Development of a *Streptococcus suis* vaccine via measurement of immune responses to four different *S. suis* vaccine preparations, using an Australian cps2 ST25 strain

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By

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Executive Summary

Streptococcus suis is a pathogen of significant concern for the Australasian and global swine production industries. To date, vaccination in the face of disease outbreaks is based on the use of predominantly formalin-inactivated autogenous bacterins, with variable levels of efficacy. Pork CRC project (2C-126) identified three predominant *S. suis* strains (serotype/ST) responsible for causing clinical infection in Australian pigs. An efficacious vaccine providing protection against these strains would result in limited disease, antimicrobial use and production loss.

This project analysed the effectiveness of four different vaccine preparations against one of the major *S. suis* strains (cps2 ST25), analysing immunoglobulin subtype responses in weaner pigs following a booster vaccination protocol. Vaccine preparations (bacterins) were developed using either formalin inactivation, heat inactivation, binary ethylenimine (BEI) inactivation or lysozyme/detergent inactivation of whole bacterial cells. The initial project proposal included sonication as an inactivation method; however, this proved to be ineffective and was removed from the trial.

Vaccines were prepared at antigenic doses of $1x10^9$ cfu/ml or $1x10^{10}$ cfu/ml, and mixed with Emulsigen®-D adjuvant at 12% v/v. This resulted in eight vaccine preparations, and a saline+adjuvant control. Approximately three-week-old, male weaner pigs were randomly assigned to treatment groups of eight pigs, and each received a 1 ml intramuscular vaccination at day 0 and day 14. Serum samples were collected from each pig at weekly intervals until the end of the trial at day 35. Following this, serum samples were tested for total immunoglobulin (Ig), IgG1, IgG2 and IgM using an indirect ELISA.

The key finding from this study was that the heat-inactivated vaccine using a dose of $1x10^{10}$ cfu/ml produced the most robust immune response as measured by total Ig and IgG1. Using sample to positive ratio as a proxy of antibody level, mean optical density (OD) levels were higher than all other treatment groups. In week 4 they were significantly ($P<0.05$) higher than heat, formalin and BEI at $1x10^9$ cfu/ml, and significantly ($P<0.05$) higher than formalin at $1x10^{10}$ cfu/ml. These results were mirrored in week 5. Higher mean OD sample/positive ratio for heat-inactivated vaccine using a dose of $1x10^{10}$ cfu/ml was seen for IgG1 subtype, with a significant ($P<0.05$) difference when compared to lysozyme and BEI at $1x10^9$ cfu/ml and BEI at $1x10^{10}$ cfu/ml. The IgG2 sample/positive ratio was also higher overall for heat-inactivated vaccine at $1x10^{10}$ cfu/ml. However, a significant ($P<0.05$) difference was only seen between this and BEI at $1x10^9$ cfu/ml. There were no statistically significant differences when assessing IgM. Finally, titration of serum samples to assess total Ig, IgG1 and IgG2 titre change between day 0 and day 35 was performed for all $1x10^{10}$ cfu/ml vaccine preparations. The heat treatment vaccine showed a significant increase in total Ig measured, with a four-fold increase in antibody titre over this period, and both the heat treatment and formalin vaccines showed ≥4-fold titre increases in IgG1 measured.

This study demonstrated that heat preparation of *S.suis* bacterins may be more effective than current formalin inactivation protocols, in producing vaccines that elicit a total Ig and IgG1 response. Of note is that these preparations perform better than other chemically inactivated bacterins tested, and heat treatment is a simple, cheap and non-toxic production method. It should be noted that this project only assessed antibody response. However, the report provides preliminary data to support assessment of formalin versus heat-inactivated

vaccines on larger scale farm trials, potentially in the face of disease outbreaks, and further assessment of cellular immune responses.

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1. Introduction

Streptococcus suis infection in pigs is an Australasian and global concern, from an economic, animal welfare and production loss viewpoint, to a public health issue, with zoonotic disease resulting in human fatalities. A recent Pork CRC project (2C-126) has determined the presence of a wide range of *S. suis* serotypes and multi-locus sequence types in Australia, although three serotype/ST strains are predominantly associated with disease (O'Dea et al., 2018). Identification of these three predominant strains will direct future efforts for targeted vaccine preparation.

Treatment for affected animals is usually reliant on administration of ß-lactams such as penicillin and amoxicillin, and, where permitted, farms may prophylactically treat all pigs at the peri-weaning stage. Another management option employed by some producers is vaccination, usually in the form of bacterins (inactivated or attenuated bacterial cell suspensions) produced as autogenous vaccines from on-farm isolates (Varela et al., 2013). Due to the highly variable antigenicity of the capsular polysaccharides (CPS), *S. suis* is currently classified into 29 serotypes (six previously classified serotypes have been reassigned to different bacterial species), and it is considered that protection is only provided by homologous vaccine serotypes (Segura, 2015). While antimicrobial therapy provides a valid and effective treatment option, the industry's direction to minimise antimicrobial use makes the implementation of effective vaccines attractive.

