Evaluation of vaccination program options against *Salmonella* Enteritidis 7A in laying hens in Australia

Final Project Report | **OCTOBER 2020**

A report for Australian Eggs Limited

by Peter C. Scott, Timothy Wilson,

José A. Quinteros, Arif Anwar, Tyrone Scott,

Pollob Shill, and Amir H. Noormohammadi

Australian Eggs Limited Publication No 31FS002

ISBN 978-1-920835-42-2

Project Title: Evaluation of vaccination program options against *Salmonella* Enteritidis 7A in laying hens in Australia

Australian Eggs Limited Project Number: 31FS002

Researcher/Author Contact Details
Name: Peter Scott
Address: Scolexia Pty Ltd, 8/19 Norwood Crescent, Moonee Ponds VIC 3039, Australia.
Phone: 03 9326 0106
Fax: 03 9372 7576
Email: info@scolexia.com.au

In submitting this report, the researcher has agreed to Australian Eggs Limited publishing this material in its edited form.

Australian Eggs Limited Contact Details:

Australian Eggs Limited
A.B.N: 66 102 859 585
Suite 6.02, Level 6, 132 Arthur St
North Sydney NSW 2060

Phone: 02 9409 6905
Fax: 02 9954 3133
Email: research@australianeggs.org
Website: www.australianeggs.org.au

Published in October 2020

Foreword

This project was conducted to assess the efficacy of three vaccination programs in the reduction of *Salmonella* Enteritidis isolate 7A faecal shedding, and also its colonisation of caecal tube 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.

Most of our publications are available for viewing or downloading through our website:

www.australianeggs.org.au

Printed copies of this report are available for a nominal postage and handling fee and can be requested by phoning 02 9409 6905 or emailing research@australianeggs.org.au.

Acknowledgments

The authors would like to acknowledge the assistance of ACE Laboratory Services (Bendigo, Victoria) for assistance in providing the challenge media and ELISA testing for this study.

Australian Eggs Limited provided the funds which supported this project.

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 Limited 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 Shill, 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.

Table of Contents

List of Tables

List of Figures

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
Rep-PCR	Repetitive element PCR
SE	*Salmonella enterica* serovar Enteritidis
SE 7A	Australian isolate *Salmonella* Enteritidis 7A
ST	*Salmonella enterica* serovar Typhimurium
TBE	Tris/Borate/EDTA
WOA	Weeks of age

Executive Summary

Salmonellosis caused by *Salmonella* Enteritidis (SE) has been historically considered an exotic disease in Australia. However, the isolation of the bacterium from food borne illness in the human population and its linkage with egg laying farms raised concern in the Australian poultry industry. In the present study, the objective was to evaluate and compare the efficacy of different vaccination programs in terms of protection against a laboratory-controlled exposure of laying hens to the Australian isolate SE 7A. The four vaccination programs included unvaccinated, only live *Salmonella* typhimurium (ST), only killed autogenous SE, and a combined program of live ST and killed autogenous SE. At 16 weeks of age, hens were exposed to SE 7A inside isolators equipped with HEPA filters. Cloacal samples were collected from all hens at 3, 7, 14, 21 and 28 days after exposure (DAE). Hens were humanely euthanised at 32 DAE. *Salmonella* cultures of ovaries and caeca from each bird were undertaken.

The present study demonstrated that high levels of seroconversion were achieved with the SE autogenous vaccine but not with the ST vaccine.

The results demonstrate that a very high level of protection against ovarian colonisation was achieved using a priming vaccination with two doses of live commercial ST vaccine in conjunction with two doses of an autogenous SE vaccine. However, a mid-lay study (at about 45 weeks of age) is essential to assess the duration of immunity conferred by this vaccination program.

1 Introduction

Salmonellae are potential zoonotic organisms, including several *Salmonella* serovars, such as *S.* Typhimurium (ST) and *S.* Enteritidis (SE). A proportion of human salmonellosis cases are closely related to the consumption of raw or partially cooked eggs[1].

The control of SE in commercial chicken production is of high importance. The mitigation of SE in table egg layers is multifactorial involving biosecurity, husbandry, hygiene, feed additives, and live and killed vaccines. In conjunction with those measures, the subsequent prevention of food safety events in humans depends on egg handling and food preparation practices.

Until 2018, the Australian commercial egg industry was free of SE that was causally associated with food safety events. Human cases of SE in Australia were historically and invariably involved with people who had recently travelled overseas[2]. In 2018, the first recognition of table egg associated SE food poisoning was recognised in several egg producers in NSW[3] and in early 2019 one producer in Victoria[4].

The recently identified SE was characterised and referred to as SE 7A, and has never been reported or observed previously in Australia or for that matter anywhere in the world. As reported by the Department of Primary Industries of New South Wales, genetic studies indicate the closest relative to 7A was an isolate from an Australian traveller returning from Southern Europe, who visited Croatia exclusively. While the source of the Australian infection is unclear, the likely mode was an introduction through human associated horizontal contacts and international travel. This is based on the epidemiology that from Infected Property 1 (IP1), where all other farms subsequently identified as SE positive had clearly identifiable trace back to the original IP1. Also, all SE 7A food safety cases were provisionally identified as related to table egg consumption. If another primary vector such as wild birds, rodents or a food source were involved, the epidemiological picture would have been different.

Currently in Australia there are two registered live ST vaccines that are marketed as an aid in the control of *Salmonella.* These are Vaxsafe ST (Bioproperties®) and Poulvac ST (Zoetis®) derived from the same vaccine candidate which is an *aroA* deletant mutant (STM-1) of a ST Phage Type 44, initially developed in Australia[5-8]. Work has shown that these vaccines confer some protection against ST (homologous) and also some protection against *S.* Infantis, *S.* Heidelberg and SE (heterologous)[8-12]. Routine vaccination protocol includes multiple administration of a live vaccine in pullets, which may be initially be primed by coarse aerosol of day-old chickens at the hatchery and/or followed up by several drinking water applications in the field. While there are several variations to the current program being implemented, the most common in the Australian layer industry is several live primes followed by incorporating the suspended freeze-dried vaccine in an inactivated vaccine (normally incorporated into EDS + NDV_1 adjuvanted killed vaccine) and given by intramuscular injection (IM).

Other vaccine options in Australia are autogenous inactivated adjuvanted bacterins that incorporate the specific *Salmonella* strain isolated from an affected farm, which are used in replacement pullets destined to production sites affected by potential food safety *Salmonella*. These vaccines are approved by the $APVMA_2$ with special conditions. In these cases, the program normally utilised is the live prime of the pullets followed by two doses of the autogenous *Salmonella* vaccine.

---

[1] EDS: Egg Drop Syndrome; NDV: Newcastle Disease.

[2] APVMA: Australian Pesticides and Veterinary Medicines Authority.

Australia has no registered live SE vaccine because of its freedom status from SE, and the industry believes there is still the potential for SE to be eradicated. At this stage, its introduction is not considered necessary and is also not supported because of potential country status interpretation and field differentiation. This could and would change if SE became endemic in the Australian egg/poultry industry.

An autogenous SE vaccine, with the consideration of a *Salmonella* bacterin vaccine Minor Use permit, is supported as a useful and risk-free option. It would assist while establishing freedom of existing positive farms wishing to restock, and other sites that consider themselves a high risk because of known horizontal contacts. Permits already exist in Australia for autogenous *Salmonella* vaccines, which include SE, and batches have already been made awaiting field application. One regulatory aspect of autogenous vaccines is that while they must be safe, their efficacy does not have to be proven and their use is at the discretion of the requesting veterinarian. Historical field data, published papers and conference presentations over the years, have determined that inactivated adjuvanted *Salmonella* bacterins are a useful and valuable tool in aiding the control of *Salmonella* in commercial layer flocks[13-19]. A peer reviewed paper indicates that live vaccines are more efficacious in the development of a broader immunological protective response, with this protection being augmented by the inactivated vaccine[20].

The objective of the present study was to evaluate the protection conferred by various vaccination programs including or excluding an autogenous SE 7A bacterin. Considering the availability of the existing live ST vaccines in Australia that provide some cross-protection, the level of protection of the ST live vaccine alone and in combination the SE autogenous vaccine were evaluated against an SE 7A oral exposure.

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 corresponds to a whole cell bacterin, where the bacterium was formalin inactivated, and using aluminium hydroxide and oil in water as adjuvants.

2.3 Source of hens and treatments

A total of 80 laying hens (Hy-Line Brown) were randomly selected from two different floor rearing farms (A and B), and randomly allocated in 5 groups (Table 1); both farms with a history of being free of SE. Hens from both farms had different parent flocks. Before being sent to the University of Melbourne, drag swabs were collected and pooled from the manure of the hens included in the trial (from both farms), and were sent to ACE laboratories for *Salmonella* isolation. Results confirmed that the hens were *Salmonella* free before the start of the laboratory phase. Also, the serological negative status of the negative controls also confirm that they were not previously exposed to *Salmonella* group B or D (see results below). As displayed in Figure 1, hens from Farm A were 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 weeks of age, and in Farm B pullets remained unvaccinated against live ST. In Farms A and B, at 8 weeks of age, and then at 12 weeks of age, 16 hens from each farm were vaccinated with the SE autogenous vaccine according to the grouping found in Table 1. 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.

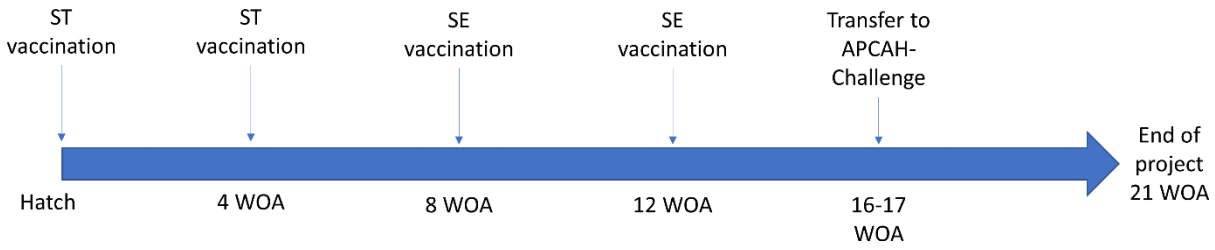


Figure 1 Experiment timeline

Birds were weighed on their arrival at the animal research facilities. Then, at 24 and 3 hours prior to exposure to SE 7A, all birds were administered 0.6 ml of the antibiotic vancomycin orally at a concentration of 100 mg/ml or approximately 30 mg/kg liveweight. Application of vancomycin has been used successfully in prior studies with ST by the authors and previously described as necessary to achieve a more stable infection rate as compared with untreated birds, and is more suitable for the study of anti-*Salmonella* interventions[21], such as vaccination. Also, when mature birds are orally

challenged with *Salmonella*, the intestinal flora of the chickens outcompete *Salmonella*[22-24], leading to the potential failure of a challenge model.

