for retrospective analysis.

The inoculum was administered to the corresponding groups at 47 WOA using the oral route of administration, 1 ml per hen. Hens in the negative control group received sterile *Salmonella* growth medium using the same route of administration (Appendix 1). After exposure, hens were monitored daily, and general health status of the birds was observed and recorded (Appendix 2).

## 2.4 Monitoring

Five hens per group were randomly selected and bled at 11 WOA (one week before SE 7A autogenous vaccine booster) and at 16, 21 and 35 WOA (6 in the vaccinated group during the last sampling). At 46

[1] Ace Laboratories: Animal Consulting Enterprise, East Bendigo Victoria 3550 Australia

WOA all the hens were tested (8 days before challenge). Each serum was used in an ELISA tests to detect Group D *Salmonella* antigens (BioChek®, Unit 5 Kings Ride Business Park, Kings Ride, Ascot, Berkshire SL5 8BP, UK), following manufacturer's directions. There was a cut-off value established by BioChek® to discriminate between positive and negative samples of 654.

After transfer to APCAH, all hens were swabbed (cloaca) for *Salmonella spp.* isolation. Then, hens were individually weighed, and the weights recorded. Once weighed the hens were placed in three isolators equipped with HEPA filters and positive pressure. Inside the isolators, feed and water were offered *ad-libitum*. Feed used was commercially formulated for laying hens (Barastoc Champion Layer, Ridley Australia). Hens were also weighed at the end of the study following euthanasia.

A cloacal swab was taken from each individual bird at 7, 14 and 28 days after exposure (DAE). Each swab was immersed in peptone water before sample collection. All swabs were sent to the microbiology laboratory at The University of Melbourne (Werribee Campus), to attempt *Salmonella spp.* isolation.

## 2.5 Post-mortem analysis

Hens were humanly euthanised at 28 DAE using an intravenous injection of barbiturates, according to the protocol approved by the animal ethics committee. The negative control group was autopsied first, followed by the vaccinated and exposed group, with the positive control group (unvaccinated and exposed) examined last. The post-mortems were performed in a biohazard cabinet. The cabinet was cleaned, and UV light sterilised between groups. During the post-mortem, cloaca, caecal contents, abdominal air sacs (inner membrane) and surface of the largest follicle (serosal surface of the epithelial lining) were swabbed from all the hens. An additional sample from the inner perivitelline membrane of the largest follicle was also collected. Prior to the collection of this swab, the surface of the follicle was seared using a hot spatula. The cloacal swabs were collected by swabbing the cloacal walls, trying to collect as much faecal material as possible. The caecal samples were taken by sterilising the surface using a hot spatula and then opening the caeca and swabbing the content. The abdominal air-sacs were swabbed closest to the F1 follicle. The samples from the ovarian surface were collected by swabbing the surface membrane as described above. For the internal F1 follicle samples, the surface of the follicle was sterilised and then a swab from contents was collected, avoiding cross contamination. All samples were sent to the microbiology laboratory at The University of Melbourne (Werribee Campus) to attempt *Salmonella spp.* isolation.

## 2.6 Statistical analysis

For the comparison of proportions, a 2×2 contingency table was used as displayed in **Error! Reference source not found.**. Calculation of the $\chi^2$ value and Fisher's exact test was undertaken. For the comparison of weight gain, a two-way analysis of variance (ANOVA) was used using the Tukey's multiple comparison test. A P-value ≤ 0.05 was considered as statistically significant. All these analyses were performed using the software package GraphPad Prism, version 8.4.2 (GraphPad Software, La Jolla California USA).

**Table 2. Two by two contingency table.**

| | Positive | Negative |
|---|---|---|
| Group X | A | B |
| Group Y | C | D |

*Probability was determined using the Chi-square (χ2) distribution with a P < 0.05 being considered significant, as determined using the Fisher's exact test.* $\chi 2 = (A-Ex_1)^2/Ex_1 + (B-Ex_2)^2/Ex_2 + (C-Ex_1)^2/Ex_1 + (D-Ex_2)^2/Ex_2$ *where the Expected values om column 1,* $Ex_1 = (A+C)/2$ *and in column 2,* $Ex_2 = (B+D)/2$.

# 3 Results

## 3.1 ELISA results after two vaccinations with live ST, and pre and post SE autogenous vaccine booster.

Five hens per group were bled and the sera were tested for the presence of antibody to *Salmonella* Group D. This sampling was undertaken before the SE autogenous vaccine booster at 11 weeks of age (11 WOA), at 16, 21 and 35 WOA, and before their transfer to the animal research facilities and exposure to SE 7A at 46 WOA, 34 weeks after the SE vaccine booster was administered. Results are displayed in Table 3.

Neither the positive nor negative control birds showed antibodies against *Salmonella* Group D, except for 1 hen at 21 weeks of age in the NC group. At 11 WOA, three hens from the Vacc group (60%) exhibited a positive reaction in the ELISA test. At 16 WOA (after the SE booster), all the serum samples from the vaccinated hens were positive. That number decreased at 21 and 35 WOA to 3 (60%) and 4 (67%) positives, respectably. The proportion of positives remain similar at 46 WOA, with 10 out of 16 sera positive (63%). The median titres per group were calculated and results can be found in Figure 2. The titres remained below the threshold in both NC and PC groups. In the Vacc group, titres increased

after booster, but gradually decreased over time, but with the average value always above the threshold.

**Table 3. Number of serums positive to *Salmonella* group D antibodies (ELISA) from blood taken before and after the booster vaccination with SE autogenous vaccine (11 WOA), and at 16, 21 and 35 WOA. Mean titre ± SD.**

| Group | Treatment | 11 WOA | 16 WOA | 21 WOA | 35 WOA | 46 WOA |
|---|---|---|---|---|---|---|
| 1 | NV (NC) | 0 of 5 (0%) | 0 of 5 (0%) | 1 of 5 (20%) | 0 of 5 (0%) | 0 of 16 (0%) |
| | | 75.4 ± 58 | 1.0 ± 0 | 203.6 ± 244 | 90.8 ± 71 | 67.8 ± 59 |
| 2 | ST+SE (Vacc) | 3 of 5 (60%) | 5 of 5 (100%) | 3 of 5 (60%) | 4 of 6 (67%) | 10 of 16 (63%) |
| | | 228.4 ± 253 | 1679.0 ± 734 | 1317.2 ± 855 | 1296.2 ± 744 | 941.8 ± 616 |
| 3 | NV (PC) | 0 of 5 (0%) | 0 of 5 (0%) | 0 of 5 (0%) | 0 of 5 (0%) | 0 of 16 (0%) |
| | | 50.6 ± 15 | 1.8 ± 2 | 78.6 ± 79 | 98.0 ± 50 | 50.8 ± 35 |

** Five hens per group were tested at each sampling day. B, D and B+D, antigens included in the ELISA tests used. NV, not vaccinated; ST, vaccinated with ST live vaccine; SE, vaccinated with SE autogenous vaccine; ST+SE, vaccinated with both live ST and autogenous SE vaccines; NC, negative control; PC, positive control. ELISA values over 654 were considered positive.*