Autogenous vaccines have long been one of the management methods used in *S. suis* associated disease; however, response is variable, and in many cases, vaccines are initiated without detailed knowledge of immunological response, resulting in poor efficacy and wasted money through both vaccine manufacture/administration costs and death of animals. Vaccination studies using whole-cell bacterins generally follow a design involving administrations of the vaccine followed by assessment of immunological response via serology, or direct challenge, or a combination of both (Buttner et al., 2012; Holt et al., 1990). Additionally, many studies and anecdotal reports indicate varying levels of protection from *S. suis* autogenous vaccines, meaning results cannot necessarily be extrapolated between strains. These studies have been completed with overseas strains, but not with Australian strains, making it difficult to reliably determine the most efficacious strain(s), doses and administration protocols for Australian piggeries experiencing outbreaks associated with the three main strains of interest. While research into subunit vaccines targeting putative virulence factors such as *mrp*, *epf* and *sly* has been undertaken (Wisselink et al., 2001), success has been limited, and whole cell vaccines are still used in the majority of cases. Currently, it has not been proven that a single subunit factor provides reliable coverage. In addition, it has been shown that serotype 2 bacterins can produce better immune responses than subunit murein-associated protein (MAP) vaccines (Baums et al., 2009). Finally, subunit vaccines are much more expensive to produce than bacterins, making them a less desirable option for vaccination of large numbers of animals.

Our laboratory recently completed analysis of 148 Australian isolates associated with clinical disease in pigs which determined that the majority were serotype 2 ST25 (17.6%), serotype 2 ST28 (6.1%) and serotype 3 ST27 (18.2%) (partially supported by Pork CRC project 2C-126). Based on this information, adoption of serological assays for determination of immune responses to *S. suis* vaccination, and development and testing of four different *S. suis* vaccine preparations, targeting one of the most common strains, serotype 2 ST25, is required to assess the most feasible and effective vaccine preparation method.

As for most swine producing countries, there are no commercially available, pre-prepared vaccines targeting *S. suis* available in Australia. Vaccination relies on strain typing from a disease outbreak, followed by autogenous vaccine production at one of a (limited) number

of facilities offering this service, using formalin inactivation. Bacterin vaccines are generally inactivated using formalin or heat treatment (Baums et al., 2009; Holt et al., 1990), both of which can have effects on the conformation of bacterial proteins, which in turn alters antigenicity of the vaccine preparation. In addition, the adjuvant used in vaccine formulation affects immunogenesis, partly through induction of type 1 or type 2 immunological responses (Segura, 2015). The Th1 cells appear to be important in promoting bacterial opsonophagocytosis, a process important in clearance of encapsulated bacteria such as *S. suis*, and this response can be improved through the use of certain adjuvants (Awate et al., 2013; Chabot-Roy et al., 2006). In order to determine the effectiveness of vaccine immunogenicity, many studies investigate IgM and IgG subclasses to determine which response is associated with a protective effect.

Formalin inactivation of bacteria occurs through a process of cross-linking proteins. In some cases, this can retain antigenic structure, and in other cases may result in loss of antigenic structure. In the case of bacterins, generally only the surface proteins are initially exposed to the immune system in the formalin inactivated preparation. Heat inactivation can be equally disruptive to antigens, as denaturation and agglutination of protein may destroy secondary structure. However, given these are classical methods for bacterial inactivation, it is prudent that they are included in this study, and there is limited information on heat treatment of *S. suis* vaccines.

Binary ethylenamine is used predominantly in preparation of viral vaccines; however, to our knowledge there have been no trials utilising it as an inactivating agent for *S. suis*. It inactivates organisms through its effects on genomic DNA via alkylating purine residues rather than denaturation of protein (Perrin and Morgeaux, 1995), and as such it may provide a valid solution in this study in retaining antigenicity of *S. suis* surface proteins. Sonication operates via production of high-frequency sound waves in a solution, leading to vibration and destruction of particles. Sonication releases internal bacterial proteins through rupture of cells, potentially exposing the immune system to antigens not available when using formalin, heat or binary ethylenamine methods. This has not been investigated to a significant degree in *S. suis* vaccines.

This study aimed to test vaccine responses using multiple vaccine formulations in a controlled environment, using multiple antibody class markers. This approach will ensure the vaccine production method giving the most significant antibody response in pigs, using Australian strains of *S. suis*, will be determined. This will potentially result in the development of a vaccine for a serotype of *S. suis* associated with a high proportion of clinical disease cases, and a significant concern for the Australasian pig industry.

We hypothesised that the antigen sparing properties of the chemical agent binary ethylenemine, and/or the antigen releasing properties of lysozyme/detergent treatment of whole bacteria, will result in more robust immune responses than traditional formalin preparation.

2. Methodology

Bacterial strains

This study used the *S. suis* isolate 199-2810, an in-house isolate which has undergone whole genome sequencing to confirm capsular type 2 and sequence type 25 (cps2 ST25). Frozen stocks were streaked onto Columbia sheep blood agar (CBSA) and subcultured twice before use.

For larger scale production, five to ten colonies were taken from blood agar and added to 400 ml Todd-Hewitt broth in 500 ml vented cap Erlenmeyer flasks (Corning). Cultures were incubated without shaking at 37°C in 5% CO_2 for 18 hours, centrifuged at 3000 x *g*, and cell pellets resuspended to required concentrations in phosphate buffered saline (PBS). Quantification of live bacteria was performed by plating 10-fold dilutions onto CBSA, and performing colony counts after 24 hours incubation at 37°C in 5% CO_2 to determine colony forming units per millilitre (cfu/ml). Stock solution cfu/ml values were used following inactivation to dilute vaccine preparations to appropriate concentrations.

Inactivation methods

Formalin inactivation was performed by incubating bacterial stock with a final formaldehyde concentration of 0.5%, for 18 hours at room temperature on a vertical rotor mixer.

Heat inactivation was performed by incubating bacterial stock at 60°C for one hour, with gentle agitation.

Binary ethylenamine (BEI) was produced by incubating 20.5 g/L of 2-Bromoethylamine hydrobromide (Sigma) in 0.175M NaOH at 37°C for 1 hour. Following this, bacterial inactivation was performed by incubating with 3mM BEI for 18 hours at 37°C. Following inactivation of bacteria, toxic BEI was inactivated by the addition of 0.1M sodium thiosulfate (Sigma).

