



Final Report

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Prepared by: Peter Groves

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Cross protection of Vaxsafe ST against a novel *S. Enteritidis* strain

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In submitting this report, the researcher has agreed to Poultry Hub Australia publishing this material in an edited form.

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Project Summary

Project Title	Cross protection of Vaxsafe® ST against a novel <i>S. Enteritidis</i> strain
Project No.	20-215
Date	Start: 31 July 2020 End: 31 August 2020
Project Leader(s)	Peter Groves and Alison Collins (EMAI)
Organisation	Zootechny Pty Ltd
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Project Aim	Evaluate the ability of a live <i>S. Typhimurium Aro-A</i> deletion mutant vaccine (Vaxsafe® ST) to provide cross-protection against intestinal and tissue colonisation by the recent <i>S. Enteritidis</i> from NSW in layer chickens.
Background	Could investing in vaccination programs develop cross-protection against <i>S. Enteritidis</i> helping to limit the number of foodborne outbreaks of the pathogen.
Research Outcome	That the existing Vaxsafe® ST vaccine used in Australia will give cross-protection against the new SE strain. It was demonstrated that Vaxsafe® ST was able to provide partial cross protection of layer hens to a <i>S. Enteritidis</i> challenge, but additional work is needed to provide higher levels of cross-protection before this research can be utilized by commercial layer farms to protect against <i>S. Enteritidis</i>
Impacts and Outcomes	The product being investigated is an established registered vaccine belonging to Bioproperties (Vaxsafe® ST). The project developed only knowledge with no expected value of new IP. The study informs the Australian poultry industry on potential cross protection of this <i>S. Typhimurium</i> -based vaccine against the novel <i>S. Enteritidis</i> strain that emerged in the industry in 2018-2019. A possible significant reduction in prevalence of <i>S. Enteritidis</i> colonization of caeca was found in vaccinated birds relative to non-vaccinated birds, but low prevalence of detection of <i>S. Enteritidis</i> in other organs in control birds made assessment of inhibition of colonization in liver, spleen and reproductive tissues difficult. There were indications of decreased pathology in duodenum, peritoneum and ovary with vaccination. A clear comparison of the standard vaccination strategy with an added oral dose of Vaxsafe® ST during pre-lay may also be possible with increased numbers of birds per vaccine treatment in future.
Publications	None

Executive Summary

Contamination of poultry products with *Salmonella* serovar Enteritidis (*S. Enteritidis*) is a significant international public health issue. Australian commercial poultry flocks are currently considered to be free from *S. Enteritidis*, but should this status change the cost to the industry and the risk to domestic public health would be great. Vaxsafe® ST is a living vaccine, being derived from *S. Typhimurium*, which is currently used in the Australian poultry industry. There are similar *aro-A* deletion mutant *S. Typhimurium* vaccines with claims to offer some cross protection against *S. Enteritidis*. This project was developed to explore whether Vaxsafe® ST vaccine could provide any cross-protection against a challenge with the novel *S. Enteritidis* strain isolated recently in NSW. This current *S. Enteritidis* strain is unique to Australia.

An earlier study evaluated a challenge model for infection and organ prevalence of the novel *S. Enteritidis* strain in layer hens at sexual maturity. This model was used in a challenge experiment (at 18 weeks of age) comparing unvaccinated birds with birds which had received either of two vaccination regimes using Vaxsafe® ST. The vaccination protocols were based on the current field use of Vaxsafe® ST: spray application at day old and drinking water application at 3 and 6 weeks of age followed by an intramuscular injection at 10 weeks of age. This was also compared with this vaccine program plus an extra oral application at 16 weeks of age. Assay of the vaccine organism present in prepared mixtures of drinking water showed that the 6-week application had failed.

Prevalence of infection in cloacal swabs and organ cultures was lower than anticipated, making determination of significant differences difficult. The vaccination regimes allowed numerically lower ($P > 0.05$) *S. Enteritidis* isolations from cloacal swabs at 5 days post infection (PI) and in liver, spleen and caecum at 14 days PI. Histopathological findings indicated less pathology in duodenum and ovary of vaccinated birds. The additional vaccination at 16 weeks did not appear to improve protection against the challenge.

