

UTILIZATION OF NEXT GENERATION SEQUENCING FOR IDENTIFYING *STREPTOCOCCUS SUI*S CLONES RESPONSIBLE FOR RECURRENT OUTBREAKS AND IDENTIFYING EFFECTIVE *S. SUI*S AUTOGENOUS VACCINE CANDIDATES

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Executive Summary

Streptococcus suis is an important cause of mortality in swine production and a zoonotic bacteria. Current management protocols consist of treatment with β -lactam antibiotics, and administration of autogenous vaccines with variable results.

This study assessed the potential for tonsil swabs from live pigs to be a suitable method for rapid identification of circulating *S. suis* clones for production of autogenous vaccines.

Tonsil swabs did not yield any isolates with genomic signatures indicative of pathogenic *S. suis* circulating in Australia, however a large number of *S. suis*-like organisms were isolated. Genomic comparison indicated that *S. suis* isolated from tonsils were genetically distinct from pathogenic *S. suis* causing invasive clinical infections in Australian pigs, and likely to be a separate subspecies. Whole genome sequencing of 148 clinical isolates from diseased pigs demonstrated serotypes 2, 3 and 1/2 are the predominant serotypes in Australia, and a trivalent vaccine incorporating these serotypes is indicated.

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Introduction

The increase in reports of infection of *Streptococcus suis* in Australian pig farms in recent years has been associated with significant production losses. Clinical disease caused by *S. suis* can result in meningitis, joint effusions, endocarditis, septicemia and sudden death. Despite these serious consequences of infection, commensal *S. suis* strains can be cultured from the tonsils, nasal cavity, genital and gastrointestinal tracts of healthy pigs. This is a complicating factor in management and treatment of infection and disease, with difficulty in determining the pathogenic potential of commensal strains, and these strains potentially masking the presence of more pathogenic strains.

Globally, *S. suis* infections also have human health impacts with infection causing endocarditis, sepsis, arthritis, endophthalmitis and meningitis. The zoonotic agent has been identified to pass to humans from the consumption of undercooked pork products and the close exposure of slaughter workers. In Australia three cases of zoonotic infection have been reported; one in an abattoir worker in 2007 and two piggery workers in 2008.

Current management techniques focus on the use of antimicrobials and autogenous vaccines, both having significant complications in controlling disease. The use of antimicrobials to prevent *S. suis* infections is decreasing in value due to the increase in resistance against antimicrobials used in veterinary medicine. Recent studies from Korea have determined relatively high rates of resistance to β -lactams, florfenicols and ceftiofur with 56.4%, 56.4% and 55.9% of *S. suis* isolated from swine [1], although resistance to ampicillin and florfenicol in Australian isolates is lower at 8.1% and 14.9% respectively (O'Dea, Abraham unpublished).

The lack of a highly discriminatory bacterial typing system for *S. suis* results in autogenous vaccines that target specific pathogenic clones however do not provide cross protection against other serogroups, or in some cases, isolates within the same serogroup [2]. It is envisaged that an increased knowledge of pathogenic and colonizing *S. suis* isolates will allow the production of wider, more effective autogenous vaccines, preventing the carriage of commensal clones belonging to serogroups with high potential of becoming pathogenic and causing systemic infection.

This study aimed to identify the dominant clones that colonise healthy pigs and cause clinical disease, and to determine whether tonsillar sampling of live pigs is a feasible diagnostic method, to develop potential autogenous vaccine candidates. Sampling was undertaken on different age groups to determine whether clonal carriage was age-associated.

Methodology

Carriage study

Tonsil swabs of 30 healthy pigs from three age groups (suckers, pre-weaners and growers) were collected from two piggeries at two sampling time points.

Round 1

- 2wk old x 30 (sucker)
- 6wk old x 30 (weaner)
- 10wk old x 30 (grower)

Round 2 - 8wk later

- 10wk old x 30 (2wk old pigs from round 1)
- 14wk old x 30 (6wk old pigs from round 1)
- 18wk old x 30 (10wk old pigs from round 1)

Immediately after collection, the swabs were sent to Murdoch University for processing.