Lysozyme/detergent inactivation was performed by resuspending cell pellets in a solution of 1.5 ml of B-Per™ reagent (Thermo) and 300 uL of 50 mg/ml lysozyme (Thermo) and incubating at 37°C for 1 hour with shaking. Following this, concentrating and buffer-exchange was performed by centrifugation using an Amicon® Ultra-15 10MWCO spin device (Merck) at 3000 x *g* for 1 hour followed by resuspension of the retentate in PBS.

Sonication was attempted using a Bandelin sonoplus sonicator with a TT 13/F2 probe. Various power setting ranging from 20%-70% amplitude for 5 sec on / 5 sec off for a period of 10-20 minutes were used. However, bacteria were not inactivated. Additional freeze-thawing of cultures prior to sonication resulted in a decrease in bacterial titre, but this was not to a safe level for vaccine production and sonication was removed from the trial.

Following inactivation, all preparations were streaked onto CSBA and incubated at 37°C in 5% CO_2 for 24 hours to ensure no colony growth was present.

Vaccine preparation

Inactivated bacterial preparations were diluted to stock solutions of $1x10^9$ cfu/ml, and $1x10^{10}$ cfu/ml in sterile PBS and frozen at -80°C. A negative control solution consisted of sterile PBS alone. Prior to use, bacterial stocks or saline were thawed, mixed with Emulsigen®-D adjuvant (MVP adjuvants) at 12% v/v on a rotor mixer for 4 hours at room temperature, and dispensed into 1 ml syringes before being stored at 4°C. Adjuvant mixed vaccine was stored for no longer than 48 hours before use (Table 1).

Table 1. Outline of vaccine treatments.

Treatment #	Inactivation method	Antigen dose
1	Heat	$1x10^9$ cfu/ml
2	Formalin	$1x10^9$ cfu/ml
3	BEI	$1x10^9$ cfu/ml
4	Lysozyme	$1x10^9$ cfu/ml
5	Heat	$1x10^{10}$ cfu/ml
6	Formalin	$1x10^{10}$ cfu/ml
7	BEI	$1x10^{10}$ cfu/ml
8	Lysozyme	$1x10^{10}$ cfu/ml
10	Neg control	n/a

Animal trials

All animal work was conducted under Murdoch University Animal Ethics Committee approval (permit R3240/20). A total of 72, three-week-old male Large-White x Landrace pigs were enrolled in the vaccine study, allowing for 8 pigs per vaccine group. Pigs were transported to the Murdoch University Isolation Animal Facility and housed in temperature-controlled rooms with *ad libitum* access to a standard commercially prepared weaner diet (FARMYARD Pig Weaner Pellets, Weston Milling) and water. Pigs underwent five days of acclimatisation prior to the trial beginning.

Two rooms with eight pens in each (Figure 1) were used; the pens in the first room had 5 pigs in four of the pens, one from each vaccine preparation and one control (these pens marked as 'C'), and four treatment pigs in each of the remaining pens; this was repeated in the second room. Each pig in each pen were given different treatments to minimise pen effect in order to make the pig the unit of measurement as opposed to the pen.

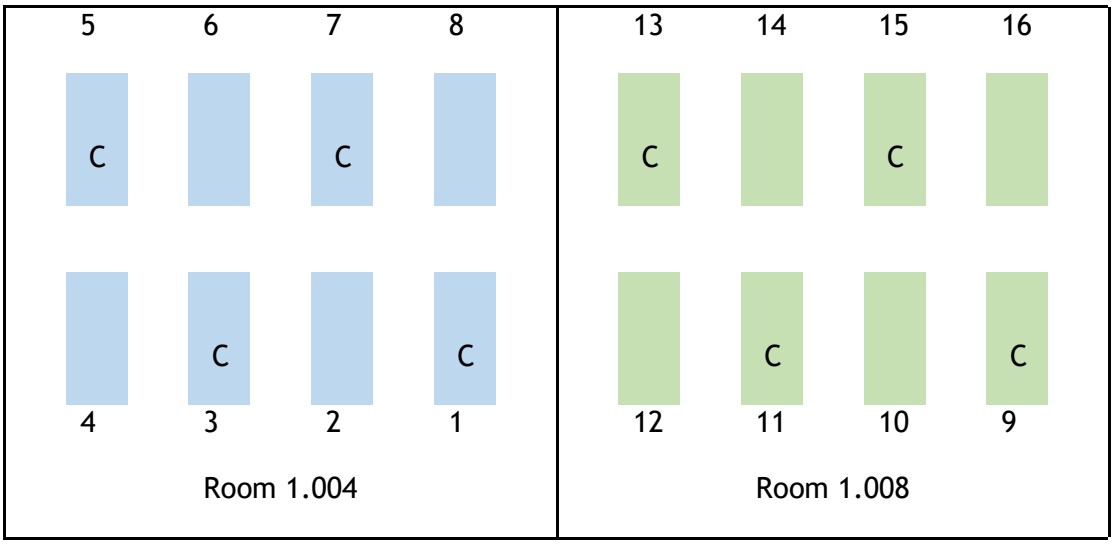

Figure 1. Block design setup for Animal Isolation House.

On day 0, pigs received a single 1 ml intramuscular vaccination on the right-hand side of the neck, and a booster dose on day 14 according to their treatment. Blood was collected from the cranial vena cava into standard clotting blood tubes (BD) on days 0, 7, 14, 21 and 28 for measurement of serological response, and pigs were weighed on days 0, 7, 14 and 21.