The findings support further studies with modifications to the vaccine methodology and protocol. The use of skim milk as a stabilizer when vaccinating by drinking water is highly recommended for the field with Vaxsafe® ST.

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Introduction

For the first time in Australia, *Salmonella* serovar Enteritidis phage type 12 (PT12) had been detected across multiple layer flocks (Fraser, 2019; Whitworth, 2019). It has caused human food poisoning cases here involving eggs as the source (NSW Health, 2019). This incursion of this strain of *S. Enteritidis* into the Australian layer industry was characterised by rapid spread amongst numerous farms in NSW and one large farm in Victoria in 2018-19. Further spread has occurred in Victoria in 2020. This was facilitated by egg trading between farms. Thirteen farms in NSW and the Victorian farm were depopulated under biosecurity orders in 2019 and none have yet been able to repopulate (Fraser, 2019). The ability to cause human salmonellosis outbreaks with this strain and the severe consequences for producers if it is detected on their farms has caused major concern in the industry and been followed by strong biosecurity orders affecting all farms in NSW.

We are just learning about how invasive the organism may be and how likely it is to contaminate eggs. At present there are no commercially available vaccines based on *S. Enteritidis* in Australia, but there is a live *S. Typhimurium* vaccine currently widely used in the

Australian layer industry. There would be considerable interest within the industry to know whether this existing vaccine, as used, confers any protection against the new *S. Enteritidis* strain. This research project is designed to determine if a registered anti-*Salmonella* vaccine (Vaxsafe® ST; Bioproperties Australia) could provide any cross-protection against *S. Enteritidis*.

The use of live attenuated *Salmonella* vaccines has become common practice in many countries (Methner, 2018). Focus has been placed on applying *Salmonella* vaccination against the serovars of major public health relevance, *S. Enteritidis* and *S. Typhimurium*. Live *Salmonella* vaccines assist in inducing protective mechanisms effective during the immunity gap period before the adaptive immune response is developed (Methner et al, 2011).

It is vital to know whether the use of the available vaccine can elicit any cross-protection against this new *S. Enteritidis* strain so that the industry can gauge its current level of protection and what immediate capabilities there are for protecting their flocks against this new strain. An initial challenge model and organ spread study, funded by Australian Eggs Ltd (Groves et al., 2019), has generated strong interest from the industry, exhibited at workshops held nationwide, and a desire to know whether any protection is afforded by existing vaccination practices.

Current programs used in Australia to eliminate *Salmonella* species from the flock environment are offering a poorly defined level of protection. The Vaxsafe® ST vaccine plays an important role in ongoing control. It is imperative to assist producers to ensure *S. Enteritidis* does not become endemic. At this time there are no vaccines specifically indicated to reduce *S. Enteritidis* infection in poultry registered for use in Australia. It is hoped that the use of Vaxsafe® ST may offer some protection to effectively reduce incursion of *S. Enteritidis*. A program developed with the use of Vaxsafe® ST to assist in reducing the spread of SE between young and adult chickens and the contamination of eggs from breeder or commercial layer flocks is a possibility. This project was proposed as the first step to establish if Vaxsafe® ST will reduce colonisation and systemic infection with SE in pullets. Reduction of the two main risk factors for egg contamination (intestinal colonisation and systemic infection with *S. Enteritidis*) will support use of the vaccine to limit vertical transmission.

Objectives

Evaluation of a live *S. Typhimurium Aro-A* deletion mutant vaccine (Vaxsafe® ST) to provide cross protection against intestinal and tissue colonisation by the recent *S. Enteritidis* from NSW in layer chickens. A measure of some cross protection was determined for caecal colonization but not for organ presence under the study duration and conditions.