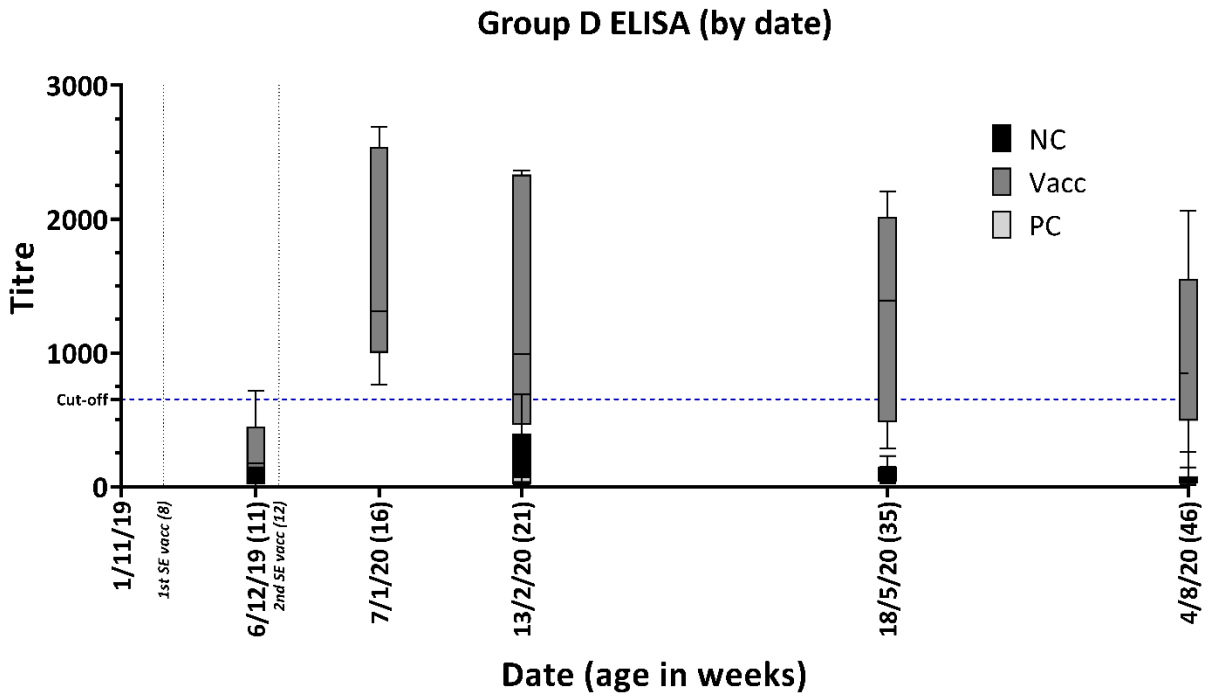


**Figure 2. Median and range (error bars) of titres measured by the ELISA group D kit from blood samples taken from the hens included in the SE trial at five different times.**

*Boxes represent the quartile range. Horizontal dotted line represents the cut-off limit determined by the ELISA test, and the dotted vertical lines the dates of first and second SE vaccinations at 8 and 12 WOA.*

## 3.2 Cloacal swabs

Results are summarised on Figure 3. At their arrival to the research facilities, as expected, all the cloacal swabs collected from the hens were negative to *Salmonella spp.* isolation (3 days before their exposure to SE 7A). At 7 DAE, the hens from the NC group remained negative, while the hens from the Vacc and PC groups had significant increase to 81.3% and 87.5% of the swabs positive to *Salmonella spp.* ($P < 0.0001$ for both groups). The proportion of positives decreased at 14 and 28 DAE in both Vacc and PC groups and remained significantly higher compared with the NC group ($P = 0.043$ for both PC and Vacc groups). There was no difference in the proportion of positive swabs between the PC and Vacc groups at any time point ($P > 0.999$).

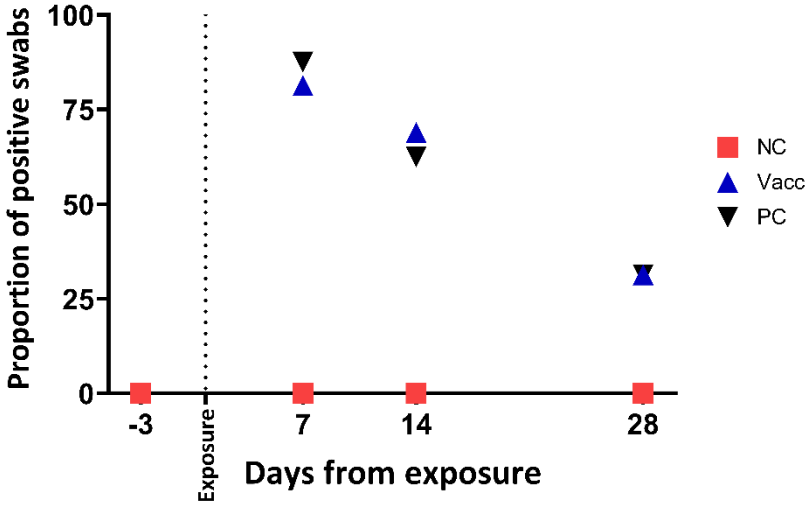


**Figure 3. Proportion of cloacal swabs positive to *Salmonella spp.* isolation.**

## 3.3 Correlation of ELISA titres and *Salmonella spp.* isolation from cloacal and caecal swabs

The correlation between the titres obtained from the Group D *Salmonella* ELISA and the SE status (positive or negative) of the hens determined by cloacal or caecal swabbing samples was examined. These ELISA results correspond to bloods collected at 46 weeks of age, just before the hens were transferred to the research facilities. In the case of caecal samples correlation, the positive or negative status of the hens and their corresponding ELISA titres at 46 WOA were evaluated from the samples collected at 7, 14 and 21 DAE as a data pool in order to increase the n-value. Air sac and ovum surface swabs were not included, as most of them were negative (2 positive from 48 air-sacs, and 1 positive

from 48 ovum surface swabs). As displayed in Figure 4, the mean titres of hens with *Salmonella spp.* negative cloacal swabs were significantly higher (1,152) than that of the *Salmonella spp.* positive hens (804), with a P-value of 0.03. The mean titres of those hens with *Salmonella spp.* negative caecal swabs (985) was slightly higher than the titres (908) of birds with *Salmonella spp.* positive caecal swabs, however this was not significant (P = 0.41).

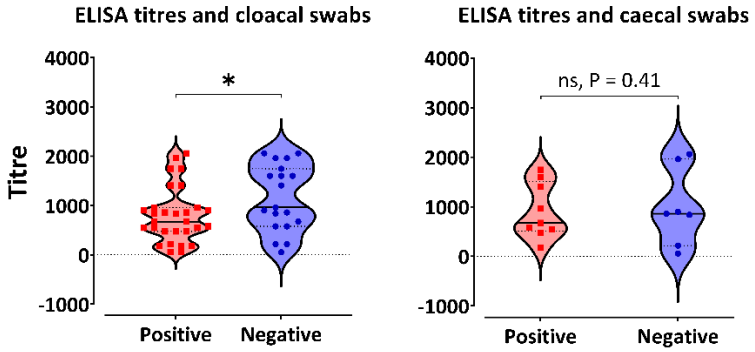


**Figure 4. Violin plots depicting correlation between the ELISA titres obtained by each hen and their status of positive or negative to *Salmonella spp.* isolation (cloacal and caecal swabs). The data on cloacal swabs correspond to those collected at 7, 14 and 21 DAE.**

*One-tailed Mann-Whitney test. *, P < 0.05.*

## 3.4 Weight gain

When weight gain was compared between groups, differences between groups were not statistically significant. The same result was obtained when comparing initial or final weights between groups, or initial versus final weight within each group (Figure 4).

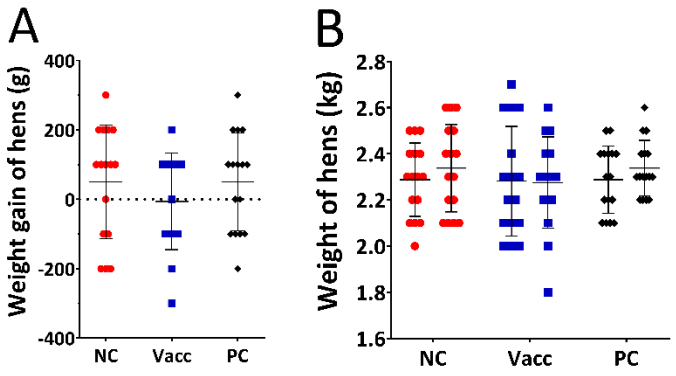


**Figure 5. A, weight gain of hens between before and after SE 7A exposure. B, initial (left) and final (right) weights of hens while in isolators and before and after challenge.**

## 3.5 Post-mortem

The number of samples positive and negative to *Salmonella spp.* isolation are both summarised on Table 4 and Figure 5 below. These samples were taken using dry swabs during the post-mortem procedure at 28 DAE. In the caeca, the proportion of positive samples in the Vacc and PC groups was significantly higher compared with the NC group. However, those differences were not significant for the air sac and ovarian samples. No SE was isolated from the inner surface of the largest follicle and its contents in the positive control group.