ELISA

To prepare antigen for coating ELISA plates, 4-5 colonies of *S. suis* were picked to inoculate a 500 ml vented cap Erlenmeyer flask of Todd-Hewitt broth, followed by incubation at 37°C in 5% CO2 for 6 hours. Cultures were then centrifuged at 3000 x *g* for 10 minutes, and the cell pellet resuspended in 40 ml of PBS/0.5% formalin. An aliquot of resuspended culture was then diluted 1:10 in formalin and the optical density read at 600 nm on a Tecan Spark plate reader. The following formula was then used to determine the dilution factor for the plate coating solution.

$$V1 = \frac{C2\ x\ V2}{C1}$$

V_1 : Volume of bacterial suspension

C_1 : original OD_{600} of the bacterial suspension

C_2 : 0.5

V_2 : nb of plates x 10 ml

Volume of ddH_2O to add: $V_2 - V_1$

Greiner 96 well flat-bottom clear Microlon High-binding plates were used, and were coated with 100 uL per well and left in a BSLII hood for 48 hours to dry. Following this, 50 uL of methanol per well was added and left to dry, before coated plates were stored at room temperature.

Prior to use, coated plates were washed three times in PBS/Tween 20 (0.05%) (PBST) and blocked with 300 uL of PBST/2% skim milk powder, before washing again three times. Pig sera were diluted 1:200 in PBST and 100 uL added to the wells. For titration studies, serum at 1:100 was added to column one, followed by a 2-fold dilution series being performed across the plate. A high positive, low positive and blank sample were also included in each assay. Serum samples were incubated on the plate for one hour at room temperature, then plates were washed three times in PBST. Following washing conjugates were added as follows:

- For total Ig, 100 uL of Goat-anti-pig IgG HRP conjugated antibody (Jackson Laboratories) diluted 1:12,000 was added to each well and incubated for 30 minutes at room temperature, before washing the plate three times and adding substrate.
- For IgM, 100 uL of Goat-anti-pig IgM HRP conjugated antibody (BioRad) diluted 1:10,000 was added to each well and incubated for one hour at room temperature, before washing the plate three times and adding substrate.
- For IgG subtypes, 100 uL of primary mouse-anti-pig IgG1 antibody (BioRad) diluted 1:5,000 or primary mouse-anti-pig IgG2 antibody (BioRad) diluted 1:9,000 were added to wells and incubated for 1 hour at room temperature, before washing the plate three times. This was followed by addition of 100 uL of goat-anti-mouse IgG/IgM HRP secondary antibody (Jackson Laboratories) at a dilution of 1:2,000 for the IgG1 assay and 1:4,000 for the IgG2 assay. Secondary antibodies were incubated for 30 minutes at room temperature, before plates were washed a further three times.
- For all assays 100 uL of K-Blue substrate (ELISA systems) was added per well, and incubated in the dark for 6 minutes at room temperature. Reactions were stopped by addition of 50 uL of 0.5M sulphuric acid, and absorbance read at 450nM on a Tecan Spark plate reader.

Analysis

For ELISA analysis, sample to positive ratios were determined by dividing sample OD values by the mean high positive control OD values. As a true positive or protective titre is unknown, an arbitrary cut-off value for the total Ig titration assay was set at a corrected OD value of 0.275, as this value allowed calculation of a titre for the pre bleed samples across all assay plates. The cut-off value for the IgG1 and IgG2 assays was calculated at the mean plus 2 SD of the negative control across all plates run (0.03 and 0.05 respectively).

Differences between treatment groups based on bodyweight or ELISA OD were determined using univariate ANOVA in SPSS (IBM). Post-hoc analysis for significant differences was performed using Tukey's HSD test.

3. Outcomes

Animal trial

Prior to beginning the trial, one pig was excluded due to the presence of a foot lesion, and this resulted in 8 pigs per treatment group (aside from the negative control group which had 7 pigs). All pigs remained healthy for the duration of the trial, and treatment groups tolerated the vaccine preparations well, with no inflammatory response noted at the injection site throughout the trial. Univariate ANOVA did not demonstrate any significant differences in mean weights across the nine treatment groups over weeks one to four.

ELISA results

Total Ig

No significant differences were seen in OD sample to positive ratios in weeks one to three.

In week 4, a significant difference ($P<0.05$) was seen between heat treatment vaccine at $1x10^{10}$ cfu/ml, and heat treatment vaccine at $1x10^{9}$ cfu/ml, formalin treatment vaccine at $1x10^{9}$ cfu/ml, BEI treatment vaccine at $1x10^{9}$ cfu/ml, formalin treatment vaccine at $1x10^{10}$ cfu/ml, and the negative control (Figure 2).

At week 5, significant differences ($P<0.05$) were seen between heat treatment vaccine at $1x10^{10}$ cfu/ml, formalin treatment vaccine at $1x10^{9}$ cfu/ml, BEI treatment vaccine at $1x10^{9}$ cfu/ml, and formalin treatment vaccine at $1x10^{10}$ cfu/ml (Figure 2). Tukey's HSD p-values are presented in Table 2.