Methodology

Forty 1-day-old ISABROWN layer pullets, vaccinated against Marek's Disease and Infectious Bronchitis, were obtained and housed at the University of Sydney in two climate-controlled rooms. Twenty-five birds were kept in one room as the vaccination group and 15 were kept in a separate room as the unvaccinated controls. Both groups received vaccinations against Newcastle Disease (Poulvac Newcastle V4, batch 332535, exp 06/06/20, Zoetis) and Infectious Bronchitis (Poulvac Bron Vic S, batch 36607, exp 11 Oct 21, Zoetis) by eye drop application at 6 and 7 weeks respectively. No other routine vaccinations were given.

The anti-*Salmonella* vaccination protocol was determined from advice from industry and the vaccine's manufacturer on general practice with this live vaccine.

These birds were allocated to treatment groups as shown in Table 1.

Table 1. Experimental design

Group	Treatment	Number Birds
UU	Unchallenged Control - birds to remain unvaccinated and unchallenged	5
SVU	Unchallenged Standard Vaccine - vaccinated by standard method and unchallenged	5
UC	Challenged Control - unvaccinated but to receive challenge	10
SVC	Challenged Standard Vaccine - vaccinated by standard method and challenged	10
TVC	Challenged Trial Vaccine - vaccinated as standard but with an extra oral dose at 16 weeks of age and challenged	10

The unchallenged control group consisted of 5 unvaccinated birds (UU) and 5 birds which received the standard commercial vaccine application (SVU).

Groups UU and UC were held in one room and groups SVU, SVC and TVC were held in a separate room. Biosecurity procedures were in place between these rooms (serviced unvaccinated group first, disinfectant foot baths, disposable overalls, gloves).

In the room holding birds to be vaccinated, Vaxsafe® ST (batch STM183491A, exp 18 Dec 21, Bioproperties) was administered to the birds as a coarse spray upon delivery to the facility from the hatchery. The same batch of Vaxsafe® ST was given again at 3 and 6 weeks via drinking water stabilized with VacPac-Plus® (blue dye preparation) mixed in the water at an equivalent rate of 100g/ 1000L at least 15 minutes prior to addition of the Vaxsafe® ST. The

tap water used was Sydney town water with a commercial stabilizer product added at least 15 minutes prior to vaccine addition. Initial dilution was in either sterile PBS (day 0 - spray) or reverse osmosis (RO) water (weeks 3, 6 and 16 oral), or prewarmed Universal diluent (week 10 intramuscular injection).

At 10 weeks of age, the vaccination group were given an intramuscular administration of Vaxsafe ST which was prepared in Universal diluent (batch 067/18, exp Jan 21, Merial), pre-warmed to 37 °C for 1 hour, and then the vaccine preparation was held at 37 °C for a further hour before administration. At 16 weeks of age, the group which were to receive an extra oral dose of Vaxsafe ST (group TV) were separated into another room and given an 1 mL oral dose via mouth drop from a syringe of Vaxsafe ST suspended in tap water, The water was stabilized with skim milk powder (SKM) for 20 minutes before adding the vaccine.

At seventeen weeks of age 30 birds (groups UC, SVC and TVC) were transferred to EMAI. Seven days later, these birds received an oral challenge of *S. Enteritidis* (PT12) at 10⁸ CFU, given using a stepper pipette and tip, of a culture suspended in sterile Phosphate Buffered Saline (PBS). Cloacal swabs were taken five days post challenge. At fourteen days post challenge samples were collected aseptically from: liver, spleen, caecum, ovary and oviduct after euthanasia. Additional swabs from peritoneum and liver were collected. Detection of *S. Enteritidis* from all samples was performed at a NATA accredited laboratory (Birling Avian Laboratories, Bringelly, NSW, Australia) in accordance with the Australian Standard AS 5013.10-2009 (equivalent to ISO6579:2002). Using Australian standard culture detection methods, subsamples from enriched initial samples (after overnight incubation in Buffered Peptone Water) were collected for *S. Enteritidis* PCR (Kasturi and Drgon, 2017) at EMAI .

Results and Discussion

Vaxsafe ST preparation and administration.

Preparation and administration details for Vaxsafe ST are shown in Table 2, along with expected cfu/mL calculations of the dilutions. Also shown in Table 2 are the bacterial counts obtained from Birling Avian Laboratories on samples of the prepared vaccine preparations. In each case a fresh vial of Vaxsafe® ST was used to prepare the media for administration.