The SE 7A challenge inoculum to be used in the experiment was prepared by ACE Laboratory Services[3] at a concentration of 0.81×10^9 CFU/ml (Appendix 1), consistent with the dose used in previous publications[21, 25]. On arrival at the animal research facilities, 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.

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	16	+	+	Yes
3	ST	16	+	-	Yes
4	SE	16	-	+	Yes
5	Positive Control (PC)	16	-	-	Yes

* The exposure to SE was conducted in the HEPA PC3 isolators located in the animal research facilities of the University of Melbourne, FVAS (Werribee Campus). Hens received a live ST vaccine, an inactivated autogenous SE vaccine, a combination of the two, or none of them (controls).

The inoculum was administered to the corresponding groups at 17 weeks of age 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. After exposure, hens were monitored daily and the 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 weeks of age (one week before SE 7A autogenous vaccine booster) and at 16 weeks of age (one week before exposure to SE 7A), and then each serum used in three different ELISA tests (BioChek®, Unit 5 Kings Ride Business Park, Kings Ride, Ascot, Berkshire SL5 8BP, UK) to detect Groups B, D and B+D *Salmonella* antigens, following the manufacturer's directions. For all ELISA tests, there was a cut-off value established by BioChek® to discriminate between positive and negative samples. The titre cut-off value for both Group B and Group D antigen ELISA tests was 654, while for Group B+D antigen ELISA, the sample to positive ratio (S/P) cut-off value was 0.5. At the time of transferring the hens to APCAH, two drag swabs embedded with peptone water were collected from the manure belts, pooled and sent to ACE Laboratory Services to attempt the isolation of *Salmonella* spp.

On arrival of the hens at the animal research facilities, hens were individually weighed, and the weights recorded. Hens were also weighed at the end of the study following euthanasia.

A cloacal swab was taken from each individual bird just before the exposure to SE 7A (day 0), and at 3, 7, 14, 21 and 28 days after exposure (DAE). Each swab was embedded in peptone water before sample collection from the cloaca. All swabs were sent to the microbiology laboratory at the University of Melbourne (Werribee Campus), to attempt *Salmonella* isolation.

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

2.5 Post-mortem analysis

Hens were humanly euthanised at 32 DAE using an intravenous injection of barbiturates, according to the protocol approved by the animal ethics committee. The post-mortem analysis started with the hens from the negative control group, followed by the vaccinated and exposed groups, with the positive control group (unvaccinated and exposed) examined last. The post-mortem of the negative control group was performed in a post-mortem room, while the same procedure for those birds exposed to SE was performed inside their corresponding HEPA filtered isolators. During the post-mortem, caecal contents and ovaries were swabbed from all the hens. The caecal samples were taken by opening the caecal tubes and swabbing the inner part of the tube, collecting its content. The ovarian samples were collected by swabbing the surface and then breaking the external membrane of the largest follicle and swabbing the follicular content. In the case of pericardium and air-sac membrane swabs, 5 birds per group were selected. In the cases of the pericardial samples, the external membrane was opened, and the swab was taken from the space between the outer and inner membrane. And in the case of the air-sac membrane samples, the sample was taken after the removal of the oblique septum, from the surface of the abdominal air-sac membrane. All samples were sent to the microbiology laboratory at the University of Melbourne (Werribee Campus) to attempt *Salmonella* isolation.

2.6 Protective index

A protective index (PI) was calculated using the data in Table 4. The PI allows the measurement of the level of protection conferred by the vaccination against the SE 7A exposure. The formula used to calculate the PI was the following:

$$PI = \frac{100 \times (\%\ positives\ in\ unvaccinated\ group - \%\ positives\ in\ vaccinated\ group)}{\%\ positives\ in\ unvaccinated\ group}$$

2.7 Rep-PCR for *Salmonella* identification

In order to confirm the identity of the *Salmonella* isolates obtained from the post-mortem samples, a repetitive element polymerase chain reaction (Rep-PCR) was conducted. A Rep-PCR utilises primers that bind randomly on the genome of the target organism generating a series of random products of different length, which are used as a 'fingerprint'. The samples selected for the Rep-PCR were one caecal sample isolate from the ST+SE group (a hen that was also positive in the pericardial sample), and one caecal and two ovarian samples isolates from ST, SE and PC groups. The hens from ST, SE and PC groups, from which these caecal isolates were selected for the Rep-PCR, were also positive by isolation from the air-sac membrane samples. Also, the hens from which these ovarian isolates were selected for the Rep-PCR were also positive by isolation from the caecal samples.

From these samples, DNA was extracted using QIAGEN® DNA mini kit. The primers used were $(GTG)_5$, which have been previously used for Rep-PCR[26, 27]. The DNA was visualised by using 1.5% agarose gel in 0.5× Tris/Borate/EDTA (TBE) at 80 volts for 60 minutes and stained with gel red. As a control for the Rep-PCR, an aliquot of the inoculum used during the exposure was utilised. This aliquot was taken during the exposure day, and saved at -80°C, as previously described. In those groups with cloacal swabs positive to *Salmonella*, one sample per group was tested. In those with ovarian swabs positive to *Salmonella*, two samples were tested.

2.8 Statistical analysis

For the comparison of proportions, a 2×2 contingency table was used as displayed in Table 2. Calculation of the χ^2 value and Fisher's exact test was undertaken using the software package GraphPad Prism, version 8.4.2 (GraphPad Software, La Jolla California USA). For the comparison of weight gain, a two-way analysis of variance (ANOVA) was used, using the Tukey's multiple comparison test. 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.

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 serum was tested for the presence of antibody to *Salmonella* Group B, D, or B+D. This sampling was undertaken before the SE autogenous vaccine booster at 11 weeks of age (11 WOA), and before transfer of the hens to the animal research facilities and exposure to SE 7A at 16 WOA, 4 weeks after the SE vaccine booster was applied. Results are displayed in Table 3. Neither the positive nor negative control birds showed antibodies against *Salmonella* Group B or D. Hens tested from group 3, which were vaccinated at the day of hatch and at 4 weeks of age with a live ST vaccine, were negative in all of the ELISA tests. On the other hand, at 11 WOA, hens from both the ST+SE and SE groups exhibited reaction in both Group D and B+D antigen ELISA tests, with only one positive hen from the ST+SE group in the Group B antigen ELISA test. At 16 WOA (after the SE booster) all the serum samples from hens allocated in both the ST+SE and SE groups were positive in both Group D and B+D antigen ELISA tests, with only one positive hen in the ST+SE group in the Group B antigen ELISA test. As stated above, at this stage the two drag swabs taken from the manure belt were negative to *Salmonella* spp. (Appendix 3).

In order to follow the course of reaction before and after the booster vaccination, the same hens were bled during both rounds of ELISA tests at 11 and 16 WOA (identified by their leg tags). The ELISA results per hen are represented in Figure 2.

Table 3 Number of serums positive to *Salmonella* antibodies (ELISA) from blood samples taken before the booster vaccination with SE autogenous vaccine, and after the booster and before their exposure to SE 7A

			Positives 11 WOA (before booster with SE)*			Positives 16 WOA (after booster with SE)*		
Group	Treatment	Farm	B+D	B	D	B+D	B	D
1	NV (NC)	B	0	0	0	0	0	0
2	ST+SE	A	3	1	3	5	1	5
3	ST	A	0	0	0	0	0	0
4	SE	B	5	0	4	5	0	5
5	NV (PC)	B	0	0	0	0	0	0

* 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.

It is possible to see that, in both NC and PC groups, and also in the ST group, there was a very slight and undistinguishable increase, but in most of the cases a decrease in titre between 11 and 16 WOA in all the ELISA tests. In those hens from the ST+SE group, there was an increase from 3 to 5 positives in the Group D ELISA test between 11 and 16 WOA. It is possible to see that there was a decrease in

titre in two out of five individuals. However, that decrease in titre is not biologically significant, as the two hens remained positive (the titres in both samples went from 3102 to 2689, and from 1288 to 1241, when the cut-off value for the test was 654). In the Group B ELISA, it is possible to see that the hen positive at 11 and 16 WOA was not the same in both sampling days, one of them with an important increase in titre between 11 and 16 WOA. In those hens from the SE group, there was not only an increase from 3 to 5 positives in the Group D ELISA between 11 and 16 WOA. In 4 out of 5 there was an important increase in titres before and after the booster with the autogenous vaccine. In the hen where it was possible to see a decrease in titre between 11 and 16 WOA, it went from 4213 to 3493, still substantially beyond the cut-off limit of 654 for the test.

A similar situation can be found in the results from the Group B+D ELISA test of the hens from the SE group, where there was an increase in titres from 11 to 16 WOA in the sera from all the hens tested. In the same group, all sera had an increase in titres between 11 and 16 WOA in the Group B ELISA test, but all remained negative.

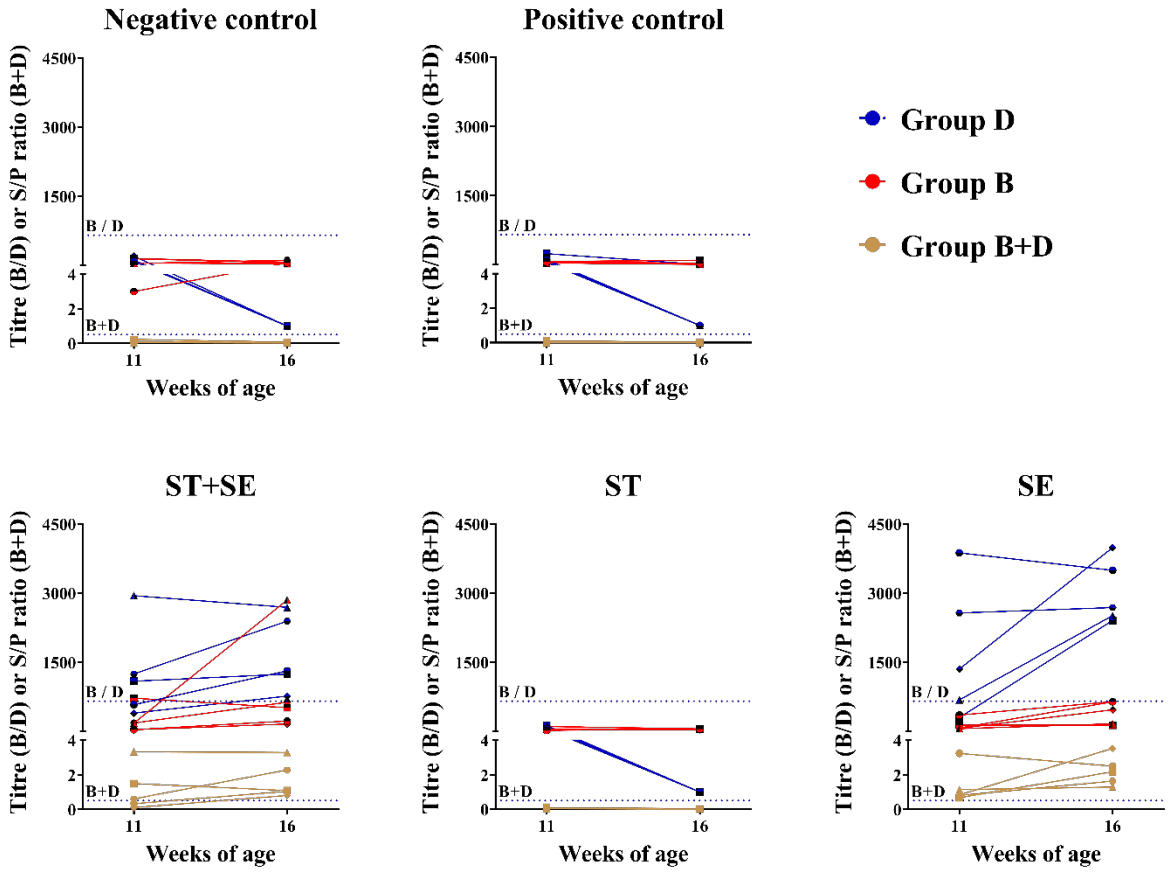


Figure 2 Difference in titres measured by the ELISA technique from blood samples taken from the hens included in the SE trial at two different times