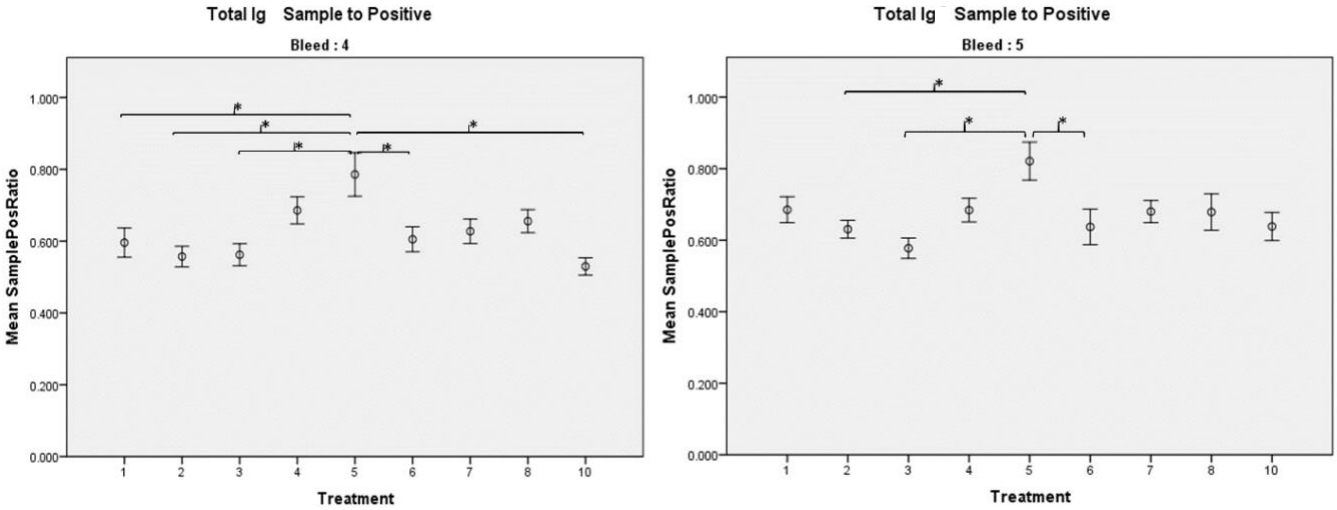

Figure 2. Total Ig sample to positive ratios for week 4 and week 5 samples. *Indicates Tukey HSD P value <0.05. Refer to Table 1 for treatments.

Table 2. Tukey HSD test multiple comparison of means for total Ig results.

Bleed	Group comparison[A]	P value
Week 1	no significant comparisons	n/a
Week 2	no significant comparisons	n/a.
Week 3	no significant comparisons	n/a.
Week 4	5 v 1	0.018
	5 v 10	0.001
	5 v 2	0.002
	5 v 3	0.002
	5 v 6	0.029
Week 5	5 v 2	0.030
	5 v 3	0.020
	5 v 6	0.041

[A]Refer to Table 1 for treatments.

Total Ig titration

To determine change in antibody titre between weeks one and five, serum samples from all pigs from each of the vaccine groups (n=8 per group) prepared with an antigen dose of $1x10^{10}$ cfu/ml and the negative control group (n=7) were diluted 2-fold, and the titre between the pre-vaccination and week 5 post-vaccination blood samples compared.

Using a cut-off OD value of 0.275, only the heat-treated vaccine preparation showed a significant four-fold increase in titre (Figure 3), with the lysozyme treatment showing a response very close to the four-fold increase. There was no significant change in titre with the formalin, lysozyme or BEI preparations, although all showed a response compared to the negative control vaccine preparations (Table 3).