As can be seen from Table 2, initial dilutions gave close to expected cfu counts where assayed, and the intramuscular preparation assays were also acceptable. However, the drinking water applications were below the expected titre. This was marginally so at 3 weeks but the 6-week application appeared to fail completely after dilution in tap water stabilized with Vac-Pac Plus® 15 minutes before addition of the Vaxsafe® ST. The oral vaccination given to the TV group at 16 weeks used skim milk powder as stabilizer with excellent titre results. At the same time, the initially diluted vaccine was also added to plain tap water tap water stabilized with Vac-Pac Plus and tap water stabilized with skim milk powder. These were not administered to the birds but were carried out to attempt to understand the earlier failed result at 6 weeks. It was apparent that plain tap water made the Vaxsafe® ST organism unable to be cultured and the use of Vac-Pac Plus was not able to provide protection against

this. The skim milk powder however allowed the vaccine organism to be cultured at the expected level. A later test of the tap water showed a chlorine level of under 0.5 ppm (using chlorine test strips). Hence protection of the vaccine organism when administering via drinking water in the field should be given high attention and the outcome of the experiment needs to take this into consideration.

Table 2. Vaxsafe ST preparation assays (batch STM183491A, exp 18 Dec 21)

Date and age	Route administered	Preparation	Expected titre (cfu/ mL)	Assayed titre (cfu/ mL)
5/11/19 Day 0	Spray	1 vial into 100 mL sterile PBS ^a Approx. 2.2 mL applied to 25 birds	10 ⁸	Not done
27/11/19 3 weeks	Drinking water	1 vial into 100 mL RO ^b water, then 8.7 mL into 900 mL tap water plus 0.1 g VacPak Plus added 15 m earlier. Allowed to drink 300 mL – at end of admin	10 ⁸ 9.7 x 10 ⁵	1.5 x 10 ⁸ 5 x 10 ³ 1.8 x 10 ⁴
18/12/19 6 weeks	Drinking water	1 vial into 100 mL RO water, then 10.7 mL into 2000 mL tap water plus 0.2 g VacPak Plus 15 m prior End of vaccination	10 ⁸ 5.35 x 10 ³ 5.35.x 10 ³	7.75 x 10 ⁷ 10 ⁷ <10 <10
14/1/20 10 weeks	Intramuscular	Pre-activation: 1 vial into 400 mL prewarmed diluent ^c , then held 37°C for 1 hr, then given at 0.4 mL i/m per bird End of vaccination	2.5 x 10 ⁸ 2.5 x 10 ⁸ 2.5 x 10 ⁸	6 x 10 ⁷ 6.65 x 10 ⁷ 1.30 x 10 ⁸
28/2/20 16 weeks	Mouth drop	1 vial into 100 mL RO water (A), then 2 mL into 20 mL tap water plus 0.05g SKM ^d – 1 mL per bird orally (B)	10 ⁸ 10 ⁷	Not tested 3.85 x 10 ⁷
28/2/20	Comparison dilutions	<ul style="list-style-type: none"> 0.5 mL A into 500 mL tap water plus 0.05g VacPak Plus (20 min prior) (C) 0.5 mL A into 500 mL tap water plus 1.25 g SKM^d (20 m prior) (D) 0.5 mL A into 500 mL tap water (E) 	10 ⁵ 10 ⁵ 10 ⁵	<10 1.8 x 10 ⁵ <10

^a Phosphate buffered saline

^b Reverse osmosis water

^c Merial universal diluent batch 067/18 exp Jan 21, warmed at 37°C for 1 hour prior to adding Vaxsafe® ST.

^d SKM – skim milk powder

Detection of S. Enteritidis in cloacal swabs and tissues

Results of cultures and qPCR outcomes of cloacal swabs and organ tissues are shown in Tables 3 and 4 respectively. Table 3A shows calculated protective indices for the vaccinated and challenged groups (SVC and TVC) compared to the challenged controls (UC).