**Table 4. Proportion of tissue samples positive to *Salmonella spp.* isolation.**

| Group | N | Caecum | | Air sac | | Ovarian surface | | Inner follicle | |
|---|---|---|---|---|---|---|---|---|---|
| | | + | % | + | % | + | % | + | % |
| NC | 16 | 0 | 0%[a] | 0 | 0.0% | 0 | 0.0% | NT | |
| Vacc | 16 | 9 | 56.3%[b] | 2 | 12.5% | 0 | 0.0% | NT | |
| PC | 16 | 11 | 68.8%[b] | 0 | 0.0% | 1 | 6.3% | 0 | 0.0% |

*Different lowercase superscript letters in the same column represent significant differences, P < 0.001. NT, not tested.*

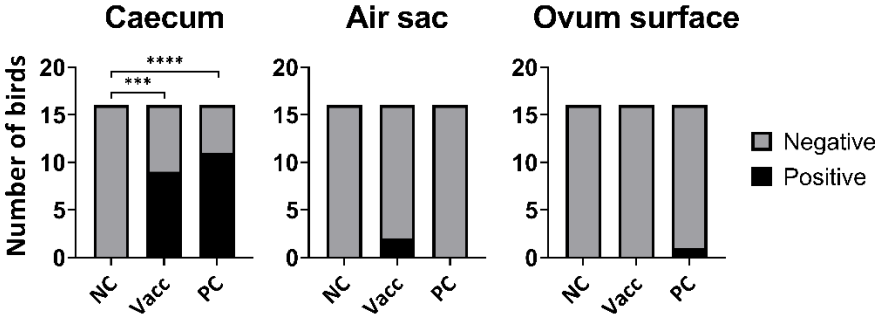


**Figure 6. Proportion of samples positive and negative to *Salmonella spp.* isolation per group. Isolations were attempted from swabs samples collected during the post-mortem procedure at 28 DAE.**

****, P < 0.001; ****, P < 0.0001*

# 4 Discussion

The present study was intended to evaluate the capacity of a *Salmonella* vaccination program to protect against a *Salmonella* Enteritidis challenge at point of mid-lay in commercial layer hens. Hens received the last vaccination booster at 12 weeks of age and were challenged at 47 weeks of age under controlled conditions. Hens were housed inside negative-pressure isolators equipped with HEPA filters.

The titres obtained with the ELISA test demonstrate a long-term memory immunity conferred by this vaccination program combining two vaccinations with a live ST vaccine at hatch and 4 weeks of age, and two vaccinations with an SE 7A autogenous vaccine at 8 and 12 weeks of age. The titres of the vaccinated hens were consistently higher than those of the unvaccinated controls. And even though there was a decrease in the titres over time, they remained above the threshold limit of 654. Experiments in mice have shown that the antibody titres in the blood against *Salmonella* can stay high for up to 6 weeks after vaccination by injection [7]. A similar result was achieved in chickens immunised against *S.* Typhimurium using a vaccination program combining live and killed ST vaccines, a similar vaccine program to the one used in the present study. That vaccination program achieved ELISA titres above the cut off value that slightly dropped in time but remained above the cut off value for up to 50 weeks of age of the hens [8]. It appears that the positive result in the ELISA test at 21 WOA in a hen in the negative control group was a false positive, despite the manufacturers claim of a specificity >99.5%. The ELISA value was only slightly above the cut-off (689 vs 654) and the hen was negative for *Salmonella* at autopsy and well below the ELISA cut-off when tested at 46 WOA (259).

Weight did not vary during the experiment in any of the groups, demonstrating that SE infection did not affect feed consumption and weight gain. According to the standards of the genetic line (Hy-Line brown), the weights of the hens do not vary between 47 and 51 weeks of age.

During the present study, hens were tested negative for *Salmonella spp.* isolation when transferred to the research facility, meaning they were free of *Salmonella*, including SE. Therefore, all posterior *Salmonella spp.* positives by isolation were assumed to be the same *Salmonella* Enteritidis included in the inoculum. The hens were placed in isolators, so there were no other possible source of *Salmonella spp.* other than that included in the inoculum. The proportion of *Salmonella spp.* positive cloacal swabs obtained during the present stage of the study was lower when compared with that obtained during the first stage of this study, when hens were exposed to an equivalent dose of SE 7A at 17 weeks of age. In the previous stage, the maximum percentage of positive samples in the unvaccinated and challenged control group reached 100% at 3 and 7 DAE and declined to 93.8% by 14, 21 and 28 DAE, whereas in the present study the maximum percentage achieved by the same group was 87.5% at 7 DAE and declined to 62.5% at 14 DAE and 31.3% at 28 DAE. Also, the caecal colonisation of the hens at 28 DAE was different between younger and older hens. In the positive control group at stage 1, 100% of caecal swabs were positive to *Salmonella* isolation, while in the present study, 68.8% of the samples were positive in the same group. Hence, older hens appeared more resistant to SE caecal colonisation, reducing the shedding of the bacterium through the faeces.

Previous studies have shown a significant decrease ($P \leq 0.001$) in CD4 and CD8 lymphocyte populations in the spleens of hens that commence at 13 weeks of age, reaching their lowest point at approximately 18 weeks of age. From that point in the development of hens, the population of both lymphocytes start to recover at a high rate until at least the 24 weeks of age, when that study concluded [9]. This decrease in lymphocyte populations is linked with the onset of laying. The lymphocyte populations also decline in the infundibulum, magnum and ovary at 18 weeks of age, with a clear recovery up to 23 weeks of age [9]. They also showed a decrease in the efficiency of the vaccination programs when birds are challenged at point of lay from around 18 weeks of age [9]. Such studies confirm the understanding of the critical importance of protecting hens during development from rearing to point of lay when changes in gut flora are common and the physiological demands on the birds are greater. If the hens can be protected from colonisation during this critical phase and as they move towards peak production, it appears from the results of this study that the hens will be more resistant to colonisation later in lay. Previous studies have demonstrated the importance of early vaccination in protecting hens from SE colonisation of internal organs and contamination of the eggs [10].

In the caecal samples collected at post-mortem, there was a numerical reduction in the number of positives in the vaccinated group compared with the positive control group from 68.8% to 56.3%, which was not statistically significant ($P = 0.72$). From the samples collected from the surface of the follicle, one sample was positive in the positive control group, while there was no positive sample in the Vacc group. The lack of SE positives in the follicles of the Vacc group confirms the earlier finding in Stage 1 that vaccinated hens were protected from follicular infection. This lends some support to the suggestion that vertical transmission of SE is not generally systemic via germinal ovarian tissue, but through the contamination of the surface of the follicle. Other studies have also shown that, in SE-infected hens, the surface of the follicles appeared contaminated but not the internal contents. They then suggested that the infection of the eggs, coincidental with the results from the present study, comes from the contaminated surface of the follicle and not from infected ovarian germinal tissue [11]. Other studies have suggested that small follicles membrane cells are more susceptible to SE invasion than mature follicles [12], with the subtle suggestion that the infection could occur directly to the ovarian tissue. However, these studies were conducted exclusively *in vitro*, not considering the proper barriers conferred by the hen immune system, and the potential role of surrounding membranes in follicle infection with SE.

The low follicular isolation rate from the positive controls in these older birds (compared to their siblings in the stage 1 study [1]) confirms the difficulty in reproducing consistent infection levels in

experimental birds and the necessity of utilising gut flora destabilisation (such as the use of vancomycin in this study) when attempting to challenge adult hens [2-5]. Whilst the dynamics of infection in the field will be different, the necessity of using small numbers of birds when experimenting with zoonotic organisms in isolators necessitates the use of such measures to give the best chance of a high proportion of positive hens in the positive control group. This by default provides more challenging conditions for vaccines to demonstrate their efficacy, and thus vaccine protection outcomes under field conditions are expected to be more favourable.