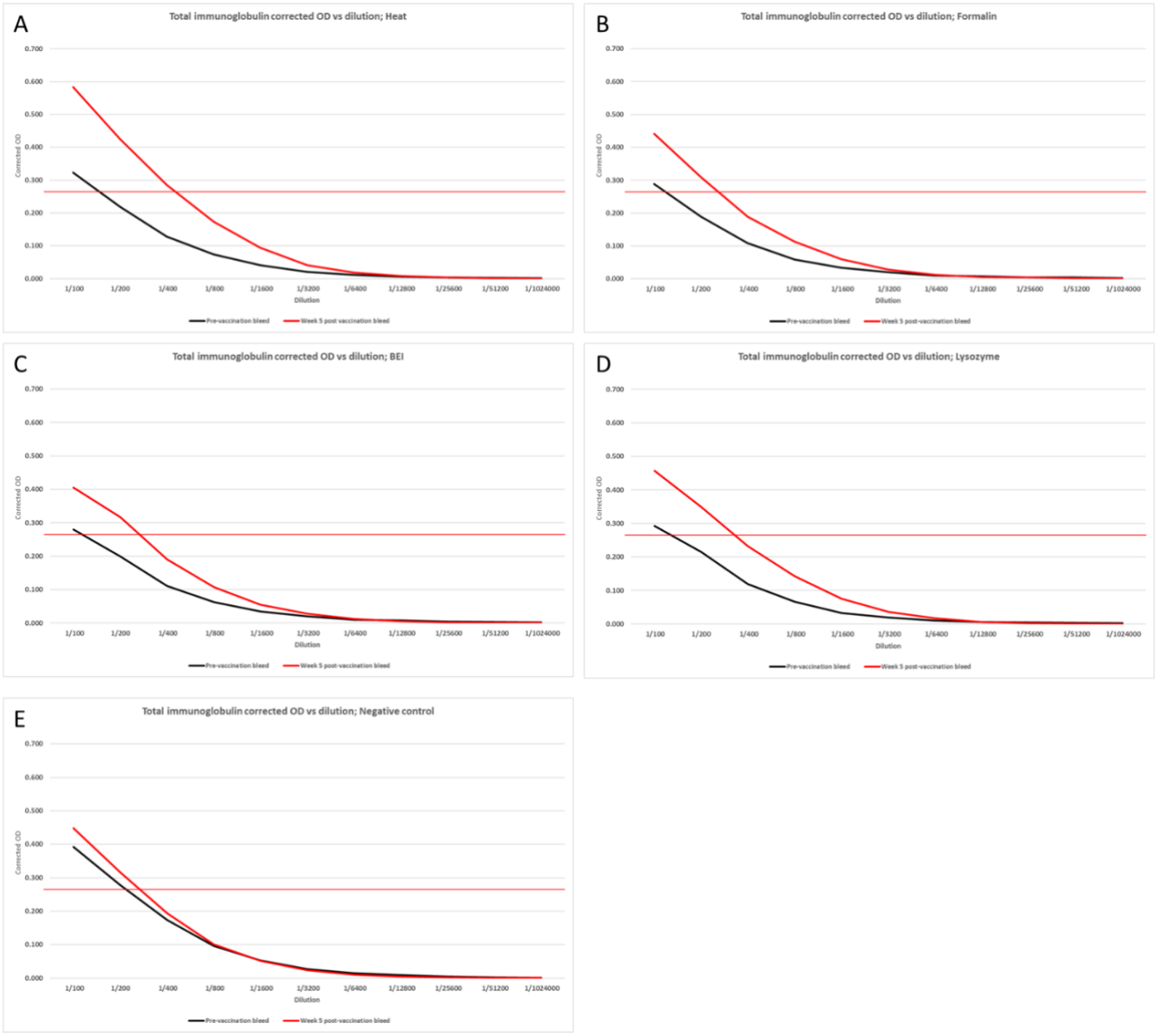

Figure 3. Titration results for heat preparation (A), formalin preparation (B), BEI preparation (C) lysozyme preparation (D) and negative control (E) vaccine serum samples. Black and red lines indicate mean OD values (+/- SD) for pre- and post-vaccination bleeds respectively. Red horizontal line indicates threshold for titre calculation.

Table 3. Titre comparisons between pre- and post-vaccination samples.

Treatment	Pre-vaccination titre	Week 5 post-vaccination titre
Heat	100	400
Formalin	100	200
BEI	100	200
Lysozyme	100	200
Neg control	100	100

IgG1

No significant differences were seen in OD sample to positive ratios in weeks one to three. In week four, a significant difference was seen between heat treatment vaccine at $1x10^{10}$ cfu/ml, and heat treatment vaccine at $1x10^{9}$ cfu/ml, BEI treatment vaccine at $1x10^{9}$ cfu/ml, lysozyme treatment vaccine at $1x10^{9}$ cfu/ml, BEI treatment vaccine at $1x10^{10}$ cfu/ml and the negative control (Figure 4).

At week five, significant ($P<0.05$) differences were seen between heat treatment vaccine at $1x10^{10}$ cfu/ml, BEI treatment vaccine at $1x10^{9}$ cfu/ml, lysozyme treatment vaccine at $1x10^{9}$ cfu/ml and BEI treatment vaccine at $1x10^{10}$ cfu/ml (Figure 4). Tukey's HSD p-values are presented in Table 3.

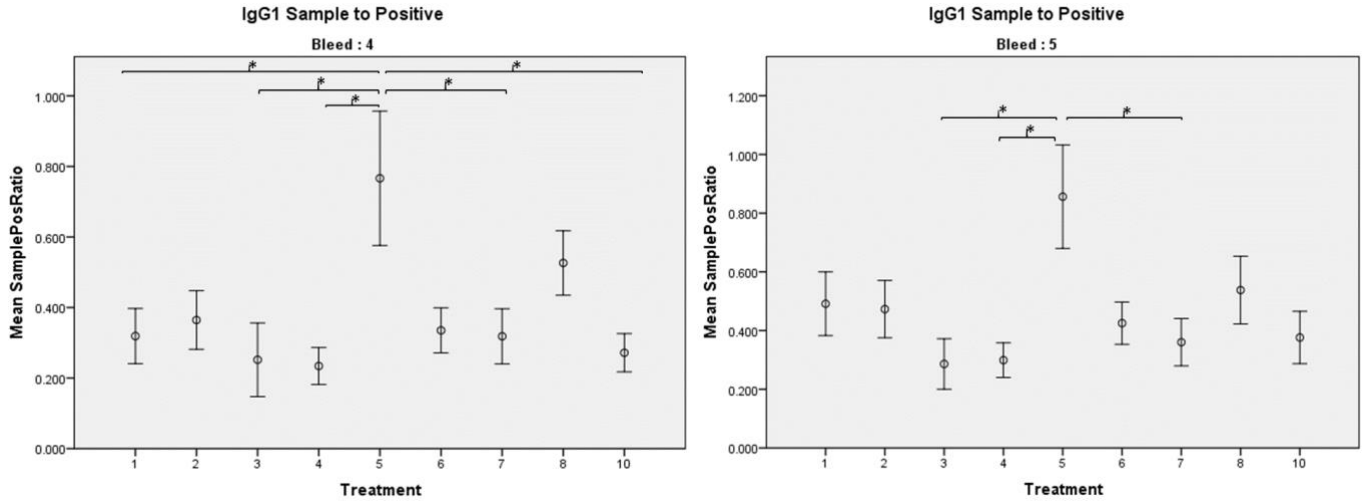

Figure 4. The IgG1 sample to positive ratios for week 4 and week 5 samples. * Indicates Tukey HSD P value <0.05. Refer to Table 1 for treatments.

Table 3. Tukey HSD test multiple comparison of means for IgG1 results.

Bleed	Group comparison[A]	P value
Week 1	no significant comparisons	n/a
Week 2	no significant comparisons	n/a
Week 3	no significant comparisons	n/a
Week 4	5 v 1	0.045
	5 v 10	0.025
	5 v 3	0.011
	5 v 4	0.008
	5 v 7	0.045
Week 5	5 v 3	0.007
	5 v 4	0.009
	5 v 7	0.031

[A]Refer to Table 1 for treatments.