Day 14 PI cloacal swabs were only tested using qPCR without enrichment culture. No *Salmonella* was detected in any of the unchallenged birds by either culture or qPCR.

Table 3. Culture results for *Salmonella* from cloacal swabs day 5 post infection (PI) and tissue cultures day 14 PI.

Group	Day 5 PI		Day 14 PI			
	Cloacal swab ^a	Liver ^a	Spleen ^a	Caecum ^a	Ovary ^a	Oviduct ^a
Unchallenged Control (UU)	0/5	0/5	0/5	0/5	0/5	0/5
Unchallenged Std Vaccine (SVU)	0/5	0/5	0/5	0/5	0/5	0/5
Challenged Control (UC)	3/10	6/10	4/10	7/10	1/10	0/10
Challenged Std Vaccine (SVC)	2/10	4/10	2/10	5/10	1/10 ^b	1/10 ^b
Challenged Trial Vaccine (TVC)	1/10	4/10	3/10	4/10	0/10	0/10

^a Number positive / number birds sampled

^b The positive result from ovary and oviduct from the standard vaccine group was from the same bird.

Protective index (PrI) can be calculated as:

$$\text{PrI} = (\text{prevalence in UC} - \text{prevalence in treatment}) / \text{prevalence in UC} \times 100 \%$$

Table 3A. Protective indices (%) of treatments (SVC and TVC) compared to challenged controls (UC) based on culture results.

Group (N)	Cloacal swab d5 PI No. Pos	Liver d14 PI No. Pos	Spleen d14 PI No. Pos	Caecum d14 PI No. Pos	Ovary d14 PI No. Pos	Oviduct d14 PI No. Pos
SVC (10)	33.3	33.3	50.0	28.6	0	undefined
TVC (10)	66.7	33.3	0	57.1	100	0

Some of these protective indices (Table 3A) look impressive but reference needs to be made to the level of prevalence in the control group (UC – see Table 3) – where these are very low a small reduction appears as a high percentage. None of these results differed significantly.

Table 4. qPCR for *S. Enteritidis* results after enrichment culture from cloacal swabs day 5 post infection (PI) and tissue cultures day 14 PI.

Group (N)	Cloacal swab d5 PI No. Pos	Cloacal Swab d14 No. Pos	Liver d14 PI No. Pos	Spleen d14 PI No. Pos	Caecum d14 PI No. Pos	Ovary d14 PI No. Pos	Oviduct d14 PI No. Pos
UU (5)	0	0	0	0	0	0	0
SVU (5)	0	0	0	0	0	0	0
UC (10)	8	3	6	4	8	2	1
SVC (10)	4	4	4	1	3*	3	1
TVC (10)	8	2	4	2	5	0	0

*differs from UC group by Fisher's exact test, 1-tailed (P<0.05).

The qPCR appeared to be more sensitive in detecting *S. Enteritidis* following enrichment than was culture. Due to the enrichment allowing overnight bacterial growth, the quantitative results for the qPCR are not meaningful, so only detection can be assessed.,

Both vaccine programs gave numerically lower *S. Enteritidis* detections than the unvaccinated challenged group for most of the samples. The outcomes for the challenged groups only were statistically compared using contingency table analysis. As expected, values in some cells in all analyses were <5, the χ^2 test was not valid hence Fisher's exact test was used to evaluate significance. As the interest here was to see what improvement was made by vaccination, the 1-tailed test was used, as only an improvement in protection was of interest. The only comparison that shows statistical significance was the number detected in the caecum between the challenged controls (UC) and the standard vaccination challenged group (SVC) (P=0.03, Fisher's exact, 1-tailed). Also related samples (i.e. cloacal swabs at 5 and 14 d, liver and spleen, reproductive tract) were compared using a stratified Mantel-Haenszel but no significant differences were detected between vaccinated and control challenged birds (data not shown).

The effect of the failed 6-week vaccine application cannot be estimated but speculatively may have produced smaller prevalence reduction than if this had been effective.