The first samples, at 11 weeks of age (WOA), were taken 3 weeks after the first vaccination with the SE autogenous vaccine in the hens from the corresponding groups and one week before the second vaccination. The second samples were taken at 16 WOA, one day before transport of the hens to the animal research facilities and one week before the exposure of the hens to SE 7A under isolated conditions. Each line represents an individual hen. Solid lines represent hens that had an increase in titre of that ELISA test between 11 and 16 WOA. Segmented lines represent hens that had a decrease in titre of that ELISA between 11 and 16 WOA. The horizontal dotted lines represent the cut-off limit determined by the ELISA tests. The upper line (at a titre of 654) represents the cut-off limit of both Group B and D ELISA tests, while the lower line (at a S/P ratio of 0.5) represents the cut-off limit of the Group B+D ELISA test.

3.2 Cloacal swabs

As expected under the controlled experimental conditions, swabs collected before the exposure to SE 7A were negative in all the groups included in the trial (Figure 3). At 3 and 7 DAE, all swabs collected from the SE 7A exposed groups were positive to *Salmonella* isolation, while the unexposed group remained negative. In hens from the ST group, there was a drop in the number of positives to 81% at 14 DAE, but then it increased to 94% and 100% at 21 and 28 DAE, respectively. In those from the SE group, the percentage of positive swabs decreased to 94% at 14 DAE, then decreased further to 81% at 21 DAE, increasing to 88% at 28 DAE. Hens from ST+SE group had a decrease in the number of positives to 88% at 14 DAE, and then to 75% at 21 and 28 DAE. In the PC group, there was a decrease in the proportion of positive samples from 100% to 94% (the sample from one hen) at 14 DAE that remained at 21 and 28 DAE (Figure 3). However, the hen that was negative on the test at day 14 was positive at day 21 and 28, and at day 21, a new hen became positive, and then again negative at day 28. Once again, a new hen became positive at day 28 that was negative at 14 and 21 days. Hens from the negative control group all remained negative during the entire duration of the experiment.

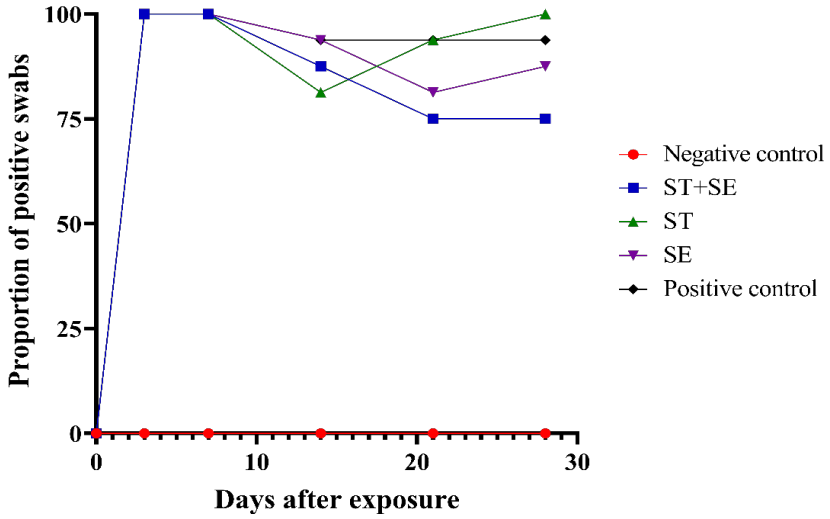


Figure 3 Proportion of cloacal swabs positive to *Salmonella* isolation

Samples were taken at 0-, 3-, 7-, 14-, 21- and 28-days after oral exposure of the hens to SE 7A. At days 3, 7 and 14, the data of the positive control group (black diamonds) overlap with the data from the SE group (purple inverted triangles).

3.3 Weight gain

The total weight gain of the hens in any of the SE 7A exposed groups (including the PC group) was not lower compared with the hens in the NC group (Figure 4). However, the total weight gain of the hens in the ST+SE group was significantly higher (405 ± 68 grams) than those of the positive control group (234 ± 79 grams). Even though the mean weights in the hens of the PC group were lower than those of the other groups (NC, ST and SE groups), those differences were not statistically significant.

3.4 Post-mortem

During the post-mortem analysis, no hen presented significant gross lesions (Appendix 4). Swabs were collected from the inner part of the caecum, the most developed follicle of the ovary, the pericardium and the air-sac membrane. While the caecal and ovarian swabs were taken in all of the hens examined,

the pericardium and air-sacs sabs were taken from 5 hens per group. The total number of positives per group and the sites tested can be found in Table 4, and graphic representation of the proportion of positives and negatives per group from the caecal and ovary samples in Figure 5. During sample collection, one caecal swab from the NC group and one ovary swab from the ST+SE were discarded for technical reasons, reducing the total number of samples to 15 instead of 16.

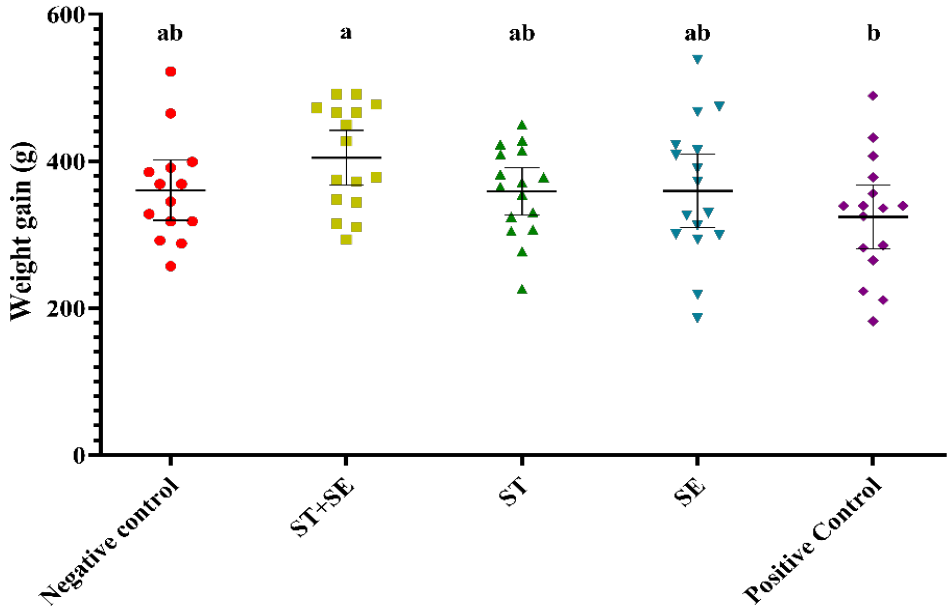


Figure 4 Total weight gain of hens in the different treatment groups

The central horizontal line and error bars indicate the mean and the 95% confidence interval, respectively. The initial weights were recorded on the arrival of the hens at the research facilities, and the final weights were recorded during the post-mortem day, 37 days after their arrival at the research facilities and 31 days after exposure. Different letters represent differences that are statistically significant ($p < 0.05$).

As expected, there were no samples positive to *Salmonella* in the NC group. In hens from the PC group, all of the swabs taken from the caecal tubes were positive to *Salmonella*, indicative of a successful challenge model, while 6 of the ovarian and 3 of the air-sac samples were positive.

Table 4 Number and proportion of samples positive to *Salmonella*, collected during post-mortem

Treatment	Number and proportion of positives					
	Caeca	%	Ovary	%	Pericardium	Air-sacs
Negative control	0 of 15^{a}	0.0%	0 of 16^{a}	0.0%	0 of 5	0 of 5
ST+SE	7 of 16^{b}	43.8%	0 of 15^{a}	0.0%	1 of 5	0 of 5
ST	15 of 16^{c}	93.8%	3 of 16^{ab}	18.8%	1 of 5	2 of 5
SE	13 of 16^{bc}	81.3%	6 of 16^{b}	37.5%	0 of 5	2 of 5
Positive control	16 of 16^{c}	100.0%	6 of 16^{b}	37.5%	0 of 5	3 of 5

Proportions in the same column labelled with different superscripts differ statistically (χ^2 and Fisher's exact tests, $p < 0.05$).

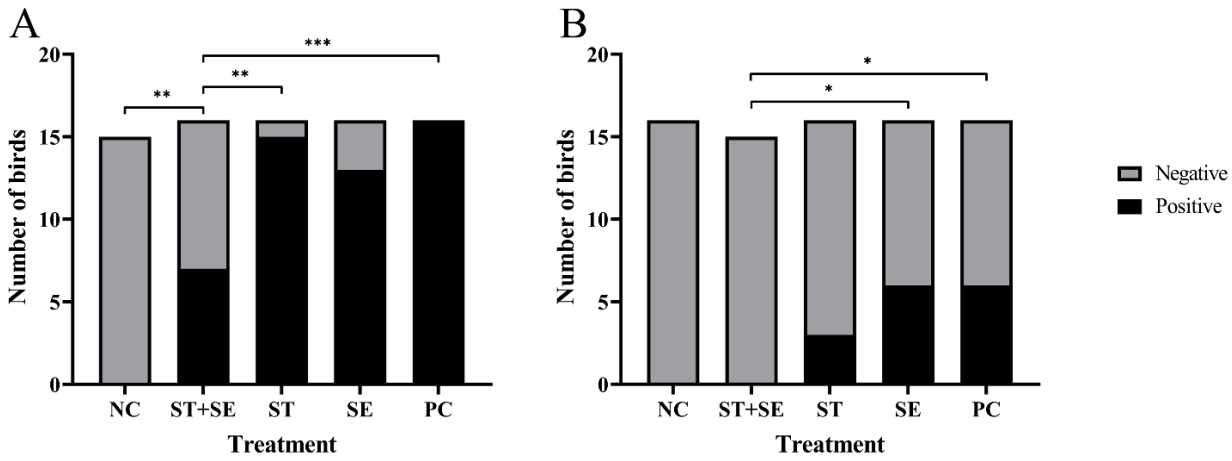


Figure 5 Proportion of samples positive and negative to *Salmonella* isolation per group

Isolations were attempted from swabs collected during the post-mortem procedure.
A – swabs collected from the interior of the caecal tubes.
B – swabs collected from the most developed follicle of the ovary.
Horizontal lines above the bars indicate when the differences in the proportions were statistically significant, using χ^2 and Fisher's exact test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

In the ST group, the situation was not significantly different to that of the PC group, with 15 of the 16 caecal swabs (93.8%) positive to *Salmonella.* The proportion of positives in the ovary was lower than in the PC group (18.8% versus 37.5%), but that difference was not statistically significant. In the SE group, the proportions of caecal and ovarian swabs positive to *Salmonella* were statistically higher than that of the NC group, and not different from that of the PC group. In hens from ST+SE group (Figure 5), the proportion of positives caecal samples was higher than the NC group. However, it was significantly lower than the proportion of positives obtained in the PC and ST groups. Vaccination with the ST+SE combination resulted in 100% prevention of colonisation of ovaries by the challenge organism. The proportion of positives (0/16) compared to the PC group (6/16) was statistically significant ($p = 0.02$) and was the same as the proportion of positives in the negative control group (Figure 5, B).