The swabs were cultured onto *Streptococcus suis* selective agar, termed NNCC medium [3]. This was prepared from Todd Hewitt broth (OXOID), 1.5% bacteriological agar, 50mg/L sodium azide, 2mg/L crystal violet, 5% sheep blood, 25mg/L nalidixic acid and 12.5mg/L colistin. All swabs from Farm A were vortexed into 5mL buffered peptone water (BPW). This solution was plated onto the NNCC medium via an automated robotic streaking method with separation of over 100 colonies/ swab developed by the antimicrobial resistance and infectious diseases laboratory, whereas swabs from Farm B were plated using traditional 16 streak methods of manual direct plating. The plates were incubated throughout the project at 37°C for 24 hours with 5% CO₂. Following overnight incubation, up to 50 colonies morphologically resembling *Streptococcus suis* from the Farm A plates were re-cultured onto sheep blood agar (Edwards) and incubated. A single colony was selected for re-culture colony from Farm B plates.

Identification of the colonies was undertaken using matrix assisted laser deionisation time of flight (MALDI-TOF) (Bruker). All isolates confirmed to be *S. suis* were transferred into microcentrifuge tubes containing BHI broth with 30% glycerol and 10% fetal calf serum. These were then stored at -80°C.

Clinical *Streptococcus suis* strains

A collection of 148 *S. suis*, isolated from clinically diseased pigs in Australian pig herds, were sent to Murdoch University from ACE Laboratory Services. Isolates spanned a seven year period with 2, 1, 29, 27, 9, 49 and 29 isolates from years 2010, 2011, 2013, 2014, 2015, 2016 and 2017 respectively, and two isolates for which the year was unknown. Initial swabs from diseased pigs were obtained from tissues outlined in Table 1. All swabs were streaked onto Columbia sheep blood

agar and plates incubated. A single colony was subcultured and incubated. Confirmation of the identification of each isolate was performed using a MALDI-TOF (Bruker) with isolates confirmed to be *S. suis* being harvested into microcentrifuge tubes and stored at -80°C.

Table 1 Number of isolates and site of origin used in the study

Isolation Site	Number of Isolates
Abdomen	2
Brain	12
Heart	12
Joint	6
Lung	93
Lymph node	1
Neck abscess	3
Upper respiratory tract	3
Unknown	16

MIC Testing

All isolates were subjected to antimicrobial susceptibility testing via broth microdilution according to the Clinical Laboratory Standards Institute (CLSI) Performance Standards.

PCR

DNA extraction for PCR reactions was performed on all the commensal isolates via chelex method. All isolates were cultured overnight in 500mL LB broth (using a 96 deep well plate) prior to extraction. The plate was centrifuged at 4000 rpm for one minute and supernatant was removed before 100 µL of MilliQ water was added to each well, and all samples transferred to a PCR 96-well plate. Centrifugation of the plate was repeated. The supernatant was removed with 100 µL of 6% chelex (BioRad) added to all samples. The plate was placed in a thermocycler at 56°C for 20 mins followed by 100°C for 5 mins and then placed straight onto ice.

The presence of the *S. suis gdh* gene and the virulence genes *mrp*, *sly* and *epf* was determined via PCR based on primers developed by Silva et al. (2006) and Liu et al. (2013) (Table 2) [4, 5].

Table 2: primer sequences used in this study for detection of *S. suis gdh*, *mrp*, *sly* and *epf* genes and capsular types 1, 2 and 3.

Gene	Primer sequence
<i>gdhF</i> <i>gdhR</i>	AAGTTCCTCGGTTTTGAGCA, GCAGCGTATTCTGTCAAACG
<i>mrpF</i> <i>mrpR</i>	ATTGCTCCACAAGAGGATGG, TGAGCTTTACCTGAAGCGGT
<i>slyF</i> <i>slyR</i>	GCTTGACTTACGAGCCACAA, CCGCGCAATACTGATAAGC

<i>epfF</i> <i>epfR</i>	CGCAGACAACGAAAGATTGA, AAGAATGTCTTTGGCGATGG
<i>cps1(14)F</i> <i>cps1(14)R</i>	TCTTATAACAGGCGTCAAAACA ATCGGTATAAAAGCAAGACACA
<i>cps2(1/2)F</i> <i>cps2(1/2)R</i>	TTCGTATTAACCTTACTTGGCGT TAAATCCCCATATGCCAAATCC
<i>cps3</i> <i>cps3</i>	ACATCCATTGCAGGAGTAGT TGCAGTTCCAAAATTCTTCGT