Swabs from peritoneum and liver were collected at day 14 post infection (during autopsy) and were cultured for salmonellae with only one culture from a challenged control bird showing growth of *S. Enteritidis* from peritoneum on all media. This result was confirmed by PCR.

Histopathological findings

A summary of the histopathological changes seen in tissues collected during post mortem on day 14 PI is included as an appendix. The Appendix – “Histopathology results summary from day 14 PI samples” is appended to show the report received from the histopathologists who examined the tissues. This report is appended “as is”, as supporting information to the full report. The pertinent information from the report is summarized below.

There were some differences detected between treatments in some tissues. Changes in the spleen were similar across groups but the pathologist commented that there was generally less lymphoid follicle formation than observed in the original challenge model study previously conducted, which may provide some insight into the lower prevalence of *S. Enteritidis* found in tissues in the present experiment. There were no significant differences in appearance of pancreas or caecum or oviduct segments in challenged or unchallenged birds. All birds had mononuclear paratyphoid nodules in the liver, but these were more frequent in challenged birds. There was an increase in vacuolation of hepatocytes in challenged but not in unchallenged birds. Inflammation of the duodenum serosa was notable in the challenged controls (UC) but this was not observed in birds which were vaccinated (SV or TV groups). In the ovary, increased inflammatory cell presence and yolk proteins were observed on the serosa of challenged birds and this appeared to be reduced by vaccination. This was also noted for thickening of the stroma with vacuolated mesothelial cells in challenged birds which was also somewhat reduced by vaccination. Increased peritonitis in multiple organs in contact with the peritoneum was also observed in non-vaccinated birds.

Conclusions

The study did not show significantly reduced prevalence of *S. Enteritidis* in cloacal swabs or tissues by culture, although levels detected in the control group were lower than experienced in the initial challenge model study. Numerical reductions were observed however. Following enrichment culture, a significantly lower prevalence of *S. Enteritidis* was detected in the caeca of standard vaccinated birds. The effectiveness of the 6-week oral vaccination was questionable and may have complicated the overall results. There were observable differences in histopathological findings in liver, duodenum and ovary with reductions in pathology noted in vaccinated birds.

The trial vaccination using the added oral dose of Vaxsafe® ST at 16 weeks (TVC) did not show any significant protection above the standard vaccination program (SVC).

These outcomes give support for further studies with a modified vaccination scheme to explore further improvement in cross-protection ability of Vaxsafe ST.

Implications

This experimental design was developed to examine if the existing Australian *Salmonella* vaccine (Vaxsafe® ST) registered to control serovar Typhimurium provides a level of protection against the new *S. Enteritidis* strain identified in Australian layers. With this work we have begun to understand how invasive the organism is and if it has the potential to infect the hen's reproductive tract. Other cross protection studies conducted overseas show some success provided by a similar vaccine against Phage Type (PT) 4 or PT13 (Beal et al., 2006) but the PT12 seems to be unique to Australia. (Fraser, 2019).

Considering the results overall, there was a numerical reduction in the number of positive cultured organs in the two Challenged Vaccinated groups, but no significant measurable difference among the results. It is important to note that this vaccination program and experimental design was conducted at sexual maturity, which is the most critical time for *Salmonella* expression and excretion in layers (Gast and Beard, 1990; Groves et al., 2016).

Extension of the results will be facilitated initially through presentations via PHA and AVPA scientific meetings and submission for journal publication.

Recommendations

This study provides presumptive evidence that some cross-protection against *S. Enteritidis* might be afforded by use of Vaxsafe® ST as currently used in the Australian poultry industry, although the full vaccination program (oral applications) was compromised by the inactivation of Vaxsafe® ST by tap water which was not protected against by a commercial stabilizer product. The application of this vaccine via drinking water by the industry needs to be carefully considered and the use of skim milk as a stabilizer is strongly recommended. This study should be the basis for future work looking at the efficacy of vaccination where Vaxsafe® ST could provide chickens with immunity against *S. Enteritidis* infection with some possible modification of the vaccination program.