There was a positive correlation between the antibody titres obtained in the last ELISA test before the SE challenge and the status of positive or negative of their cloacal swabs (SE shedding). Antibody titres of those hens negative to *Salmonella spp.* isolation (non-shedders) were significantly higher compared with the titres of those positive (shedders), indicating that vaccination could reduce the faecal shedding of the bacterium. Previous studies have indicated an important role of humoral immune system in the clearance of SE after infecting bursectomised chickens [13]. On the other hand, results obtained from other studies are slightly different. In these studies, it has been proposed that cell mediated immunity could play a more significant role in the clearance of SE and other *Salmonellas* compared with antibodies [14, 15]. Also, results from the Stage 1 of this vaccine study showed that while the hens vaccinated with only the SE killed vaccine exhibited a significant increase in antibody levels, they did not exhibit a reduction in caecal and ovarian colonisation compared with the unvaccinated and challenged group [1]. This lack of agreement demonstrate that more studies are required to fully understand the immune response and protection against *Salmonella* infections in chickens.

The presence of the bacterium in the air sacs was relatively low, only in 2 out of 16 samples of the Vacc group. However, the exact route of infection of the air sacs remains unknown. Previous studies have shown that hens infected via intravenous injection of SE demonstrated a higher rate of air sac contamination (33%) than an oral challenge (2.5%). It is possible that the air sac infection comes not from those multiplying in the intestinal mucosa, but from particles in the air. *Salmonella* particles could have been aerosolised from the faeces secreted by infected hens. Airborne infection with SE under experimental and farm conditions has been reported before [16-18]. In one of these experiments, 70% of the air samples collected were positive to SE isolation [18].

In conclusion, the present vaccination program including two vaccinations with the live ST vaccine and two vaccinations with the SE autogenous vaccine provided a long-term humoral immune response at least until 47 weeks of age. As distinct from the outcome in the earlier study, the low level of SE positive follicles in the positive control group did not allow the demonstration of a significant difference in hens

of this age, so the evidence of protection of ovarian and caecal tissues by the vaccine was inconclusive in the present study. The study also demonstrated a correlation between humoral antibodies and cloacal shedding, which was not shown in the earlier study, probably due to the complex nature of protection which appears to involve more than only humoral protection [19-21].

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# Plain English Summary

| **Project Title:** | |
|---|---|
| Australian Eggs Limited Project No | 1FS002 |
| Researchers Involved | Peter C. Scott[a], Timothy Wilson[a], José A. Quinteros[a], Arif Anwar[a], Tyrone Scott[a], Pollob Shill[b], Amir H. Noormohammadi[b]. |
| Organisations Involved | [a] Scolexia Pty Ltd, 8/19 Norwood Crescent, Moonee Ponds VIC 3039. [b] Asia-Pacific Centre for Animal Health (APCAH), Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Werribee VIC 3030, Australia |
| Phone | 03 9326 0106 |
| Fax | 03 9372 7576 |
| Email | pscott@scolexia.com.au |
| **Objectives** | To assess the long-term protection conferred by a SE vaccination program. Parameters measured were faecal shedding, caecal, air sac and ovarian surface (abdominal cavity) colonisation of *Salmonella* Enteritidis (SE) after challenge. Vaccines included were a commercial live S. Typhimurium (ST) and a SE autogenous vaccine (which is made with the causal organism isolated on the farm). |
| **Background** | In a previous stage of our research, a significant protection against a challenge with SE strain 7A was detected in hens vaccinated with a vaccination program combining ST live and autogenous SE vaccinations at different time points. Protection over the life of the hen was not assessed in the previous study. This study was designed to assess the duration of immunity provided by the vaccination program |
| **Research** | A vaccination group received a program consisting of two live ST vaccinations (at hatch and 4 weeks of age) followed by two killed SE autogenous vaccinations at 8 and 12 weeks of age. A comparison was made with a negative control (unvaccinated and unchallenged) and a |

| | |
|---|---|
| | positive control (unvaccinated and challenged) groups. To assess the efficacy of the vaccination program, vaccinated hens were orally exposed to the Australian isolate SE 7A in a controlled environment (isolators). Cloacal swabs were obtained at -5, 7, 14 and 28 days after exposure. At the end of the study, hens were humanly euthanised and caecal content, and air sac and largest follicle surfaces swabbed and cultured. |
| **Outcomes** | The vaccination program tested during this study was demonstrated to be capable of inducing SE antibody levels at least until 47 weeks of age. Also, vaccination numerically reduced the colonisation of caeca and the largest follicle. The immune response induced by vaccination were able to numerically reduce the bacterial shedding in the hens, even though results were not statistically significant.<br>A lower number of positives in the PC group could have prevented the results to be more conclusive. It seems possible that hens become more resistant to SE as they get older. |
| **Implications** | The present vaccination program using two applications of the live ST vaccine at hatch and 4 weeks of age, and two vaccinations with SE autogenous vaccine can confer a long-lasting antibody response that could be related with protection against an SE challenge. It could be a useful tool in the control of SE in Australia. |
| **Key Words** | *Salmonella* Enteritidis<br>Salmonellosis<br>Autogenous vaccine<br>Immunity |
| **Publications** | NA |

# Appendix

## Appendix 1: ACE laboratories inoculum concentration report

**Laboratory Results**

To: Tim Wilson

**Final report date: 18/08/2020**
Date testing commenced: 12/08/2020
Date samples received: 12/08/2020
Date samples collected: 12/08/2020


ACE Laboratory Services
12 Gildea Lane, Bendigo East, VIC 3550
PO Box 6101, White Hills, Vic 3550
Phone: (03) 5443 9665 Fax: (03) 5443 9669
Email: info@acelabservices.com.au
ABN 92115191056


**MICROBIOLOGY REPORT**

Laboratory Reference: V9102
No. Samples Submitted: *Salmonella enteritidis* for *in vivo* challenge X 1
Sterile Media only X 1
Submitter: Tim Wilsom
Owner/Farm: Scolexia

| Lab No. | Sample Details | Count * CFU/mL | Purity and identity |
|---|---|---|---|
| 1 | *Salmonella enteritidis* Tested at 12:00 am 12/08/2020 | $0.85 \times 10^9$ | Pure growth- *Salmonella enteritidis* |
| 2 | Media only Tested at 12:00 am 12/08/2020 | No growth | Sterile media |
| 3 | | | |

Report authorised by: Yousef Abs EL-Osta
ACE Laboratory Services actively seeks and welcomes your feedback, phone 03 54439665.

**Comments:**

Identification and serotyping of the seed was performed by ACE and confirmed by Melbourne Diagnostic Unit (MDU) prior to isolate storage (laboratory reference: 01914/19 5RXi).

Challenge identity as *Salmonella enteritidis* was confirmed by MALDI-TOF.

Viable cell count was performed in accordance with GMP method (SOP 1167).

* = This testing is not covered by the scope of our NATA accreditation.