IgG1 titration

To determine change in antibody titre between weeks one and five, serum samples from all pigs from each of the vaccine groups prepared with an antigen dose of $1x10^{10}$ cfu/ml (n=8 per group) and the negative control group (n=7) were diluted 2-fold, and the titre between the pre-vaccination and week 5 post-vaccination blood samples compared (Table 4). Using a cut-off OD value of 0.03, interpretation of the results was confounded by the reactivity of the negative control group, with an assay titre rising from 100 to 400 post-vaccination, indicating the development of non-specific IgG1 with age.

Despite this, heat treated vaccines demonstrated the best response, with an antibody titre increase of ≥ 8x post-vaccination. However, the final titre of 800 was not significantly different from the final titre of the negative control group.

Table 4. The IgG1 titre comparisons between pre- and post-vaccination samples.

Treatment	Pre- vaccination titre	Week 5 post-vaccination titre
Heat	negative	800
Formalin	negative	400
BEI	200	800
Lysozyme	100	800
Neg control	100	400

IgG2

No significant differences were seen in OD sample to positive ratios in weeks one to three. In week four a significant (P<0.05?) difference was seen between heat treatment vaccine at $1x10^{10}$ cfu/ml and the negative control (p-value = 0.048) (Figure 5).

In week five, the only significant difference was between heat treatment vaccine at $1x10^{10}$ cfu/ml and BEI treatment vaccine at $1x10^{9}$ cfu/ml (p-value = 0.009) (Figure 5).

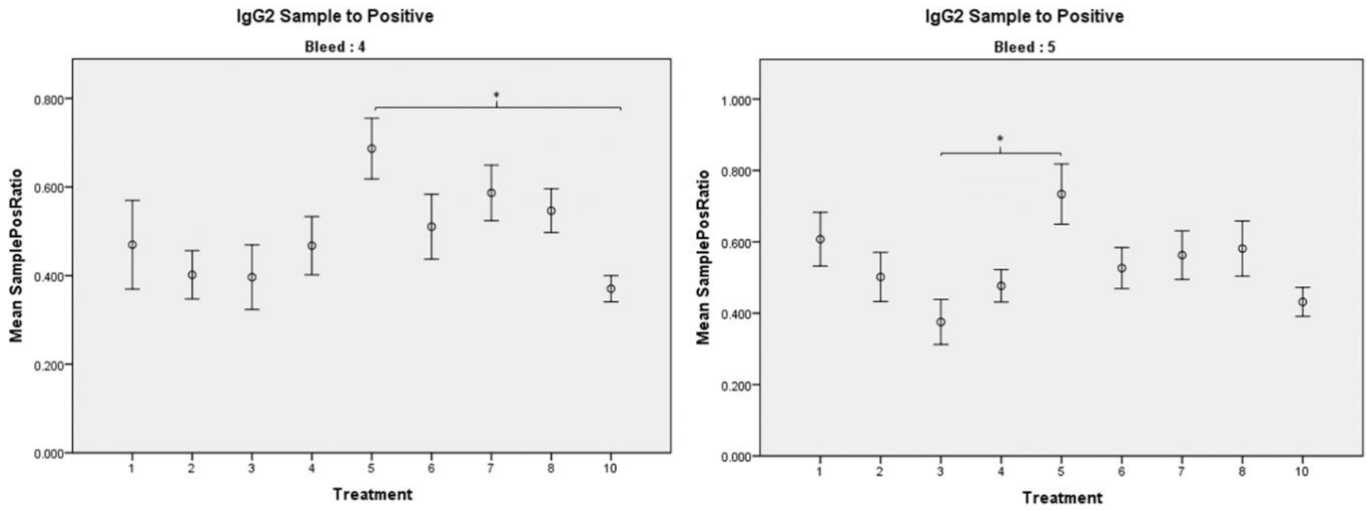

Figure 5. The IgG2 sample to positive ratios for week 4 and week 5 samples. *Indicates Tukey HSD P value <0.05. Refer to Table 1 for treatments.

IgG2 titration

To determine change in antibody titre between weeks one and five, serum samples from pigs each the heat treatment (n=8), formalin treatment (n=8), lysozyme (n=7) and BEI treatment (n=7) groups prepared with an antigen dose of $1x10^{10}$ cfu/ml, and from the negative control group (n=6), were diluted 2-fold. The titre between the pre-vaccination and week 5 post-vaccination blood samples were then compared (Table 5). No antibody response was seen in any of the treatment groups.

Table 5. The IgG2 titre comparisons between pre- and post-vaccination samples.

Treatment	Pre- vaccination titre	Week 5 post-vaccination titre
Heat	1600	1600
Formalin	800	800
BEI	800	800
Lysozyme	800	800
Neg control	800	400

IgM

No significant differences were seen in IgM sample to positive OD ratios between treatment groups across the total length of the trial.

4. Application of Research

This research has demonstrated that *S. suis* heat-treated vaccine preparations can elicit IgG antibody responses that are superior to other preparations tested, including the currently used formalin inactivation method. The use of heat inactivation is a simple and cheap method for bacterin production, and would likely be a feasible option for large scale vaccine

production following further tests. If this proved to be efficacious in on-farm trials, adoption by producers would not require any further labour costs or production costs, as existing formalin vaccines could be swapped out for heat-treated vaccines. It would also minimise costs associated with formalin inactivated vaccine production as there are no chemical costs or residual formalin testing required.

5. Conclusion

This study has confirmed that heat-treated *S. suis* vaccines can elicit serotype specific IgG responses, with a predominant IgG1 subtype response. This response is greater than that for the commonly used formalin inactivated vaccine when assessing total Ig, although on-par when assessing IgG1 and IgG2 subclasses. Heat is also superior to the previously untested inactivation methods using BEI or lysozyme/detergent.