Whole genome sequencing

DNA extraction was performed using MagMax DNA multi sample kit (ThermoFisher Scientific) with the only variation from the manufacturer's instructions being the omission of the RNase treatment step. Whole genome sequencing library preparation was conducted using a Nextera XT kit (Illumina). The tagmentation time was increased to 7 minutes with all steps completed as per the manufacturer's instructions. Sequencing of the isolates was performed with a mid output V2 (2 x 150 cycles) kit on an Illumina Nextseq 500 platform.

Analysis of whole genome sequencing data was performed using the Nullarbor bioinformatics pipeline (v1.20). Determination of capsular serotypes was performed by querying contig files against a database of reference serotype genes using Abricate (0.4) at a 90% cutoff. To distinguish serotype 2 from serotype 1/2 and serotype 1 from serotype 14, analysis was manually performed using CLC genomics. All contig files from the tonsillar strains were also run through the species identification online tool JSpecies (v3.0.17).

Results

Isolation of *Streptococcus suis*

The total number of swabs processed was 300, inclusive of 150 (30 from suckers and 60 from weaners and growers) from each farm. A total of 326 and 4 *S. suis* strains as confirmed by MALDI-TOF were isolated from Farm A and Farm B respectively, with the automatic robotic plating and picking method (Farm A) leading to significantly higher numbers of *S. suis* isolated. From Farm A, 40 isolates were from suckers (note only a single sample point), 209 isolates were obtained from weaners and 77 isolates were obtained from growers. Farm B isolates were all from weaners.

AMR Testing

A high proportion of the isolates were resistant to both tetracycline (99.3%) and erythromycin (83.8%). Low levels of resistance were observed for florfenicol (14.9%), penicillin G (8.1%), ampicillin (0.7%) and trimethoprim/ sulphamethoxazole (0.7%). None of the isolates were resistant to enrofloxacin (Table 3)

Table 3: Distribution of minimum inhibitory concentrations for *S. suis*.

drug	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	ns_ci	cr_ci
Ampicillin		99.3 (147)						.7 (1)					0.7(0,3.7)	0.7(0,3.7)
Ceftriaxone		88.5 (131)	7.4 (11)	2 (3)	1.4 (2)					.7 (1)			.	.
Ciprofloxacin		37.2 (55)	37.2 (55)	22.3 (33)	3.4 (5)								.	.
Enrofloxacin	14.2 (21)	44.6 (66)	40.5 (60)	.7 (1)									0.7(0,3.7)	0(0,2.5)
Erythromycin	11.5 (17)	4.1 (6)	.7 (1)	1.4 (2)			2 (3)	1.4 (2)	79.1 (117)				84.5(77.6,89.9)	83.8(76.8,89.3)
Florfenicol				2 (3)	4.1 (6)	79.1 (117)	11.5 (17)		.7 (1)	1.4 (2)	.7 (1)	.7 (1)	93.9(88.8,97.2)	14.9(9.6,21.6)
Gentamicin					6.1 (9)	40.5 (60)	43.2 (64)	9.5 (14)					.	.
PenicillinG	81.8 (121)	4.7 (7)	4.7 (7)	3.4 (5)	3.4 (5)		.7 (1)		.7 (1)				12.8(7.9,19.3)	8.1(4.3,13.7)
Tetracycline				.7 (1)		.7 (1)	1.4 (2)	2 (3)	1.4 (2)	93.9 (139)			100 (97.5,100)	99.3(96.3,100)
TMS			91.9 (136)	5.4 (8)	2 (3)		.7 (1)						8.1(4.3,13.7)	0.7(0,3.7)

Percentage of isolates classified as non-susceptible (ns ci) and/or clinically resistant (cr ci) with corresponding 95% confidence intervals for those where breakpoints are available. Shaded areas indicate the range of dilutions evaluated. Vertical bars indicate clinical breakpoint (where available). TMS; trimethoprim/sulfamethoxazole

Determination of species and presence of virulence genes via PCR

In total, 342 isolates underwent PCR screening for *gdh* and virulence factors. 273 (79.8%) of isolates were positive on the *gdh* PCR, indicating *S. suis* or closely related species. Putative virulence factor PCRs for *mrp*, *sly* and *epf* were positive for 146 (42.7%), 19 (5.6%) and 5 (0.5%) isolates respectively. No isolates were positive on the *cps1* PCR, and 3 (1.1%) and 22 (6.4%) were positive on *cps 2* and *cps 3* PCRs respectively. All other samples returned negative or indeterminate results. It should be noted that no PCR results were confirmed by sequencing.