The proportion of *Salmonella* positive pericardial and air-sac membrane swabs was not significantly different between any of the groups.

3.5 Rep-PCR for *Salmonella* identification

Results from the Rep-PCR (Figure 6 and Appendix 5) show that the amplicons obtained were of the same molecular weight compared with those obtained using the aliquot of inoculum saved at -80°C during the SE exposure day. On the other hand, the amplicons obtained from other bacteria used as control (two ST isolated from a chicken and a horse, a *Salmonella* Anatis isolated from a duck and E. coli) clearly differ from that of SE. None of the bands found in these controls were found in the SE samples.

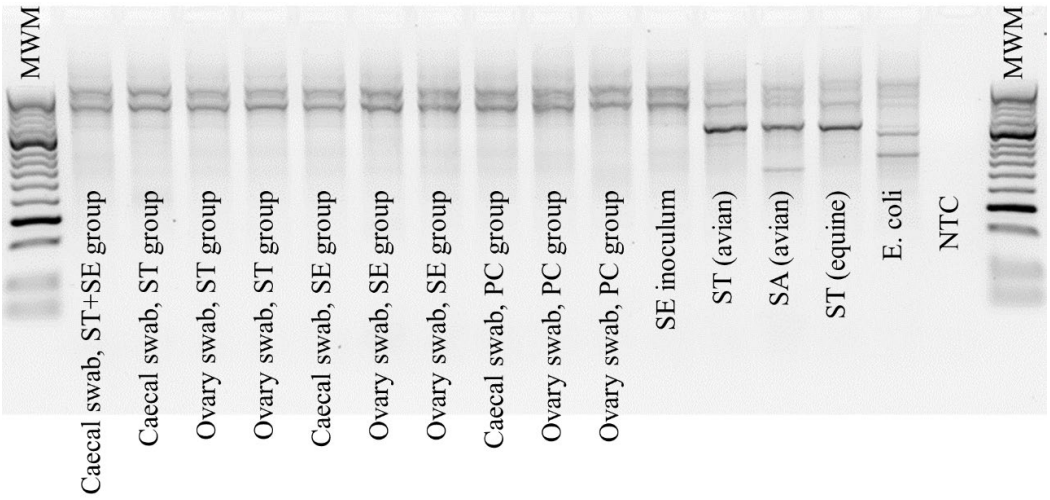


Figure 6 Agarose gel (1.5%) containing the amplicons obtained after performing a Rep-PCR from *Salmonella* isolates obtained from caecal and ovarian samples

MWM – molecular weight marker.
SA – *Salmonella* Anatis.
NTC – non-template control (negative control).

3.6 Protective index

Protective index results are presented in Table 5. The calculated PI was higher in the ST+SE group compared with that obtained in the ST and SE groups for both the caeca and ovary.

Table 5 Protective index calculated using the data obtained from the *Salmonella* isolations

Treatment (vaccines)	Protective index	
	Caeca	Ovary
ST+SE	**56.2%**	**100.0%**
ST	6.2%	49.9%
SE	18.8%	0.0%

4 Discussion

The challenge model used in the present study was demonstrated to be successful, as it was possible to reproduce the colonisation of the internal organs in all the unvaccinated hens exposed to SE 7A. The vancomycin treatment of the hens before exposure aided SE 7A colonisation in mature birds, as previously demonstrated[22]. In previous studies conducted by this group and others, a pre-exposure treatment with vancomycin was demonstrated to be necessary to obtain a reliable successful high level of SE or ST challenge in mature birds[21, 22]. Results from this experiment have shown that there was no detectable Group B or D antibodies in the samples from the ST group. However, live *Salmonella* vaccines induce a significant increase in local mucosal immunity, but not parenteral immunity[28, 29]. It was demonstrated that detectable antibody levels against Group D *Salmonella* can be achieved with two vaccinations with an SE autogenous killed vaccine alone. Also, detectable Group D antibodies were achieved with a program that included two vaccinations with a live ST vaccine and two injections of the SE autogenous vaccine. This is consistent with previous studies, where chickens immunised with only a killed vaccine had a significant increase in the number of parenteral antibodies against *Salmonella* compared with those vaccinated with the live vaccine (both flagellar and envelope antibodies)[30]. However, the results obtained in the present study suggest that the increase in the level of serum antibodies at best only partially prevents SE colonisation of internal organs. Hens in the SE group had a notable increase in antibody levels after two vaccinations, in some cases with titres higher than those obtained in hens from the ST+SE group (Figure 2 and Table 3). However, the results presented in Table 4 and Figure 5 demonstrated that the colonisation of the internal organs, especially caeca and ovary, was not prevented by these parenteral antibodies, suggesting that the protection observed in the ST+SE vaccinated birds involved more than the humoral response. Previous studies suggest that, in the case of *Salmonella* vaccinations, the use of a live vaccine is essential, as the cell-mediated immune response generated by these vaccines confers an adequate protection against the disease[30, 31]. The local mucosal immunity conferred by the live vaccine would have also played a role in the significant level of protection conferred by the ST+SE vaccination program. As discussed above, live *Salmonella* vaccines are capable of inducing mucosal immunity by enhancing the IgA levels[28]. However, the intestine mucosal immunity was not assessed in the present study, and it should be considered in future vaccine research. The results obtained by Tran*, et al.* [32], where two *Salmonella* Enteritidis bacterin vaccines were tested should also be considered. One of the inactivated vaccines was able elicit local intestinal IgA immunity, while the other bacterin did not accomplish that level of IgA in the mucosa.

In terms of the isolation of *Salmonella* from cloacal swabs, there was no difference between the challenged groups (PC, ST, SE and ST+SE) at any of the sampling times (3-, 7-, 14-, 21- and 28-DAE). Previous studies in broilers showed that more than 70% of the cloacal swabs in vancomycin-pre-treated birds and challenged with ST remained positive for *Salmonella* for 3 weeks after the infection[21]. There was a lower proportion of *Salmonella* positive swabs in the ST+SE group (75%) compared with the PC group (94%) although this was not statistically significant ($p = 0.33$). The results of *Salmonella* isolation from the cloacal swabs in the PC group, where a different hen per week stopped shedding SE, suggests that the excretion of *Salmonella* is intermittent in some individuals.

During the present study, no birds challenged with SE 7A demonstrated any overt clinical signs or morphological pathology (Appendix 6). While this has been the field observations of SE 7A infection in commercial layers, there was one report of mortalities associated with peritonitis from which SE 7A was recovered (personal communications, Alfirevich 2020).

In the present study, colonisation of the organs was assessed by a generic *Salmonella* isolation from the internal organ swabs collected during the post-mortem analysis. The results from the Rep-PCR confirm that the isolates correspond to the original SE inoculum used during the exposure.

In terms of colonisation of the internal organs of the hens with SE, a reduction in the caecal colonisation of 56.2% was achieved by the combination of the live ST and autogenous killed SE 7A vaccines. Under these high challenge conditions, this level of reduction was considerably higher than that conferred exclusively by the application of the SE autogenous vaccine (18.8%). Even though a cross-protection to SE has been reported in chickens vaccinated with the ST live vaccines, a low level of protection was achieved in this trial, with a PI of only 6.2% for caecal colonisation, lower than the PI for caecal colonisation of 54.7% calculated from the data reported in the Zoetis technical information sheet of their live ST vaccine[12]. In the Zoetis ST live vaccine trials, the chickens used were broilers. Also, the live vaccine was applied to day-old chickens at the hatchery followed by a challenge with SE at 4 days of age, and the attempt of *Salmonella* isolation from the internal organs performed at 21 days of age (testing an early infection). The results presented in the present study demonstrate that this level of protection does not remain until the point of lay (POL). Similar results were obtained in another study[33], where broiler breeders were vaccinated with a combination of ST live and killed ST vaccines. In that study, there was no reduction of the caecal colonisation in their progeny after a challenge with 1×10^6 cfu of SE/chick at 1 day of age[33], indicating that the antibodies transferred from the breeders to their progeny were not protective. A more long-lasting protection should be considered and studied, in order to protect the birds until their POL and beyond.

The results presented here show that the prime vaccination with a live vaccine and a booster with a live and two killed vaccinations can achieve a level of protection against an SE challenge. However, a future study challenging layers in mid lay (at around 45 weeks of age) is necessary to assess the duration of immunity following the live ST/killed SE vaccination schedule.

In terms of caecal and ovarian colonisation, there was a significant reduction in those hens vaccinated with the combined vaccination schedule with both ST and SE vaccines. While 37.5% of the swabs were positive to *Salmonella* (6 out of 16) in the unvaccinated and challenged group, there was no positive isolation in the ST+SE vaccinated group (PI = 100%). The χ^2 and a two-tailed Fisher's exact test showed that the difference in the proportion of positives in the ST+SE vaccinated group compared with challenged group was significant, with a p-value = 0.02 (Figure 5). A similar result was achieved in a previous study combining ST and SE vaccines[34]. The colonisation of the ovary after a SE challenge was lower in hens vaccinated with both ST and SE vaccines compared with the positive control. Also, the number of positives in the ST only or SE only vaccinated hens was not significantly lower than the positive control[34].

The isolation of SE from both pericardial and air-sac membrane swabs confirms that the Australian SE 7A isolate causes a systemic infection in the infected layers, as previously described for *Salmonella* spp. [35, 36] This also indicates that SE 7A may, with intercurrent stressors, manifest with clinical signs and morphological lesions not unlike chronic respiratory disease as observed by Alfirevich.