# Appendix 2: APCAH animal and isolator monitoring sheet

Asia-Pacific Centre Animal Health, Faculty of Veterinary & Agricultural Sciences, The University of Melbourne

Animal Facility (AF)

WORKSHEET: Animal and Isolator Monitoring Sheet

WS No.: AH/AMLS/05C — Date Issued: 05/02/2018 — Page 1 of 1

Building No.: 423 Isolator No.: 413 Isolator Pressure: NEGATIVE Start Date: 11 AUG 20 End Date: 9 SEP 20

Experiment Procedure: EFFICACY OF SALMONELLA ENTERITIDIS AUTOGENOUS VACCINE IN COMMERCIAL LAYERS (SEP 20-10)

Experiment Group: (1) VACCINATED Researcher: SCIENTIST

Species: CHICKEN Source: CAMDEN Egg Access No.: 218 Age: 47 WKS Animal Numbers: 20 FOR SPECIMEN TAKES

APCAH Accession No.: 218 Animal House Accession No.: 0211/2020 AEEC No.: 19/043.1 AQIS/OGTR No.: 218

Feed Type: CRUMBLE Water Type: TAP Waste and Faecal Disposal: COMPOST

| DATE | TIME | Bird Number | Clinical Signs (tick appropriate boxes and adhere to the Intervention Criteria sheet) | | | | | | | | | | Comments or Procedures performed (including comments on any clinical signs seen) | FEED | | WATER | | FILTER | | FAECES | Sign |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Normal | Alertness | Posture | Movement | Feathers | Respiratory signs | Eyes | Body condition | Diarrhoea | Other | | New bag | Top up | Fill | HCL | Clean | New | Roll up | Removed | |
| 7/8/20 | 1227 | ANC | OK | | | | | | | | | 16 BIRDS WEIGHED COLOUR SWAB + FAECES | | | | | | | | | AE |
| 9/8/20 | 945 | ALL | OK | | | | | | | | | | | | | | | | | | AE |
| 10/8/20 | 952 | ALL | OK | | | | | | | | | | | | | | | | | | AE |
| 11/8/20 | 910 | ANC | OK | | | | | | | | | BIRDS GIVEN VACCINATION | | ✓ | | | | | ✓ | | PS/AE/AK |
| 12/8/20 | 850 | ANC | OK | | | | | | | | | BIRDS GIVEN VACCINATION / MAN REMOVED | | | ✓ | ✓ | | | | | PS/AE/AK |
| 12/8/20 | 1106 | ANC | | | | | | | | | | BIRDS GIVEN MEDIA (OMA) | | | | | | | | | PS/AE/AK |
| 13/8/20 | 926 | ANC | OK | | | | | | | | | | | ✓ | | | | ✓ | | ✓ | SD |
| 14/8/20 | 8:00 | ANC | OK | | | | | | | | | MAN REMOVED FAECES | | ✓ | ✓ | | | | | | SD |
| 15/8/20 | 829 | ALL | OK | | | | | | | | | | | | | | | | | | AE |
| 16/8/20 | 934 | ALL | OK | | | | | | | | | MAN REMOVED FAECES | | | | | | | | | AE |
| 17/8/20 | 957 | ANC | OK | | | | | | | | | | | ✓ | ✓ | | | ✓ | ✓ | ✓ | SD |
| 18/8/20 | 948 | ANC | OK | | | | | | | | | MAN REMOVED FAECES | | ✓ | ✓ | | | ✓ | ✓ | ✓ | SD |
| 19/8/20 | 943 | ANC | OK | | | | | | | | | ALL BIRDS CLEANED SWABS | | ✓ | ✓ | | | ✓ | ✓ | ✓ | PS/AE/AK |
| 20/8/20 | 815 | ANC | OK | | | | | | | | | | | | ✓ | | | ✓ | ✓ | ✓ | PS/AE/AK |
| 21/8/20 | 723 | ANC | OK | | | | | | | | | MAN REMOVED FAECES | | ✓ | ✓ | | | ✓ | ✓ | ✓ | SD |
| 22/8/20 | 911 | ALL | OK | | | | | | | | | | | | | | | | | | AE |
| 23/8/20 | 933 | ALL | OK | | | | | | | | | MAN REMOVED FAECES | | ✓ | | | | | ✓ | | AE |
| 24/8/20 | 955 | ALL | OK | | | | | | | | | | | | | | | | | | AE |
| 25/8/20 | 902 | ANC | OK | | | | | | | | | MAN REMOVED FAECES | | ✓ | ✓ | | | ✓ | ✓ | ✓ | SD |
| 26/8/20 | 941 | ANC | OK | | | | | | | | | ALL BIRDS CLEANED SWABS | | ✓ | ✓ | | | ✓ | ✓ | ✓ | PS/AE/AK |
| 27/8/20 | 938 | ANC | OK | | | | | | | | | MAN REMOVED FAECES | | ✓ | ✓ | | | ✓ | ✓ | ✓ | SD |
| 28/8/20 | 918 | ANC | OK | | | | | | | | | | | | | | | | | | SD |

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Animal Facility (AF)

# WORKSHEET: Animal and Isolator Monitoring Sheet

WS No.: AH/AIMLS/05C | Date issued: 05/02/2018 | Page 1 of 1

Building No.: 423 Isolator No.: L13 Isolator Pressure: NEGATIVE Start Date: 11 AUG 20 End Date: 9 SEP 2020

Experiment Procedure: EFFICACY OF SALMONELLA ENTERITIDIS AUTOGENOUS VACCINE IN COMMERCIAL LAYERS (SCA 20-10)

Experiment Group: (1) NEGATIVE CONTROL. Researcher: SOLEKHA.

Species: CHICKEN Source: CARNABOOK Egg Access No.: NIL Age: 47 WKS Animal Numbers: AS PER SPLOTD SHEET

APCAH Accession No.: N/A Animal House Accession No.: 024/2020 AEEC No.: 1915043-1 AQIS/OGTR No.: N/A

Feed Type: COMMERCIAL Water Type: POTABLE Waste and Faecal Disposal: GENERAL WASTE

| DATE | TIME | Bird Number | Clinical Signs (tick appropriate boxes and adhere to the Intervention Criteria sheet) | | | | | | | | | | Comments or Procedures performed (including comments on any clinical signs seen) | FEED | | WATER | | FILTER | | FAECES | | Sign |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Normal | Alertness | Posture | Movement | Feathers | Respiratory signs | Eyes | Body condition | Diarrhoea | Other | | New bag | Top up | Fill | HCL | Clean | New | Roll up | Removed | |
| 29/8/20 | 833 | ALL | OK | | | | | | | | | | Filled non drinker | | | ✓ | | | | | | AC |
| 30/8/20 | 827 | ALL | OK | | | | | | | | | | | · | ✓ | | | | | | | AC |
| 31/8/20 | 736 | ALL | OK | | | | | | | | | | MAN DRINKER FILLED | ✓ | | ✓ | | ✓ | | ✓ | ✓ | JD |
| 1/9/20 | 903 | ALL | OK | | | | | | | | | | | | ✓ | | | | | | | JD |
| 2/9/20 | 731 | ALL | OK | | | | | | | | | | | | | ✓ | | | | ✓ | | JD |
| 3/9/20 | 809 | ALL | OK | | | | | | | | | | MAN DRINKER FILLED | ✓ | ✓ | ✓ | | | | | | JD |
| 4/9/20 | 746 | ALL | OK | | | | | | | | | | | ✓ | | ✓ | | ✓ | | ✓ | ✓ | JD |
| 5/9/20 | 826 | ALL | OK | | | | | | | | | | MAN drinker filled | | | | | | | | | AC |
| 6/9/20 | 934 | ALL | OK | | | | | | | | | | | | ✓ | | | | | | | AC |
| 7/9/20 | 1029 | ALL | OK | | | | | | | | | | MAN DRINKER FILLED | ½ | ✓ | ✓ | | | | ✓ | | PD/JD |
| 8/9/20 | 716 | ALL | OK | | | | | | | | | | | | | | | | | | | JD |
| 9/9/20 | 751 | | | | | | | | | | | ALL | BIRDS CULLED + SENT FOR POST MORTEM | | | | | | | | | PD/JD |

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Animal Facility (AF)

# WORKSHEET: Animal and Isolator Monitoring Sheet

WS No.: AH/AIMLS/05C

Date issued: 05/02/2018



Building No.: 423 Isolator No.: 49 Isolator Pressure: NEGATIVE Start Date: 11 AUG 20 End Date: 9 SEP 20

Experiment Procedure: EFFICACY OF SALMONELLA ENTERITIDIS AUTOGENOUS VACCINE IN COMMERCIAL LAYERS

Experiment Group: (2) ST + VE VACCINATED

Species: CHICKEN Source: CARLSBROOK Researcher: SCOLEMA

APCAH Accession No.: A14 Animal House Accession No.: 024/2020 AEEC No.: 1915043-1 Animal Numbers: 40 PER SPREADSHEET