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Christine Clark¹, Alison Collins², Karen Gao¹, Greg Underwood³, Sarah Williamson⁴, Sue Sharpe⁴ and Peter Groves^{1,5} provided scientific and laboratory inputs. Anne Jordan² carried out the histopathology examinations.

Bird care and husbandry was provided by Joy Gill¹, Wade Chen¹ and Jadranka Velnic⁵.

¹The University of Sydney, ²Elizabeth Macarthur Agricultural Institute, ³Bioproperties Australia, ⁴Birling Avian Laboratories, ⁵Zootechny Pty Ltd.

Media and Publications

No publications have been released to date. The project will be presented at the Poultry Hub Australia Ideas Exchange in September, 2020. Following further presentation is intended at the scientific meeting of the Australasian Veterinary Poultry Association expected in February, 2021. Journal publication will be considered.

Intellectual Property Arising

The study produced knowledge only; no commercialisable output was generated. There is no reason for any part of this report to be embargoed.

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Appendices

Appendix – Histopathology results summary from day 14 PI samples

Summary observed histopathology shown by tissue:

Liver pathology:

- Mononuclear paratyphoid nodules (lymphocytes and macrophages) and occasional lymphoid aggregates are present in 80% of non *S. Enteritidis* challenge controls (UU & SVU) and also in SE challenged birds (UC, SVC & TVC), so unlikely to be a significant result of ST vaccination or SE challenge. However, lymphocyte aggregates are expanded or more frequent in 40-50% of Se challenged birds. This wasn't reduced by vaccination.
- Suppurative hepatitis with hepatocyte degeneration was present in 20% negative controls (one UC and one SVU) and also in one (10%) positive controls (no vacc, but SE challenge) but not in any vaccinated & challenged birds (SVC and TVC). Not significant to ST vaccination or SE challenge.
- Reactive lymphoid hyperplasia only found in one bird's liver (TVC).
- Vacuolation of hepatocytes occurs to some degree in all hepatocytes – however, significant cytoplasmic vacuolation occurred in 30%-80% vacc/challenged birds (80% SVC and 30% TVC) as well as SE challenge controls (50% of UC), but not in any

unchallenged birds (checked by Anne). Cytoplasmic vacuolation of hepatocytes is a well-known phenomenon in mammalian cells after exposure to bacterial or viral pathogens. Vacuolation is also indicative of altered hepatocellular metabolism due to metabolic liver disease, toxins, protein malnutrition, anorexia (increased fatty acid mobilization from peripheral stores), extrahepatic visceral inflammation, and anoxia (inhibits fatty acid oxidation). Vacuolar hepatopathy observed in dogs given iv infusions of large doses of attenuated ST to reduce tumour growth (spontaneous neoplasia). In pullets, vacuolation of muscle cells (positive stain for neutral lipids) at site of im injection of bacterin SE (breast muscle) seen. Liver and intestinal cells also stained for neutral lipids.

Spleen:

- Generally, there was less follicle formation in spleen of these birds compared with previous trial and wondered what might explain the reduction in chronic infection. Challenge dose and age of birds the same, along with 2 week infection period before necropsy, but maybe vaccine slowed or suppressed immune response to challenge.
- Periarteriolar lymphoid tissue (PALS) populated by T lymphocytes and periellipsoidal macrophage sheet (PEMS) of B cells were present in birds of all treatments but their abundance increased from being prominent to mild, moderate or marked expansion. Moderate to marked expansion of PALS was also observed in most treatments, ranging from 20% to 50% of negative controls, vaccinated and challenged birds. Occasional to numerous lymphoid follicles were also observed in the spleen of 10% to 30% of SE challenged birds.
- The presence of tangible body macrophages in the spleen of a small proportion of both SE challenged and non-challenged birds indicates non-specific antigenic stimulation somewhere in the body, leading to lymphocyte stimulation in the spleen, followed by macrophage ingestion of the lymphocytes.
- Normally *Salmonella* replicate in macrophages and their replication isn't inhibited by limited availability of iron and zinc. Therefore *Salmonella* infected macrophages don't form free radicals and aren't lysed by NF-kB activation, unlike *Escherichia coli* infected macrophages.