The results obtained in the present study confirm the systemic nature of the *Salmonella* Enteritidis isolate 7A. They also demonstrate the transovarian (vertical) transmission of this Australian isolate. The calculated PI for both caeca and ovary suggested an important level of protection conferred by the ST+SE vaccination program against the colonisation with SE, suggesting that this could be the best vaccination program conferring the highest level of protection against SE. However, more studies will be required to confirm this finding and to assess a long-lasting protection against SE conferred by this vaccination program.

5 References

1. Hope B, Baker A, Edel E, et al. An overview of the *Salmonella* Enteritidis risk assessment for shell eggs and egg products. *Risk Anal.* 2002;22(2): 203-218.

2. Chousalkar K, Gast R, Martelli F, Pande V. Review of egg-related salmonellosis and reduction strategies in United States, Australia, United Kingdom and New Zealand. *Crit Rev Microbiol.* 2018;44(3): 290-303.

3. *Salmonella* Enteritidis outbreak linked to contaminated eggs; https://www.health.nsw.gov.au/Infectious/alerts/Pages/enteritidis-eggs-2018.aspx. Accessed 17/06/2020.

4. *Salmonella* Enteritidis (SE) linked to eggs; 2019. https://www.foodstandards.gov.au/consumer/safety/Pages/Salmonella-Enteritidis-linked-to-eggs.aspx. Accessed 17/06/2020.

5. Coloe PJ. Live in ovo vacicne. US6231871B1. Zoetis WHC 2 LLC; 1994.

6. Alderton MR, Fahey KJ, Coloe PJ. Humoral responses and salmonellosis protection in chickens given a vitamin-dependent *Salmonella* typhimurium mutant. *Avian Dis.* 1991;35(3): 435-442.

7. APVMA. A Study to Assess the Safety of Vaxsafe ST (Living, Strain STM-1); 2016. https://portal.apvma.gov.au/pubcris;jsessionid=i7b+cZhyI6h4txbD0bjvwMRc?p_auth=TjiNg871&p_p_id=pubcrisportlet_WAR_pubcrisportlet&p_p_lifecycle=1&p_p_state=normal&p_p_mode=view&p_p_col_id=column-1&p_p_col_pos=2&p_p_col_count=4&_pubcrisportlet_WAR_pubcrisportlet_id=40728&_pubcrisportlet_WAR_pubcrisportlet_javax.portlet.action=viewProduct. Accessed 30th of March, 2020 2020.

8. Hayashi R, Tujimoto-Silva A, Muniz E, Verdi R, Santin E. *Salmonella* typhimurium vaccine to control a brazilian *Salmonella* heidelberg strain in broiler chickens. *Ars Veterinaria.* 2018;34(3): 105-114.

9. Abs El-Osta Y, Mohotti S, Carter F, et al. Preliminary analysis of the duration of protection of Vaxsafe® ST vaccine against *Salmonella* shedding in layers. *26th annual Australian Poultry Science Symposium.* Sydney, NSW, Australia; 2015:163-166.

10. Muniz EC, Verdi R, Leão JA, Back A, Nascimento VPd. Evaluation of the effectiveness and safety of a genetically modified live vaccine in broilers challenged with *Salmonella* Heidelberg. *Avian Pathol.* 2017;46(6): 676-682.

11. Zoetis. Poulvac® ST: for reduced *Salmonella* risk; 2020. https://www.zoetisus.com/products/poultry/poulvac-st.aspx.

12. Karakonji B, Lehrbach P. Technical information update - Poulvac® ST and *Salmonella* spp. Zoetis, NSW, Australia; 2019.

13. Gast RK, Stone HD, Holt PS, Beard CW. Evaluation of the efficacy of an oil-emulsion bacterin for protecting chickens against *Salmonella* enteritidis. *Avian Dis.* 1992;36(4): 992-999.

14. Gast RK, Stone HD, Holt PS. Evaluation of the efficacy of oil-emulsion bacterins for reducing fecal shedding of *Salmonella* enteritidis by laying hens. *Avian Dis.* 1993;37(4): 1085-1091.

15. Woodward MJ, Gettinby G, Breslin MF, Corkish JD, Houghton S. The efficacy of Salenvac, a *Salmonella* enterica subsp. Enterica serotype Enteritidis iron-restricted bacterin vaccine, in laying chickens. *Avian Pathol.* 2002;31(4): 383-392.

16. Nakamura M, Nagamine N, Takahashi T, Suzuki S, Sato S. Evaluation of the efficacy of a bacterin against *Salmonella* enteritidis Infection and the effect of stress after vaccination. *Avian Dis.* 1994;38(4): 717-724.

17. Desin TS, Köster W, Potter AA. *Salmonella* vaccines in poultry: past, present and future. *Expert Review of Vaccines.* 2013;12(1): 87-96.

18. Groves P, Sharpe S, Muir W, Pavic A, Cox J. Live and inactivated vaccine regimens against caecal *Salmonella* Typhimurium colonisation in laying hens. *Aust Vet J.* 2016;94(10): 387-393.

19. Jackson C, Underwood G. Development of *Salmonella* vaccination strategies for the Australian poultry industry. *Bioproperties Article.* 2006.

20. Nassar T, Al-Nakhli H, Al-Ogaily Z. Use of live and inactivated *Salmonella* enteritidis phage type 4 vaccines to immunise laying hens against experimental infection. *Revue Scientifique et Technique-Office International des Epizooties.* 1994;13: 855-855.

21. Marcq C, Cox E, Szalo IM, Thewis A, Beckers Y. *Salmonella* Typhimurium oral challenge model in mature broilers: Bacteriological, immunological, and growth performance aspects. *Poult Sci.* 2011;90(1): 59-67.

22. Stern N. *Salmonella* species and *Campylobacter jejuni* cecal colonization model in broilers. *Poult Sci.* 2008;87(11): 2399-2403.

23. Crhanova M, Hradecka H, Faldynova M, et al. Immune response of chicken gut to natural colonization by gut microflora and to *Salmonella enterica* serovar enteritidis infection. *Infect Immun.* 2011;79(7): 2755-2763.

24. Stavric S, Gleeson TM, Blanchfield B, Pivnick H. Role of adhering microflora in competitive exclusion of *Salmonella* from young chicks. *J Food Prot.* 1987;50(11): 928-932.

25. Deguchi K, Yokoyama E, Honda T, Mizuno K. Efficacy of a novel trivalent inactivated vaccine against the shedding of *Salmonella* in a chicken challenge model. *Avian Dis.* 2009;53(2): 281-286.

26. Rasschaert G, Houf K, Imberechts H, Grijspeerdt K, De Zutter L, Heyndrickx M. Comparison of five repetitive-sequence-based PCR typing methods for molecular discrimination of *Salmonella* enterica isolates. *J Clin Microbiol.* 2005;43(8): 3615-3623.

27. Gevers D, Huys G, Swings J. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiol Lett.* 2001;205(1): 31-36.

28. Lalsiamthara J, Kamble NM, Lee JH. A live attenuated *Salmonella* Enteritidis secreting detoxified heat labile toxin enhances mucosal immunity and confers protection against wild-type challenge in chickens. *Vet Res.* 2016;47(1): 60.

29. Cooper GL, Venables LM, Woodward MJ, Hormaeche CE. Vaccination of chickens with strain CVL30, a genetically defined *Salmonella* enteritidis aroA live oral vaccine candidate. *Infect Immun.* 1994;62(11): 4747-4754.

30. Babu U, Dalloul R, Okamura M, et al. *Salmonella* enteritidis clearance and immune responses in chickens following *Salmonella* vaccination and challenge. *Vet Immunol Immunopathol.* 2004;101(3-4): 251-257.

31. Babu U, Scott M, Myers MJ, et al. Effects of live attenuated and killed *Salmonella* vaccine on T-lymphocyte mediated immunity in laying hens. *Vet Immunol Immunopathol.* 2003;91(1): 39-44.

32. Tran TQL, Quessy S, Letellier A, Desrosiers A, Boulianne M. Immune response following vaccination against *Salmonella* Enteritidis using 2 commercial bacterins in laying hens. *Can J Vet Res.* 2010;74(3): 185-192.

33. Young SD, Olusanya O, Jones KH, Liu T, Liljebjelke KA, Hofacre CL. *Salmonella* incidence in broilers from breeders vaccinated with live and killed *Salmonella*. *Journal of Applied Poultry Research.* 2007;16(4): 521-528.

34. Gantois I, Ducatelle R, Timbermont L, et al. Oral immunisation of laying hens with the live vaccine strains of TAD *Salmonella* vac® E and TAD *Salmonella* vac® T reduces internal egg contamination with *Salmonella* Enteritidis. *Vaccine.* 2006;24(37): 6250-6255.

35. Jones MA, Hulme SD, Barrow PA, Wigley P. The *Salmonella* pathogenicity island 1 and *Salmonella* pathogenicity island 2 type III secretion systems play a major role in pathogenesis of systemic disease and gastrointestinal tract colonization of *Salmonella* enterica serovar Typhimurium in the chicken. *Avian Pathol.* 2007;36(3): 199-203.

36. Barrow P, Huggins M, Lovell M, Simpson J. Observations on the pathogenesis of experimental *Salmonella* typhimurium infection in chickens. *Res Vet Sci.* 1987;42(2): 194-199.

6 Plain English Summary

Project Title:	**Evaluation of vaccination program options against *Salmonella* Enteritidis 7A in laying hens in Australia**
Australian Eggs Limited Project No	31FS002
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, Australia [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 efficacy of different *Salmonella* vaccination programs in reducing faecal shedding, and caecal and ovarian colonisation of *Salmonella* Enteritidis (SE). Vaccines included were a commercial live S. Typhimurium (ST) and a SE autogenous vaccine (which is made with the causal organism isolated in the farm).
Background	Recently, public concerns have been raised after cases of gastroenteritis associated with the consumption of table eggs in Australia. These cases have been traced back to farms where the infection has been confirmed. Using the experience of other countries, the best measure to prevent the infection of chickens is by vaccinating the hens with a program including both live and attenuated SE vaccines. However, Australia does not have approved live or attenuated SE vaccines.
Research	Three different vaccination programs were tested. The first vaccination program included two applications of commercial live ST vaccines (ST vaccinated); the second, two applications of SE autogenous vaccine made at ACE laboratories (SE vaccinated); and the third vaccination program used a combination of the first two programs (ST+SE vaccination). To assess the efficacy of the different vaccination programs, vaccinated hens were orally exposed to the Australian isolate SE 7A in a controlled environment (isolators). Cloacal swabs were obtained at 0, 3, 7, 14, 21 and 28 days after exposure. At the end of the study, hens were humanly euthanised and caecal content and ovary contents cultured.