The use of lysozyme/detergent, while demonstrating an increased titre response on the IgG1 ELISA compared to BEI and the negative control, did not result in a significant total Ig titre increase between pre- and post-vaccination sera. Furthermore, it did not show significant responses as measured by sample/positive ratios to warrant further investigation.

6. Limitations/Risks

There are three key limitations which must be taken into account from this study. First, the sample sizes are small, with 8 pigs per treatment group, and thus this must be viewed as a pilot study with the response to the heat-treated vaccine indicating that this is suitable for advancement to larger scale field trials.

Second, despite the farm of origin not reporting *S. suis*-associated disease, this is a common organism, and all piglets had some level of reactivity on the ELISAs at the beginning of the trial. This indicates the possibility of cross-reactive maternal antibodies being present. While this should be noted as a confounding factor, the randomisation of pigs into treatment groups and the comparison of responses between groups allows conclusions to be drawn.

Finally, this trial was designed only to assess immunoglobulin responses, precluding the ability to determine protectiveness of the vaccine preparations upon exposure to virulent *S. suis*, as would be possible with a challenge trial. It should also be noted that the predominant increase in Ig levels was IgG1 based, which has less complement-activating capacity and involvement in opsonisation, a key response to clearing encapsulated bacteria, than IgG2 (Crawley and Wilkie, 2003). However, this IgG1 based response is similar to formalin-based vaccine responses, and the significant increases in IgG1 titres across weeks four and five of the study may deliver a level of protection not achieved with current formalin preparations. This inactivation method, coupled with an appropriate adjuvant formulation to promote immunoglobulin isotype switching to favour IgG2 production, may be a suitable vaccine preparation for more effective autogenous vaccine preparations in Australia.

7. Recommendations

Given the total Ig response seen with the heat-inactivated vaccine preparation, particularly in comparison to the currently used formalin preparation, it is recommended that a further small-scale trial mimicking that in this report is performed using several different adjuvant

preparations (e.g., Emulsigen, Allhydrogel, oil-in-water) to determine that which produces the greatest titre increase and the highest IgG2 switch. This trial could be much smaller than the current trial as only four vaccine groups using $1x10^{10}$ cfu/ml doses and a negative control group of 8 weaners each would be required.

Following this, scale up to an on-farm trial comparing formalin with the optimal heat preparation should be performed using significantly larger numbers of pigs, to assess efficacy in preventing disease. *Streptococcus suis* challenge trials are very difficult to perform, and require development of an infection model based on a certain serotype. This is not feasible for evaluation of vaccine efficacy in the current context. It is proposed that the protective effect of the optimum vaccine preparation is tested on a commercial piggery with ongoing *S. suis* disease outbreaks, typed as a cps2 ST25 strain.

Based on an expected incidence of disease in the herd of 5%, and a reduction of disease to 1% in vaccinated animals, an 80% power calculation would suggest approximately 200 animals are needed per treatment group in comparison to the general population. As such we would propose vaccinating 200 weaners with a standard formalin vaccine, 200 weaners with the optimum heat-treated vaccine following the protocol used in the current study, and compare the disease incidence against the general population for evidence of protection.

Finally current Australian autogenous vaccine manufacturers can be engaged to assess switching from formalin to heat based vaccine production.

8. References

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Appendices

Appendix 1: Treatment groups and sow parity.

PEN	Tag No	Treatment	Parity	Sow
1	1	1	2	Y2797
1	4	2	4	PI669
1	25	6	4	Y2502
1	43	8	2	T4910
1	59	-ve	2	T4886
2	2	2	2	Y2797
2	5	1	4	PI669
2	26	8	4	Y2483
2	44	7	2	T4910
3	3	3	2	Y2797
3	6	4	4	PI669
3	10	-ve	4	B5561
3	27	5	4	Y2483
3	45	6	2	T4910
4	7	3	4	PI669
4	16	4	2	Y2794
4	28	7	4	Y2483
4	46	5	2	W1602
5	8	6	4	B5569
5	17	5	2	Y2794
5	29	3	4	Y2483
5	47	4	2	W1602
5	60	-ve	2	T1809
6	9	5	4	B5569
6	18	6	2	Y2794
6	30	2	4	B5556
6	48	3	2	W1602
7	11	8	4	B5561
7	19	7	2	Y2794
7	31	1	4	B5556
7	61	2	2	T1809
7	71	-ve	4	T4344
8	12	7	4	Y2510
8	32	4	4	B5556
8	34	8	2	Y2781
8	50	1	2	W1586

PEN	Tag No	Treatment	Parity	Sow
9	13	2	4	Y2510
9	33	6	4	B5556
9	35	1	2	Y2781
9	51	8	2	W1586
9	54	-ve	2	T4942
10	14	1	4	Y2510
10	36	2	2	Y2781
10	52	7	2	W1586
10	63	8	4	T4287
11	15	4	4	Y2510
11	37	3	2	Y2781
11	53	6	2	T4942
11	64	5	4	T4287
11	70	-ve	4	T4205
12	20	3	4	Y2476
12	38	4	2	W1594
12	62	5	2	T1809
12	65	7	4	T4287
13	21	6	4	Y2476
13	39	5	2	Y2795
13	49	-ve	2	W1602
13	55	4	2	T4942
13	66	3	4	T4287
14	22	5	4	Y2476
14	40	6	2	Y2795
14	56	3	2	T4886
14	67	2	4	T4205
15	41	7	2	Y2795
15	57	2	2	T4886
15	68	1	4	T4205
15	72	8	4	B5567
16	24	7	4	Y2502
16	42	8	2	Y2795
16	58	1	2	T4886
16	69	4	4	T4205