Feed Type: COMMERCIAL Water Type: POTABLE Waste and Faecal Disposal: GENERAL WASTE

| DATE | TIME | Bird Number | Clinical Signs (tick appropriate boxes and adhere to the Intervention Criteria sheet) | | | | | | | | | | Comments or Procedures performed (including comments on any clinical signs seen) | FEED | | WATER | | FILTER | | FAECES | | Sign |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Normal | Alertness | Posture | Movement | Feathers | Respiratory signs | Eyes | Body condition | Diarrhoea | Other | | New bag | Top up | Fill | HCL | Clean | New | Roll up | Removed | |
| 7/8/20 | 1303 | ALL | OK | | | | | | | | | | | | | | | | | | | |
| 8/8/20 | 849 | ALL | OK | | | | | | | | | 16 | BIRDS WEIGHED CLOACAL SWAB + PLACES | | | | | | | | | JD |
| 9/8/20 | 846 | ALL | OK | | | | | | | | | | | | | | | | | | | JD |
| 10/8/20 | 809 | ALL | OK | | | | | | | | | | | | | | | | | | | AC |
| 11/8/20 | 933 | ALL | OK | | | | | | | | | | | | | | | | | | | AC |
| 12/8/20 | 928 | ALL | OK | | | | | | | | | ALL | BIRDS GIVEN VANCOMYCIN | | ✓ | ✓ | | | | | | JD |
| 12/8/20 | 1127 | ALL | OK | | | | | | | | | ALL | BIRDS GIVEN VANCOMYCIN / MAN DRINKER FILLED | ✓ | ✓ | ✓ | | | | | | PS/JD/AC |
| 13/8/20 | 806 | ALL | OK | | | | | | | | | ALL | BIRDS CHALLENGED (OLAL) | | | | | | | | | PD/JD/AC |
| 14/8/20 | 822 | ALL | OK | | | | | | | | | | | ✓ | | | | | | | | PS/AC/JD |
| 15/8/20 | 834 | ALL | OK | | | | | | | | | | | ✓ | ✓ | | | ✓ | | ✓ | ✓ | JD |
| 16/8/20 | 838 | ALL | OK | | | | | | | | | | | | | | | | | | | JD |
| 17/8/20 | 812 | ALL | OK | | | | | | | | | | | | | | | | | | | AC |
| 18/8/20 | 801 | ALL | OK | | | | | | | | | | | | | | | | | | | AC |
| 19/8/20 | 957 | ALL | OK | | | | | | | | | | MAN DRINKER FILLED | | ✓ | ✓ | | ✓ | | ✓ | ✓ | JD ? |
| 20/8/20 | 819 | ALL | OK | | | | | | | | | | ALL BIRDS CLOACAL SWAB | | | ✓ | | | | | | JD |
| 21/8/20 | 837 | ALL | OK | | | | | | | | | | | ✓ | ✓ | ✓ | | | ✓ | | | JD/AK/D |
| 22/8/20 | 815 | ALL | OK | | | | | | | | | | | | | ✓ | | | | | | JD |
| 23/8/20 | 946 | ALL | OK | | | | | | | | | | | ✓ | | ✓ | | ✓ | | ✓ | ✓ | JD |
| 24/8/20 | 1014 | ALL | OK | | | | | | | | | | | | | | | | | | | AC |
| 25/8/20 | 812 | ALL | OK | | | | | | | | | | MAN DRINKER FILLED | | ✓ | | | | | | | AC |
| 26/8/20 | 957 | ALL | OK | | | | | | | | | | | ✓ | | ✓ | | ✓ | | ✓ | ✓ | AC |
| 27/8/20 | 744 | ALL | OK | | | | | | | | | | ALL BIRDS CLOACAL SWAB | | ✓ | ✓ | | | | | | JD |
| 28/8/20 | 831 | ALL | OK | | | | | | | | | | | ✓ | ✓ | ✓ | | | ✓ | | | PS/JD/AC |
| | | | | | | | | | | | | | | | | | | | | | | JD |

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# WORKSHEET: Animal and Isolator Monitoring Sheet

WS No.: AH/AIMLS/05C | Date issued: 05/02/2018 | Page 1 of 1

Building No.: 423 Isolator No.: L9 Isolator Pressure: NEGATIVE Start Date: 11 AUG 20 End Date: 9 SEP 20

Experiment Procedure: EFFICACY OF SALMONELLA ENTERITIDIS AUTOGENOUS VACCINE IN COMMERCIAL LAYERS (SCX 20-10

Experiment Group: (2) ST + SE VACCINATED Researcher: SCOLEXIA.

Species: CHICKEN Source: GREENWICH Egg Access No.: N/A Age: 47 WKS Animal Numbers: AS PER SPLOTD SHEET

APCAH Accession No.: N/A Animal House Accession No.: 024/2020 AEEC No.: 1915043-1 AQIS/OGTR No.: N/A

Feed Type: COMMERCIAL Water Type: POTABLE Waste and Faecal Disposal: GENERAL WASTE

| DATE | TIME | Bird Number | Clinical Signs (tick appropriate boxes and adhere to the Intervention Criteria sheet) | | | | | | | | | | Comments or Procedures performed (including comments on any clinical signs seen) | FEED | | WATER | | FILTER | | FAECES | | Sign |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Normal | Alertness | Posture | Movement | Feathers | Respiratory signs | Eyes | Body condition | Diarrhoea | Other | | New bag | Top up | Fill | HCL | Clean | New | Roll up | Removed | |
| 29/8/20 | 840 | ALL | OK | | | | | | | | | | MAN DRINKER FILLED | | | ✓ | | | | | | AE |
| 30/8/20 | 833 | ALL | OK | | | | | | | | | | | | ✓ | | | | | | | AE |
| 31/8/20 | 752 | ALL | OK | | | | | | | | | | | ✓ | ✓ | ✓ | | ✓ | | ✓ | ✓ | JD |
| 1/9/20 | 908 | ALL | OK | | | | | | | | | | | | | | | | | | | JD |
| 2/9/20 | 737 | ALL | OK | | | | | | | | | | | | | ✓ | | | | ✓ | | JD |
| 3/9/20 | 823 | ALL | OK | | | | | | | | | | MAN DRINKER FILLED | ✓ | ✓ | ✓ | | | | | | JD |
| 4/9/20 | 758 | ALL | OK | | | | | | | | | | | ✓ | ✓ | ✓ | | ✓ | | ✓ | ✓ | JD |
| 5/9/20 | 830 | ALL | OK | | | | | | | | | | | | | | | | | | | AS |
| 6/9/20 | 840 | ALL | OK | | | | | | | | | | | ✓ | | | | | | | | AS |
| 7/9/20 | 1040 | ALL | OK | | | | | | | | | | MAN DRINKER FILLED | | ✓ | ✓ | | | | ✓ | | PS/JD |
| 8/9/2 | 720 | ALL | OK | | | | | | | | | | | | | | | | | | | JD |
| 9/9/20 | 830 | ALL | OK | | | | | | | | | ALL | BIRDS CULLED - SENT FOR POST MORTEM | | | | | | | | | PS/JD |

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Asia-Pacific Center for Animal Health, Faculty of Veterinary & Agricultural Sciences, The University of Melbourne

Animal Facility (AF)

# WORKSHEET: Animal and Isolator Monitoring Sheet

WS No.: AH/AIM/LS/05C | Date issued: 05/02/2018 | Page 1 of 1

Building No.: 423 Isolator No.: L8 Isolator Pressure: NEGATIVE Start Date: 12 AUG 2020 End Date: 9 SEP 2020

Experiment Procedure: EFFICACY OF SALMONELLA ENTERITIDIS AUTOGENOUS VACCINE IN COMMERCIAL LAYERS

Experiment Group: (3) POSITIVE CONTROL Researcher: SCHLEGER.