Outcomes	The SE autogenous vaccine was able to induce a significant immune response in the vaccinated hens. There was a partial decrease in bacterial shedding in the vaccinated groups, but differences were not significant. The ST+SE vaccination program caused a significant reduction in the SE colonisation of the caecal tube, and a 100% reduction in the SE isolated from the ovarian follicle.
Implications	The results of this study indicated that SE control is possible using a combination of the current registered live ST vaccines and an SE killed autogenous vaccine. However, the long-term protection conferred by this vaccination program still needs to be proven.
Key Words	*Salmonella* Enteritidis, salmonellosis, autogenous vaccine, immunity
Publications	NA

7 Appendix

Appendix 1: ACE laboratories inoculum concentration report

Laboratory Results

To: Tim Wilson

Final report date: 16/01/2020
Date samples received & tested: 14/01/2020

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

No. Samples Submitted: Salmonella enteritidis *in vivo* challenge x 2
Submitter: Tim Wilson
Farm: Bridgewater / Scolexia

Lab No.	Sample Details	Count CFU/mL	Purity
1	9:30am 15/01/2020	0.75×10^9	Pure growth – Salmonella enteritidis
2	5:00pm 15/01/2020	0.87×10^9	Pure growth – Salmonella enteritidis

Average count per trial:

15th January – 0.81×10^9 CFU/ml

Reported by: Elizabeth Hickey
ACE Laboratory Services actively seeks and welcomes your feedback, phone 03 54439665.

Comments:

Isolate identification and serotyping performed by ACE and confirmed by MDU, Melbourne, prior to isolate storage (Laboratory Reference: 01914/19 5RXi).

Challenge growth bacterial species confirmed by MALDI-TOF.

Viable cell counts of samples determined using methods not accredited by NATA but in accordance with GMP methods (SOP 1167).

NATA ACCREDITED FOR TECHNICAL COMPETENCE

Page 1 of 1 Report prepared by: EH date 16/01/2020 Report checked by date
This report may not be reproduced except in full. This testing was performed in accordance with SOP 353. This analysis relates to the samples submitted and it is the submitters responsibility to ensure that the sample is representative of the material tested.
Accreditation Number: 15636 Accredited for compliance with ISO/IEC 17025 - Testing

Appendix 2: APCAH animal and isolator monitoring sheet

Asia-Pacific Center fo[r Ani]mal Health, Faculty of Veterinary & Agricultural Sciences, The [Uni]versity of Melbourne

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.:** L7 **Isolator Pressure:** NEGATIVE **Start Date:** 14 JAN 2020 **End Date:** 31 JAN 2020

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

Experiment Group: (1) NEGATIVE CONTROL **Researcher:** SCOLEXIA (PULOS SH...)

Species: CHICKEN **Source:** CARISBROOK **Egg Access No.:** N/A **Age:** 16 WKS **Animal Numbers:** 0855 to 0870

APCAH Accession No.: N/A **Animal House Accession No.:** 003/2020 **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	
8/1/20	1310	All	OK									All	Birds (W) tagged, weighed + placed									PJ/JD/AC
9/1/20	736	All	OK																			JD
10/1/20	748	All	OK																			JD
11/1/20	726	All	OK																			ac
12/1/20	825	All	OK																			ac
13/1/20	759	All	OK															✓		✓		JD
13/1/20	1232	All	OK									All	All birds given Vancomycin (oral)									PJ/JD/AC
14/1/20	1626	All	OK										Man drinker filled / All birds given	✓		✓						JD/PJ
—	—	—	—										Vancomycin + given mgoit / cloacal swabbed		✓							JD/AC
15/1/20	754	All	OK																			ac
16/1/20	739	All	OK															✓		✓		JD
17/1/20	927	All	OK									All	All birds cloacal swab		✓	✓						JD/PS
18/1/20	862	All	OK																			ac
19/1/20	825	All	OK																			ac
20/1/20	739	All	OK										Man drinker filled	✓		✓		✓		✓	✓	JD
21/1/20	937	All	OK										All birds cloacal swab		✓							PJ/JD/JJ AC
22/1/20	1101	All	OK																			JD
23/1/20	753	All	OK											✓				✓		✓		JD
24/1/20	842	All	OK												✓	✓						JD
25/1/20	802	All	OK																			ac
26/1/20	759	All	OK										man drinker filled			✓						ac
27/1/20	724	All	OK											1/4	✓	✓		✓		✓		JD
28/1/20	912	All	OK									All	All birds cloacal swab	✓	✓							PJ/JD/AC

COPY

18/3/2020

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

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.: A7 Isolator Pressure: NEGATIVE Start Date: 14 JAN 2020 End Date: 31 JAN 2020
Experiment Procedure: EFFICACY OF SALMONELLA ENTERITIDIS AUTOGENOUS VACCINE IN COMMERCIAL LAYERS
Experiment Group: (1) NEGATIVE CONTROLS Researcher: POULTRY STAFF FOR SCIENTIST
Species: CHICKEN Source: CARISBROOK Egg Access No.: N/A Age: 16 WKS Animal Numbers: 0855 to 0870
APCAH Accession No.: N/A Animal House Accession No.: 005/2020 AEEC No.: 1915C43.1 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	
29/1/20	759	ALL	OK										MAN DRINKER FILLED			✓						JD
30/1/20	813	ALL	OK											1/4	✓			✓		✓	✓	JD
31/1/20	902	ALL	OK										MAN DRINKER FILLED	✓✓	✓	✓						JD
1/2/20	8.35	ALL	OK																			ac
2/2/20	8.34	ALL	OK										man drinker filled		✓	✓						ac
3/2/20	814	ALL	OK															✓		✓		JD
4/2/20	946	ALL	OK									ALL	BIRDS CLOACAL SWAB / MAN DRINKER FILLED	✓	✓	✓						PS/DP/AK
5/2/20	744	ALL	OK													✓		✓		✓	✓	JD
6/2/20	826	ALL	OK																			JD
7/2/20	821	ALL	OK										MAN DRINKER FILLED	✓✓	✓	✓		✓		✓		JD
8/2/20	852	ALL	OK																			ac
9/2/20	844	ALL	OK										man drinker filled			✓						ac
10/2/20	838	ALL	OK												1/2	✓		✓		✓	✓	JD
11/2/20	943	ALL	OK										CLOACAL SWABS TAKEN / MAN DRINKER FILLED	✓	✓	✓						JD/AK/PS
12/2/20	943	ALL	OK															✓		✓		ac
13/2/20	831	ALL	OK											1/4	✓	✓						JD
14/2/20	920	ALL										ALL	BIRDS CULLED + SENT FOR POST MORTEM									RS/JD

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/AIMLS/05C | **Date issued: 05/02/2018** | **Page 1 of 1**

Building No.: 423 **Isolator No.:** A10 **Isolator Pressure:** NEGATIVE **Start Date:** 14 JAN 2020 **End Date:** 31 JAN 2020
Experiment Procedure: EFFICACY OF SALMONELLA ENTERITIDIS ANTIGENICITY VACCINE IN COMMERCIAL LAYERS
Experiment Group: (2) ST + SE VACC + CHALLENGE **Researcher:** POULTRY UNIT FOR SCIENCE
Species: CHICKEN **Source:** CARINGBAH **Egg Access No.:** N/A **Age:** 16 WKS **Animal Numbers:** 0871 TO 0886
APCAH Accession No.: **Animal House Accession No.:** 05/12/20 **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	
8/1/20	1347	ALL	OK									ALL	BIRDS (W) TAGGED, WEIGHED + PLACED									PS/JD/A
9/1/20	745	ALL	OK																			JD
10/1/20	755	ALL	OK																			JD
11/1/20	832	ALL	OK																			AC
12/1/20	842	ALL	OK										MAN DRINKER FILLED			✓						AC
13/1/20	1321	ALL	OK									ALL	BIRDS GIVEN VANCOMYCIN (OVAL)					✓		✓		JD/AC/PS/JD
14/1/20	1137	ALL	OK									ALL	BIRDS GIVEN VANCOMYCIN (OVAL) + CLOACAL SWABS	✓	✓							PS/JD/JD/AC
14/1/20	1313	ALL	OK									ALL	BIRDS CHALLENGED									PS/JD/JD/AC
15/1/20	810	ALL	OK										MAN DRINKER FILLED			✓						AC
16/1/20	754	ALL	OK													✓		✓		✓		JD
17/1/20	1020	ALL	OK										ALL BIRDS CLOACAL SWAB / MAN DRINKER FILLED	✓	✓							JD/PS/JD
18/1/20	808	ALL	OK																			AC
19/1/20	838	ALL	OK										MAN DRINKER FILLED			✓						AC
20/1/20	806	ALL	OK											✓				✓		✓	✓	JD
21/1/20	1025	ALL	OK									ALL	BIRDS CLOACAL SWAB / MAN DRINKER FILLED		✓	✓						JD/JD/AC
22/1/20	1117	ALL	OK																			JD
23/1/20	811	ALL	OK											½				✓		✓		JD
24/1/20	859	ALL	OK										MAN DRINKER FILLED		✓	✓						JD
25/1/20	808	ALL	OK																			AC
26/1/20	823	ALL	OK										MAN DRINKER FILLED			✓						AC
27/1/20	747	ALL	OK											¼	✓			✓		✓		JD
28/1/20	957	ALL	OK									ALL	BIRDS CLOACAL SWAB / MAN DRINKER FILLED	✓	✓	✓						PS/JD/AC
29/1/20	821	ALL	OK																			JD

Asia-Pacific Center f nimal Health, Faculty of Veterinary & Agricultural Sciences, T niversity of Melbourne

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.:** L10 **Isolator Pressure:** NEGATIVE **Start Date:** 14 JAN 2020 **End Date:** 31 JAN 2020
Experiment Procedure: EFFICACY OF SALMONELLA ENTERITIDIS AUTOGENOUS VACCINE IN COMMERCIAL LAYERS
Experiment Group: (2) ST + SE VACC + CHALLENGE **Researcher:** SCELERIA (FULLED DAIL)
Species: CHICKEN **Source:** CALISBZOOIL **Egg Access No.:** N/A **Age:** 16 WKS **Animal Numbers:** 0871 TO 0886
APCAH Accession No.: N/A **Animal House Accession No.:** 003/2020 **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	
30/1/20	842	All	ok															✓		✓	✓	JD
31/1/20	932	All	ok										MAIN DRINKER FILLED	✓	✓	✓						JD
1/2/20	848	All	ok																			ac
2/2/20	953	All	ok										man drinker filled			✓						ac
3/2/20	835	All	ok												1/2			✓		✓		JD
4/2/20	1035	All	ok									Au	Bird. Cloacal Swab / Main Drinker Filled	✓	✓	✓						PS/AC/JD
5/2/20	812	All	ok															✓		✓	✓	JD
6/2/20	845	All	ok										MAIN DRINKER FILLED			✓						JD
7/2/20	851	All	ok											✓✓	✓			✓		✓		JD
8/2/20	919	All	ok										man drinker filled			✓						ac
9/2/20	909	All	ok											✓								ac
10/2/20	922	All	ok										MAIN DRINKER FILLED			✓		✓		✓	✓	JD
11/2/20	1058	All	ok									AU	Birds Cloacal Swab	✓	✓							PS/AC/JD
12/2/20	10:38	All	OK										man drinks filled	✓		✓		✓		✓		ac
13/2/20	913	All	ok										MAIN DRINKER FILLED		✓	✓						JD
14/2/20	1110	All										Au	Birds Culled + Post Mortem (Metford)									B/JD/