Pancreas:

- Multifocal serositis with mild expansion of macrophages, lymphocytes and heterophils was observed in the pancreas of approximately half of the birds in each treatment, whether vaccinated or challenged or controls. This was complicated by inflammation of multiple organs in contact with the peritoneum, classified as peritonitis, in 10-20% birds in most treatments except for non-vaccinated SE challenged birds where 40% showed peritonitis with occasional lymphoid follicles.

Caecum:

- Small number of lymphocytes and plasma cells in LP and lymphoid aggregates in mucosa found in all treatments. Along with protozoa (maybe coccidia) in caecal epithelium, therefore not significant in SE challenge or ST vaccination (supported by Rod and Anne).

- Heterophils are normal in the mucosal LP of the duodenum and caecum, but were considered significant if they moved out of LP and into epithelium and if they aggregated in crypts.
- Heterophilic typhlitis observed in 30% of non-vaccinated and SE challenged birds (UC), so both vaccination strategies appeared to reduce typhlitis incidence. Check for clusters of heterophils, especially around blood supply indicating they're coming out of circulation and also if they pass from LP to epithelium.
- Possible oedema in LP of 30% TVC, 30% SVC. Probably not.

Duodenum:

- Inflammation of serosa or mucosa (enteritis) in 40% of positive controls (no vaccine + SE) was reduced to occasional lymphoid aggregates in 10% of vaccinated birds or not present in unchallenged birds.

Ovary:

- Extramedullary haematopoiesis (EMH) is normal in birds, but can suggest immune stimulation if number of heterophils significantly increases, cluster or move into epithelium.
- Frequent to numerous clusters of large mononuclear cells and granulocytes found in all negative control birds (UU and SVU) except for vaccinated R1510(PM #9) with large number of heterophils in stroma clustered with mononuclear cells and R1508 (PM10) – yolk protein mixes with macrophages on serosa. Single larger follicle with thickened granuloma layer and cell debris in lumen.
- Yolk protein on serosa surface and inflammatory cells observed in SE challenged birds, but not in any unchallenged birds. Presence of vacuolation associated with serositis or peritonitis does indicate immune activation, especially with yolk protein around those with serositis and multiple organs affected with peritonitis. Vaccination reduced incidence and intensity of serositis or peritonitis from 50% of non-vaccinated birds to 20% of vaccinated birds (SVC or TVC).
- Vacuolated mesothelial cells may be an indication of follicle atresia, a normal process. Thickened stroma with vacuolated mesothelial cells and clusters of granulocytes (i.e. EMH) in 70% SE challenge controls (UC), 70% of SVC and 40% of TVC (\pm serositis/peritonitis) with additional 20% SVC and 10% TVC described as moderate scattered and clustered heterophils within stroma (normal and not significant). Vacuolation of cells not reported in non-challenged birds (SVU and UU), but they would be there, just not in high abundance.

Infundibulum:

- Few lymphocytes and plasma cells in muscularis and serosa reported in all treatments with occasional clusters of granulocytes and lymphocytes within muscularis (EMH) reported in both control birds and infected birds. Not significant.

- Possible oedema (sub-epithelial or LP clear spaces in tips of mucosal folds) in infundibulum, isthmus or magnum of many birds may be an indication of recent passing of egg, therefore unlikely to be significant. It could also be a histological processing artefact.
- Struggling to see any significant treatment effects except for occasional lymphoid aggregates in LP (normal) and increased perivascular cellularity in serosa, including clusters of granulocytes and MNC in birds 31 and 35 (SVC). Increased abundance of immune cells around blood supply (perivascular) is indicative of recent immune reaction and infiltration of lymphocytes etc, compared to the normal presence of immune cells in LP.
- Occasional defects in mucosal surface of magnum with secretory cells extruded into lumen - could be artifact

Magnum:

- Epithelial cells replete with eosinophilic granules and small numbers of MNC including macrophages in serosa in all treatments, so not significant to vaccination or SE challenge.

Shell gland:

- Metritis observed in one positive control (# 23, UC) and one negative control (UU)