Species: CHICKEN Source: GOONDIWINDI Egg Access No.: N/A Age: 47 WKS Animal Numbers: AS PER SPLITS SHEET

APCAH Accession No.: N/A Animal House Accession No.: 024/2020 AEEC No.: 1915043-1 AQIS/OGTR No.: N/A

Feed Type: COMMERCIAL Water Type: POTABLE Waste and Faecal Disposal: GENERAL WASTE

| DATE | TIME | Bird Number | Clinical Signs (tick appropriate boxes and adhere to the Intervention Criteria sheet) | | | | | | | | | | Comments or Procedures performed (including comments on any clinical signs seen) | FEED | | WATER | | FILTER | | FAECES | | Sign |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Normal | Alertness | Posture | Movement | Feathers | Respiratory signs | Eyes | Body condition | Diarrhoea | Other | | New bag | Top up | Fill | HCL | Clean | New | Roll up | Removed | |
| 7/8/20 | 1245 | ALL | OK | | | | | | | | | 16 | BIRDS WEIGHED, CLOACAL SWAB + PLACED | | | | | | | | | PS/JD |
| 8/8/20 | 858 | ALL | OK | | | | | | | | | | | | | | | | | | | AC |
| 9/8/20 | 856 | ALL | OK | | | | | | | | | | MAN DRINKER FILLED | | | ✓ | | | | | ✓ | AC |
| 10/8/20 | 759 | ALL | OK | | | | | | | | | | | | ✓ | ✓ | | | | | | JD |
| 11/8/20 | 921 | ALL | OK | | | | | | | | | ALL | BIRDS GIVEN VANCOMYCIN | | | ✓ | | | | | | PS/JD/AC |
| 12/8/20 | 910 | ALL | OK | | | | | | | | | ALL | BIRDS GIVEN VANCOMYCIN | ✓ | ✓ | ✓ | | | | | | PS/JD/AC |
| 12/8/20 | 1117 | ALL | OK | | | | | | | | | ALL | BIRDS CHALLENGED (OLAC). | | | | | | | | | PS/AC/JD |
| 13/8/20 | 818 | ALL | OK | | | | | | | | | | | ✓ | | ✓ | | ✓ | | ✓ | ✓ | JD |
| 14/8/20 | 830 | ALL | OK | | | | | | | | | | MAN DRINKER FILLED | ✓ | ✓ | ✓ | | | | | | JD |
| 15/8/20 | 838 | ALL | OK | | | | | | | | | | | | | | | | | | | AC |
| 16/8/20 | 842 | ALL | OK | | | | | | | | | | | | | | | | | | | AC |
| 17/8/20 | 922 | ALL | OK | | | | | | | | | | | | ✓ | ✓ | | ✓ | | ✓ | ✓ | JD |
| 18/8/20 | 809 | ALL | OK | | | | | | | | | | MAN DRINKER FILLED | | | ✓ | | | | | | JD |
| 19/8/20 | 1009 | ALL | OK | | | | | | | | | | ALL BIRDS CLOACAL SWABS | ✓ | ✓ | ✓ | | | | ✓ | | PS/JD/AC |
| 20/8/20 | 824 | ALL | OK | | | | | | | | | | | | | ✓ | | | | | | JD |
| 21/8/20 | 853 | ALL | OK | | | | | | | | | | MAN DRINKER FILLED | ✓ | ✓ | ✓ | | ✓ | | ✓ | ✓ | JD |
| 22/8/20 | 919 | ALL | OK | | | | | | | | | | | | | | | | | | | AC |
| 23/8/20 | 850 | ALL | OK | | | | | | | | | | | | | | | | | | | AC |
| 24/8/20 | 1034 | ALL | OK | | | | | | | | | | MAN drinker filled | ✓ | | ✓ | | ✓ | | ✓ | ✓ | AC |
| 25/8/20 | 819 | ALL | OK | | | | | | | | | | | | ✓ | ✓ | | | | | | JD |
| 26/8/20 | 1012 | ALL | OK | | | | | | | | | | ALL BIRDS CLOACAL SWABS | ✓ | ✓ | | | | | ✓ | | PS/JD/AC |
| 27/8/20 | 748 | ALL | OK | | | | | | | | | | MAN DRINKER FILLED | | | ✓ | | | | | | JD |
| 28/8/20 | 840 | ALL | OK | | | | | | | | | | | ✓✓ | ✓ | ✓ | | ✓ | | ✓ | ✓ | JD |

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Asia-Pacific Centre for Animal Health, Faculty of Veterinary & Agricultural Sciences, The University of Melbourne

Animal Facility (AF)

# WORKSHEET: Animal and Isolator Monitoring Sheet

WS No.: AH/AIMLS/05C

Date issued: 05/02/2018



Building No.: 423 Isolator No.: L 8 Isolator Pressure: NEGATIVE Start Date: 11 AUG 20 End Date: 9 SEP 20

Experiment Procedure: EFFICACY OF SALMONELLA ENTERITIDIS AUTOGENOUS VACCINE IN COMMERCIAL LAYERS.

Experiment Group: (3) POSITIVE CONTROL

Species: CHICKEN Source: CARLSBROOK Researcher: SCELETHA

APCAH Accession No.: N/A Egg Access No.: N/A Age: 47 WKS. Animal Numbers: AS PER SPREADSHEET

Feed Type: COMMERCIAL - Water Type: POTABLE Waste and Faecal Disposal: GENERAL WASTE

| DATE | TIME | Bird Number | Clinical Signs (tick appropriate boxes and adhere to the Intervention Criteria sheet) | | | | | | | | | | Comments or Procedures performed (Including comments on any clinical signs seen) | FEED | | WATER | | FILTER | | FAECES | | Sign |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Normal | Alertness | Posture | Movement | Feathers | Respiratory Signs | Eyes | Body condition | Diarrhoea | Other | | New bag | Top up | Fill | HCL | Clean | New | Roll up | Removed | |
| 29/8/20 | 846 | ALL | OK | | | | | | | | | | MAN DRINKER FILLED | | | ✓ | | | | | | |
| 30/8/20 | 838 | ALL | OK | | | | | | | | | | | | ✓ | | | | | | | AC |
| 31/8/20 | 801 | ALL | OK | | | | | | | | | | | | | | | | | | | AC |
| 1/9/20 | 911 | ALL | OK | | | | | | | | | | | ✓ | ✓ | ✓ | | ✓ | | ✓ | ✓ | JO |
| 2/9/20 | 748 | ALL | OK | | | | | | | | | | MAN DRINKER FILLED | | | ✓ | | | | | | JO |
| 3/9/20 | 828 | ALL | OK | | | | | | | | | | | | | ✓ | | | | ✓ | | JO |
| 4/9/20 | 814 | ALL | OK | | | | | | | | | | MAN DRINKER FILLED | | | ✓ | | | | | | JO |
| 5/9/20 | 834 | ALL | OK | | | | | | | | | | | ✓ | ✓ | ✓ | | ✓ | | ✓ | ✓ | JO |
| 6/9/20 | 903 | ALL | OK | | | | | | | | | | MAN DRINKER FILLED | | | ✓ | | | | | | AC |
| 7/9/20 | 1046 | ALL | OK | | | | | | | | | | | ✓ | ✓ | ✓ | | | | | | AC |
| 8/9/20 | 723 | ALL | OK | | | | | | | | | | | | | | | | | ✓ | | PS/JO |
| 9/9/20 | 915 | ALL | | | | | | | | | | ALL | BIRDS CULLED + SENT FOR POST MORTEM | | | | | | | | | JO |
| | | | | | | | | | | | | | | | | | | | | | | PS/JO |

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## Appendix 3: Results of *Salmonella* spp. isolation from cloacal swabs.

**Veterinary Microbiology Laboratory**

The University of Melbourne
Faculty of Veterinary and Agricultural Sciences
250 Princes Highway Werribee VIC 3030
Phone: 03 9731 2044
Fax: 03 9731 2377
Email: vet-micro@unimelb.edu.au

17th September 2020

**Client:** Scolexia Pty LTD
**Contact:** Dr. Jose Quinteros

Salmonella Vaccine Trial August September 2020

**Salmonella isolation**

Samples were received in swab form. Salmonella isolation involved a two stage enrichment process in Buffered Peptone Water and Rappaport Vassiliadis media before plating on chromogenic selective media. Supect Salmonellae were then confirmed by latex agglutination testing.