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/AIMLS/05C | Date issued: 05/02/2018 | Page 1 of 1

Building No.: 423 Isolator No.: L-8 Isolator Pressure: NEGATIVE Start Date: 14 JAN 2020 End Date: 31 JAN 2020
Experiment Procedure: EFFICACY OF SALMONELLA ENTERITIDIS AUTOGENOUS VACCINE IN COMMERCIAL LAYERS
Experiment Group: (3) ST VACC + CHALLENGE Researcher: POULT SIL FOR SCIENTIA
Species: CHICKEN Source: CARISBROOK Egg Access No.: N/A Age: 16 WKS Animal Numbers: 0887 to 0902
APCAH Accession No.: Animal House Accession No.: 05/3/2020 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	Normal	Alertness	Posture	Movement	Feathers	Respiratory signs	Eyes	Body condition	Diarrhoea	Other	Comments or Procedures performed (Including comments on any clinical signs seen)	FEED New bag	FEED Top up	WATER Fill	WATER HCL	FILTER Clean	FILTER New	FAECES Roll up	FAECES Removed	Sign
8/1/20	1336	ALL	OK									AL	BIRDS (w) TAGGED + WEIGHED + [illegible]									PJ/JQ/AC
9/1/20	740	ALL	OK																			JQ
10/1/20	731	ALL	OK																			JQ
11/1/20	828	ALL	OK																			AC
12/1/20	832	ALL	OK										man drinker filled			✓						AC
13/1/20	1110	ALL	OK									AL	BIRDS GIVEN VACCINATION					✓		✓		JQ, AC, PJ/JQ
14/1/20	1119	ALL	OK									AL	BIRDS GIVEN VACCINATION + CLOACAL SWAB	✓	✓							PJ/JQ JQ/AC
14/1/20	1308	ALL	OK									AL	BIRDS CHALLENGED									PJ/JQ JQ/AC
15/1/20	802	ALL	OK										filled man drinker			✓						AC
16/1/20	747	ALL	OK															✓		✓		JQ
17/1/20	1004	ALL	OK									AL	BIRDS CLOACAL SWAB / MAN DRINKER FILLED		✓	✓						PJ/JQ/JQ
18/1/20	806	ALL	OK																			AC
19/1/20	829	ALL	OK																			AC
20/1/20	759	ALL	OK										MAN DRINKER FILLED	✓		✓		✓		✓	✓	JQ
21/1/20	1008	ALL	OK									AL	BIRDS CLOACAL SWAB		✓							PJ/JQ JQ/AC
22/1/20	1115	ALL	OK													✓						JQ
23/1/20	806	ALL	OK										MAN DRINKER FILLED	✓		✓		✓		✓		JQ
24/1/20	850	ALL	OK												✓							JQ
25/1/20	906	ALL	OK																			AC
26/1/20	815	ALL	OK										man drink filled			✓						AC
27/1/20	740	ALL	OK											1/4	✓			✓		✓		JQ
28/1/20	938	ALL	OK									AL	BIRDS CLOACAL SWAB	✓	✓	✓						PJ/JQ/AC
29/1/20	819	ALL	OK										MAN DRINKER FILLED			✓						JQ

COPY 18/3/2020

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/AIMLS/05C	Date issued: 05/02/2018	Page 1 of 1

Building No.: 423 **Isolator No.:** L8 **Isolator Pressure:** NEGATIVE **Start Date:** 14 JAN 2020 **End Date:** 31 JAN 2020
Experiment Procedure: EFFICACY OF SALMONELLA ENTERITIDIS AUTOGENOUS VACCINE IN COMMERCIAL LAYERS
Experiment Group: (3) ST VACC + CHALLENGE **Researcher:** SCELERIA (FOLLOW SHIL)
Species: CHICKEN **Source:** CARISBROOK **Egg Access No.:** N/A **Age:** 16 WKS **Animal Numbers:** 0887 to 0902
APCAH Accession No.: **Animal House Accession No.:** 003/2020 **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): Normal	Alertness	Posture	Movement	Feathers	Respiratory signs	Eyes	Body condition	Diarrhoea	Other	Comments or Procedures performed (Including comments on any clinical signs seen)	FEED: New bag	FEED: Top up	WATER: Fill	WATER: HCL	FILTER: Clean	FILTER: New	FAECES: Roll up	FAECES: Removed	Sign
30/1/20	834	All	OK											½	✓			✓		✓	✓	JD
31/1/20	919	All	OK										MAN DRINKER FILLED	✓✓	✓	✓						JD
1/2/20	845	All	OK																			ac
2/2/20	944	All	OK										Man drinker filled		[illegible]	✓						ac
3/2/20	827	All	OK															✓		✓		JD
4/2/20	1018	All	OK									All	BIRDS CLOACAL SWAB	✓	✓	✓						PS/AC/JD
5/2/20	803	All	OK										MAN DRINKER FILLED					✓		✓	✓	JD
6/2/20	835	All	OK											✓	✓	✓						JD
7/2/20	843	All	OK											✓		✓		✓		✓		JD
8/2/20	909	All	OK										Man drinker filled		✓							ac
9/2/20	857	All	OK																			ac
10/2/20	905	All	OK											½	½	✓		✓		✓	✓	JD
~~11~~	~~906~~																					
11/2/20	1024	All	OK									All	BIRDS CLOACAL SWAB | MAN DRINKER FILLED	✓	✓	✓						PS/AC/JD
12/2/20	10:16	All	OK											✓				✓		✓		ac
13/2/20	843	All	OK												✓	✓						JD
14/2/20	1048	All										All	BIRDS CULLED + POST MORTEM PERFORMED									PS/JD

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/AIMLS/05C | Date issued: 05/02/2018 | Page 1 of 1

Building No.: 423 Isolator No.: A-9 Isolator Pressure: NEGATIVE Start Date: 14 JAN 2020 End Date: 31 JAN 2020

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

Experiment Group: (4) DE VAC + CHALLENGE Researcher: POULTRY SECTION FOR SCIENTIA

Species: CHICKEN Source: CARINDALE Egg Access No.: N/A Age: 16 WKS Animal Numbers: 0903 to 0918

APCAH Accession No.: Animal House Accession No.: 05/2020 AEEC No.: 1915C43:1 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	
8/1/20	1322	ALL	OK									ALL	BIRDS (N) TAGGED, WEIGHED + PLACED									PS/JD/AK
9/1/20	744	ALL	OK																			JD
10/1/20	753	ALL	OK																			JD
11/1/20	836	ALL	OK																			AK
12/1/20	834	ALL	OK																			AK
13/1/20	1300	ALL	OK									ALL	BIRDS GIVEN VANCOMYCIN (CLAL) / MAN DRINKER FILLED			✓		✓		✓		JD/PS/JD/AK
14/1/20	1102	ALL	OK									ALL	BIRDS GIVEN VANCOMYCIN (CLAL)	✓	✓							JD/PS/AK/JD
15/1/20	1259	ALL	OK									ALL	BIRDS CHALLENGED									JD/AK/JP/PS
15/1/20	756	ALL	OK																			AK
16/1/20	743	ALL	OK															✓		✓		JD
17/1/20	948	ALL	OK									ALL	BIRDS CLOACAL SWAB / MAN DRINKER FILLED		✓	✓						PS/JD
18/1/20	904	ALL	OK																			AK
19/1/20	827	ALL	OK																			AK
20/1/20	748	ALL	OK											✓				✓		✓	✓	JD
21/1/20	954	ALL	OK									ALL	BIRDS CLOACAL SWABS		✓							JD/PS
22/1/20	1107	ALL	OK										MAN DRINKER FILLED			✓						JP
23/1/20	757	ALL	OK											½				✓		✓		JD
24/1/20	846	ALL	OK												✓	✓						JD
25/1/20	804	ALL	OK																			AK
26/1/20	857	ALL	OK										MAN DRINKER FILLED			✓						AK
27/1/20	732	ALL	OK											¼	✓			✓		✓		JD
28/1/20	925	ALL	OK									ALL	BIRDS CLOACAL SWAB	✓	—							PS/JD/AK
29/1/20	811	ALL	OK										MAN DRINKER FILLED			✓						JD

COPY 15/5/2020

Asia-Pacific Center nimal Health, Faculty of Veterinary & Agricultural Sciences, T niversity of Melbourne

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.:** L9 **Isolator Pressure:** NEGATIVE **Start Date:** 14 JAN 2020 **End Date:** 31 JAN 2020

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

Experiment Group: (4) SE VACC + CHALLENGE **Researcher:** JOANNA (POULTRY ...)

Species: CHICKEN **Source:** CARISBROOK **Egg Access No.:** N/A **Age:** 16 WKS **Animal Numbers:** 0903 to 0918

APCAH Accession No.: **Animal House Accession No.:** 003/2020 **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): Normal	Alertness	Posture	Movement	Feathers	Respiratory signs	Eyes	Body condition	Diarrhoea	Other	Comments or Procedures performed (Including comments on any clinical signs seen)	FEED: New bag	Top up	WATER: Fill	HCL	FILTER: Clean	New	FAECES: Roll up	Removed	Sign
30/1/20	824	All	OK											½	✓			✓		✓	✓	JD
31/1/20	907	All	OK											✓✓	✓	✓						JD
1/2/20	842	All	OK										Man drinker filled									AC
2/2/20	838	All	OK																			AC
3/2/20	820	All	OK															✓		✓		JD
4/2/20	1002	All	OK									All	Birds cloacal swab / Man drinker filled	✓	✓	✓						PS/JD/AC
5/2/20	752	All	OK															✓		✓	✓	JD
6/2/20	829	All	OK																			JD
7/2/20	836	All	OK										Man drinker filled	✓✓	✓	✓		✓		✓		JD
8/2/20	857	All	OK																			AC
9/2/20	851	All	OK																			AC
10/2/20	851	All	OK										Man drinker filled		½	✓		✓		✓	✓	JD
11/2/20	1006	All	OK									All	Birds cloacal swab	✓	✓							PS/AC/JD
12/2/20	1003	All	OK										Man drinker filled			✓		✓		✓		AC
13/2/20	836	All	OK											¼	✓							JD
14/2/20	1027	All										All	Birds culled + post mortemed									PS/JD

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/AIMLS/05C

Date issued: 05/02/2018

Building No.: 423 Isolator No.: A11 Isolator Pressure: NEGATIVE Start Date: 14 JAN 2020 End Date: 31 JAN 2020

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

Experiment Group: (5) POSITIVE CONTROL. Researcher: POLLY SMIT FOR SCIENTIA

Species: CHICKEN Source: CARISBROOK Egg Access No.: N/A Age: 16 WKS Animal Numbers: 0919 TO 0934