Results are summarised in the following tables.

Table 1: Cloacal swab Pools (7/8/2020)

3 Groups of 16 swabs pooled.

| Group | *Salmonella spp.* detected |
|---|---|
| Group 1 | No |
| Group 2 | No |
| Group 3 | No |

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Table 2: Cloacal Swabs (19/8/2020) *Salmonella spp* Detected.

| Group 1 | Tags | Result | Group 2 | | Group 3 | |
|---|---|---|---|---|---|---|
| Pool 1 | 13 | No | 7 | Yes | 2 | Yes |
| | 26 | | 11 | Yes | 7 | No |
| | 27 | | 13 | No | 9 | Yes |
| | 31 | | 24 | Yes | 10 | Yes |
| Pool 2 | 34 | No | 33 | No | 16 | Yes |
| | 43 | | 54 | Yes | 25 | Yes |
| | 44 | | 59 | No | 33 | Yes |
| | 50 | | 69 | Yes | 35 | Yes |
| Pool 3 | 52 | No | 70 | Yes | 51 | Yes |
| | 56 | | 72 | Yes | 61 | No |
| | 67 | | 74 | Yes | 62 | Yes |
| | 69 | | 76 | Yes | 74 | Yes |
| Pool 4 | 73 | No | 79 | Yes | 78 | Yes |
| | 87 | | C99 | Yes | 79 | Yes |
| | 88 | | White 29 | Yes | 84 | Yes |
| | 89 | | Yellow 29 | Yes | 86 | Yes |



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Table 3: Cloacal Swabs (26/8/2020) *Salmonella spp* Detected.

| Group 1 | Tags | | Group 2 | | Group 3 | |
|---|---|---|---|---|---|---|
| Pool 1 | 31 | No | 7 | Yes | 2 | No |
| | 89 | | 11 | No | 7 | Yes |
| | 56 | | 13 | No | 9 | Yes |
| | 13 | | 24 | Yes | 10 | Yes |
| Pool 2 | 26 | No | 33 | No | 16 | No |
| | 53 | | 54 | Yes | 25 | No |
| | 88 | | 59 | No | 33 | No |
| | 50 | | 69 | Yes | 35 | Yes |
| Pool 3 | 34 | No | 70 | Yes | 51 | Yes |
| | 69 | | 72 | Yes | 61 | No |
| | 67 | | 74 | Yes | 62 | Yes |
| | 27 | | 76 | Yes | 74 | Yes |
| Pool 4 | 43 | No | 79 | Yes | 78 | Yes |
| | 44 | | C99 | Yes | 79 | Yes |
| | 73 | | White 29 | Yes | 84 | Yes |
| | 87 | | Yellow 29 | No | 86 | No |

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Table 4: Cull swabs (9/9/2020) Group 1. *Salmonella spp.* Detected

| Group 1 | Tags | Site | | | |
|---|---|---|---|---|---|
| | | Cloaca | Caecum | Air Sac | Ovarian Surface |
| Pool 1 | 69, 44, 67, 43 | No | No | No | No |
| Pool 2 | 73, 53, 13, 89 | No | No | No | No |
| Pool 3 | 34, 26, 31, 50 | No | No | No | No |
| Pool 4 | 88, 56, 27, 87 | No | No | No | No |

Table 5: Cull swabs (9/9/2020) Group 2. *Salmonella spp.* Detected

| Group 2 | Site | | | |
|---|---|---|---|---|
| Tags | Cloaca | Caecum | Air Sac | Ovarian Surface |
| 7 | Yes | Yes | No | No |
| 11 | No | No | No | No |
| 13 | Yes | No | No | No |
| 24 | No | Yes | No | No |
| 33 | Yes | No | No | No |
| 54 | No | Yes | No | No |
| 59 | No | Yes | No | No |
| 69 | No | Yes | No | No |
| 70 | Yes | Yes | No | No |
| 72 | No | No | Yes | No |
| 74 | No | No | No | No |
| 76 | No | Yes | No | No |
| 79 | Yes | Yes | Yes | No |
| C99 | No | No | No | No |
| Yellow 29 | No | Yes | No | No |
| White 29 | No | No | No | No |

Table 6: Cull swabs (9/9/2020) Group 3. *Salmonella spp.* Detected

| Group 3 | Site | | | | |
|---|---|---|---|---|---|
| Tags | Cloaca | Caecum | Air Sac | Ovarian Surface | Ovum |
| 2 | No | Yes | No | No | No |
| 7 | No | No | No | No | No |
| 9 | No | Yes | No | No | No |
| 10 | No | Yes | No | No | No |
| 16 | Yes | Yes | No | No | No |
| 25 | No | Yes | No | No | No |
| 33 | No | Yes | No | No | No |
| 35 | No | Yes | No | No | No |
| 51 | No | Yes | No | No | No |
| 61 | No | Yes | No | No | No |
| 62 | No | No | No | No | No |
| 74 | Yes | No | No | No | No |
| 78 | Yes | No | No | No | No |
| 79 | Yes | Yes | No | No | No |
| 84 | Yes | Yes | No | Yes | No |
| 86 | No | No | No | No | No |

Report prepared by R. Bushell

## Appendix 4: Asia-Pacific Centre for Animal Health postmortem findings and end of trial report

ASIA-PACIFIC CENTRE FOR ANIMAL HEALTH

APCAH

THE UNIVERSITY OF MELBOURNE

**15th September 2020**

**Peter Scott**
**Scolexia Animal and Avian Health Consultancy**
**21 Slater Parade,**
**Keilor East VIC 3033**

Dear Peter,
Here is the final report for the requested testing "Efficacy of *Salmonella enteritidis* autogenous vaccine in commercial layers".

**Efficacy of *Salmonella enteritidis* autogenous vaccine in commercial layers**
The study was conducted between 11th August 2020 and 09th September 2020.

**Source of chicken**
Commercial Layers of 47 weeks of age were supplied by a Victorian poultry farm for this experiment.

**Animal facility accession number**
This experiment was documented under accession number 024/2020.

**Experimental outline**
On 07th August 2020, chickens were received and placed into separate isolators as outlined in the following table. The birds were challenged on 12th August 2020, with live *Salmonella enteritidis* culture as mentioned in the following table.

**Bird groups and treatment in the experiment**

| Group | Treatment | No. of birds/group | *Salmonella typhimurium* (ST) vaccine (given on the farm) | *Salmonella enteritidis* (SE) vaccine (given on the farm) | Challenge with SE (conducted in APCAH animal facilities) | Isolator |
|---|---|---|---|---|---|---|
| 1 | Negative Control | 16 | - | - | No (media only) | L13 |
| 2 | ST+SE vaccination | 16 | + | + | Yes | L9 |
| 3 | Positive Control | 16 | - | - | Yes | L8 |

**Feeding**

Birds were provided with free access to feed and water throughout the study.

**Swab collection**

Cloacal swabs were collected from all the treatment groups before challenge and on day 7,14 and 28-days post challenge and sent to clinical microbiology laboratory for culture.
Swabs from other tissues were collected on day 28 post challenge and sent to clinical microbiology laboratory for processing.

**Clinical signs and post-mortem findings**

Chickens were monitored daily. All birds remained in good health throughout the experiment period with no clinical signs noted. All birds in the experimental groups, were euthanised on 09th September 2020 and subjected to post-mortem analysis. No gross lesions or adverse reactions were noted in any of the birds examined.

Sincerely Yours,

Amir H. Noormohammadi, DVM, PhD, MACVS
Professor in Avian Diseases
Faculty of Veterinary & Agricultural Sciences
The University of Melbourne
250 Princes Highway, Werribee, Victoria - 3030
Ph: (03) 9731 2275, Mobile: 0428 502 324
Fax: (3) 9731 2366, Email: amir.hadjinoormohammadi@unimelb.edu.au