APCAH Accession No.: Animal House Accession No.: OC3/2020 AEEC No.: 1915C43.1 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	
8/1/20	1256	ALL	OK									ALL	BIRDS (W) TAGGED, WEIGHED + PLACED									PJ/JD/AC
9/1/20	733	ALL	OK																			JD
10/1/20	745	ALL	OK																			JD
11/1/20	824	ALL	OK																			AC
12/1/20	823	ALL	OK										MAN DRINKER FILLED			✓						AC
13/1/20	754	ALL	OK															✓		✓		JD
13/1/20	1243	ALL	OK									ALL	BIRDS GIVEN VACCINATION (OCAC)									PJ/PJ/JD
14/1/20	1044	ALL	OK									ALL	BIRDS, CLOACAL SWABBED + GIVEN VACCINATION	✓	✓							PJ/JD/AC/JD
15/1/20	1251	ALL	OK									ALL	BIRDS, CHALLENGED									PJ/JD/JD/AC
15/1/20	815	ALL	OK										MAN DRINKER FILLED			✓						AC
16/1/20	759	ALL	OK															✓		✓		JD
17/1/20	1057	ALL	OK										ALL BIRDS, CLOACAL SWAB									JD/PJ/JD
18/1/20	810	ALL	OK																			AC
19/1/20	842	ALL	OK										MAN DRINKER FILLED			✓						AC
20/1/20	814	ALL	OK											✓				✓		✓	✓	JD
21/1/20	1044	ALL	OK									ALL	BIRDS CLOACAL SWABS		✓							JD/PJ/AC
22/1/20	1121	ALL	OK													✓						JD
23/1/20	820	ALL	OK										MAN DRINKER FILLED	½		✓		✓		✓		JD
24/1/20	903	ALL	OK												✓							JD
25/1/20	810	ALL	OK																			JD
26/1/20	836	ALL	OK										MAN DRINKER FILLED	¼	✓	✓						AC
27/1/20	756	ALL	OK											¼	✓			✓		✓		JD
28/1/20	1016	ALL	OK									ALL	BIRDS CLOACAL SWAB / MAN DRINKER FILLED	✓	✓	✓		✓		✓		PJ/AC/JD

Asia-Pacific Center f[illegible] nimal Health, Faculty of Veterinary & Agricultural Sciences, T[illegible] iversity of Melbourne

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.:** L 1 **Isolator Pressure:** NEGATIVE **Start Date:** 14 JAN 2020 **End Date:** 31 JAN 2020

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

Experiment Group: (5) POSITIVE CONTROL. **Researcher:** SOLEHA (FOLLOW DAIL)

Species: CHICKEN **Source:** CALISBZOOIL **Egg Access No.:** N/A **Age:** 16 WKS **Animal Numbers:** 0919 to 0934

APCAH Accession No.: ______ **Animal House Accession No.:** 003/2020 **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): Normal	Alertness	Posture	Movement	Feathers	Respiratory signs	Eyes	Body condition	Diarrhoea	Other	Comments or Procedures performed (Including comments on any clinical signs seen)	FEED: New bag	FEED: Top up	WATER: Fill	WATER: HCL	FILTER: Clean	FILTER: New	FAECES: Roll up	FAECES: Removed	Sign
29/1/20	825	All	OK																			SP
30/1/20	353	All	OK											1/2	✓			✓		✓	✓	JP
31/1/20	943	All	OK										MAN DRINKER FILLED	✓✓	✓	✓						JP
1/2/20	651	All	OK																			ac
2/2/20	904	All	OK										man drinker filled		✓	✓						ac
3/2/20	844	All	OK															✓		✓		JP
4/2/20	1055	All	OK									All	BIRD CLOACAL SWAB / MAN DRINKER FILLED	✓	✓	✓						PS/JP/AK
5/2/20	822	All	OK															✓		✓	✓	JP
6/2/20	853	All	OK										MAN DRINKER FILLED			✓						JP
7/2/20	903	All	OK										REPLACED FAECES GLOVE (R)	✓✓	✓			✓		✓		JP
8/2/20	927	All	OK										man drinker filled			✓						ac
9/2/20	9.19	All	OK												✓							ac
10/2/20	938	All	OK										MAN DRINKER FILLED			✓		✓		✓	✓	JP
11/2/20	1054	All	OK									All	BIRD CLOACAL SWAB	✓	✓							PS/AK/JP
12/2/20	1059	All	OK										man drinker filled	✓		✓		✓		✓		ac
13/2/20	920	All	OK												✓	✓						JP
14/2/20	1133	All										All	BIRDS CULLED + POST MORTEM									

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

Laboratory Results

To: Scolexia Pty Ltd

Scolexia

Final report date: 14/01/20
Date testing commenced: 08/01/20
Date samples received: 08/01/20
Date samples collected: 07/01/20

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

AVIAN MICROBIOLOGY REPORT

Laboratory Reference: 00127/20 No Samples Submitted: 1 x Drag Swab
Owner: N/A
Submitter: Scolexia Pty Ltd – 2/21 Slater Parade, Keilor East VIC 3033

Lab No.	Sample Details	Culture Results
1	SCARF Age: 16 weeks	No *Salmonella* spp. isolated from the sample.

Report authorised by: Trenton Seager - Scientist
ACE Laboratory Services actively seeks and welcomes your feedback, phone 03 5443 9665.

Comments:

Appendix 4: Report of findings during the post-mortem procedure

Dr Amir H. Noormohammadi, DVM, PhD, MACVSc (Avian Health)
Professor and Head of Avian Medicine

THE UNIVERSITY OF MELBOURNE

Dr Jose A. Quinteros
Intensive Animal R&D and Regulatory Affairs
Scolexia Animal and Avian Health Consultancy

25th March 2020

Re: Efficacy of *Salmonella enteritidis* autogenous vaccine in commercial layers

Dear Jose,

Birds from groups 1-5 as listed below were subjected to a brief postmortem examination of visceral tissues including heart, liver, ovary and intestinal tract during collection of specimens within isolators on Friday 14th February.

Group	Treatment	n	ST vaccine	SE vaccine	Exposure* to SE
1	Negative Control	16	-	-	No
2	ST+SE	16	+	+	Yes
3	ST	16	+	-	Yes
4	SE	16	-	+	Yes
5	Positive Control	16	-	-	Yes

No significant gross lesions were detected in any of the birds sampled.

Amir H. Noormohammadi
DVM, PhD, MACVS (Poultry Health)

FACULTY OF VETERINARY AND AGRICULTURAL SCIENCES
VETERINARY CLINICAL CENTRE
THE UNIVERSITY OF MELBOURNE
250 PRINCES HIGHWAY, WERRIBEE
VICTORIA, AUSTRALIA, 3030
TELEPHONE: +61 3 9737 2275. FACSMILE: +61 3 9731 2366
E-MAIL: Amirh@unimelb.edu.au

Appendix 5: Asia-Pacific Centre for Animal Health Rep-PCR report

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

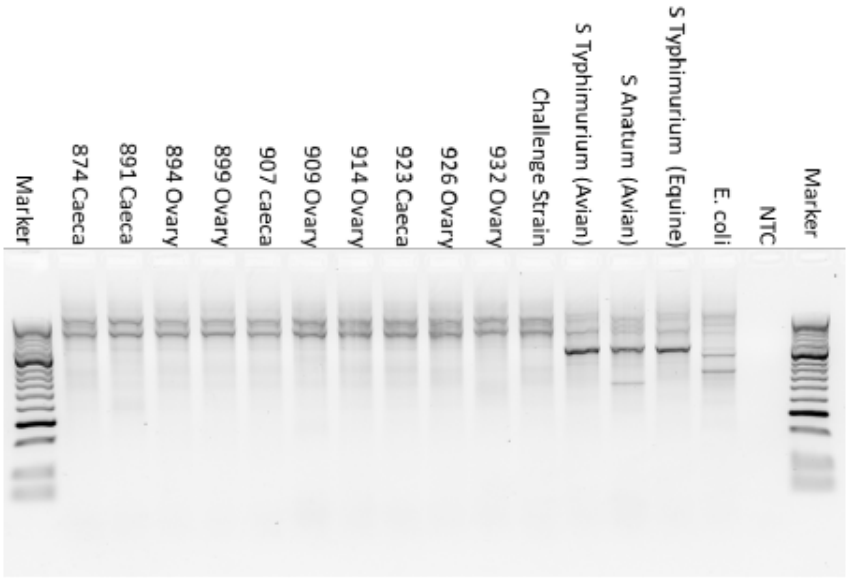
1 April 2020

Client: Scolexia Pty LTD
Contact: Dr. Jose Quinteros

Test: Salmonella Rep-PCR (GTG)

Interpretation

As is grossly observable in the gel picture below the 10 Salmonellae selected from the cull samples, and the challenge strain, produced highly similar banding patterns in the Rep-PCR and are suspected to be the same organism. Larger differences indicating dissimilar organisms can be observed in the control lanes labelled S Typhimurium (avian), S Anatum (avian), S Typimurium (Equine), and .E coli. These lanes contain strong bands that do not exist in the test group.



Appendix 6: Asia-Pacific Centre for Animal Health end of trial report

APCAH
Asia Pacific Centre for Animal Health

THE UNIVERSITY OF MELBOURNE

19th February 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 14th January 2020 and 14th February 2020.

Source of chicken
Commercial Layers of 16 weeks of age were supplied by a Victorian poultry farm for this experiment.

Animal facility accession number
This experiment was documented under accession number 003/2020.

Experimental outline
On 08th January 2020, chickens were wing tagged, weighed and placed into separate isolators as outlined in the following table. The birds were challenged on 14th January 2020, with live *Salmonella enteritidis* culture as mentioned in the table, according to the method outlined in the University of Melbourne Animal Ethics Committee approved project 1915043.

Bird groups and treatment in the experiment

Group	Treatment	Wing tag No.	*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	0855-0870	-	-	No (media only)	L7
2	ST+SE vaccination	0871-0886	+	+	Yes	L10
3	ST vaccination	0887-0902	+	-	Yes	L8
4	SE vaccination	0903-0918	-	+	Yes	L9
5	Positive Control	0919-0934	-	-	Yes	L11

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

Cloacal swab collection
Cloacal swabs were collected from all the treatment groups before challenge and on day 3,7,14,21 and 28 days post challenge and sent to clinical microbiology laboratory for culture.

Clinical signs throughout the experiment
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 14th February 2020 and subjected to post-mortem analysis. No gross lesions or adverse reactions were noted in any of the birds examined.

Sincerely Yours,

Pollob K. Shil, DVM, PhD
Animal Experimentation Facilities Manager
Asia Pacific Centre for Animal Health
Faculty of Veterinary & Agricultural Sciences
The University of Melbourne
250 Princes Highway, Werribee, Victoria - 3030
Ph: (03) 9731 2036
Fax: (03) 9731 2366, Email: pollob.shil@unimelb.edu.au