WGS and bioinformatics analysis

A representative subsample of isolates (n=42) that was identified as *S. suis* by MALDI-TOFF underwent whole genome sequencing. 37 (37/42) sequenced strains identified most similarly to *S. suis* however the mean level of identification was low at 37% (range 5-70%), with the other strains identified as most similar to *Streptococcus pneumoniae*.

Genomic analysis of the 37 commensal *Streptococcus suis* isolates by WGS identified only 6 capsular serotypes across 10 isolates (Table 3). These 6 serotypes were 5, 9, 19, 28, 29 and 30. Twenty seven (73%) isolates had unidentifiable serotypes. None of the commensal isolates had identifiable multi locus sequence types. 30/37 (81%) were positive for the *gdh*, which was consistent with the proportion identified by targeted PCR.

Table 4: Frequency of capsular serotypes detected from tonsillar swab isolates in this study

Capsular serotype	Frequency
5	1
9	1
19	1
28	4
29	2
30	1

Three isolates were sent to DPIRD WA for Vitek biochemical analysis. Species identifications of *S. mitis*/*S. oralis* grouping was returned for two isolates and the third isolate could not be identified by Vitek analysis.

The dominant capsular serotypes detected amongst the clinical isolates were *cps 2* (39/148), 3 (37/148) and 1/2 (11/14) (Figure 1). Other common serotypes include *cps 16* (n=8), 19 (n=8), 4 (n=7) and 8 (n=7) with a total of 20 different serotypes detected. Genomic analysis of the 148 clinical disease isolates identified 11 different known MLST with 39 (26.2%) isolates resulting in 26 newly identified MLSTs, designated ST1031-ST1056 inclusive. The most common MLSTs were ST27 (n=27), ST25 (n=26), ST28 (n=11) and ST483 (n=11) (Figure 2).

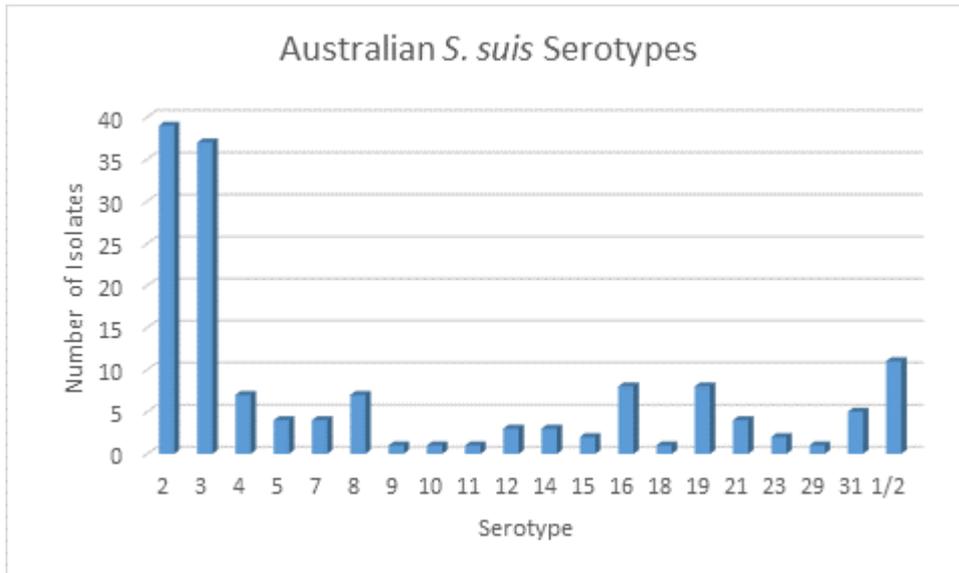


Figure 1: *S. suis* serotypes from Australian clinical swine isolates (n=148)

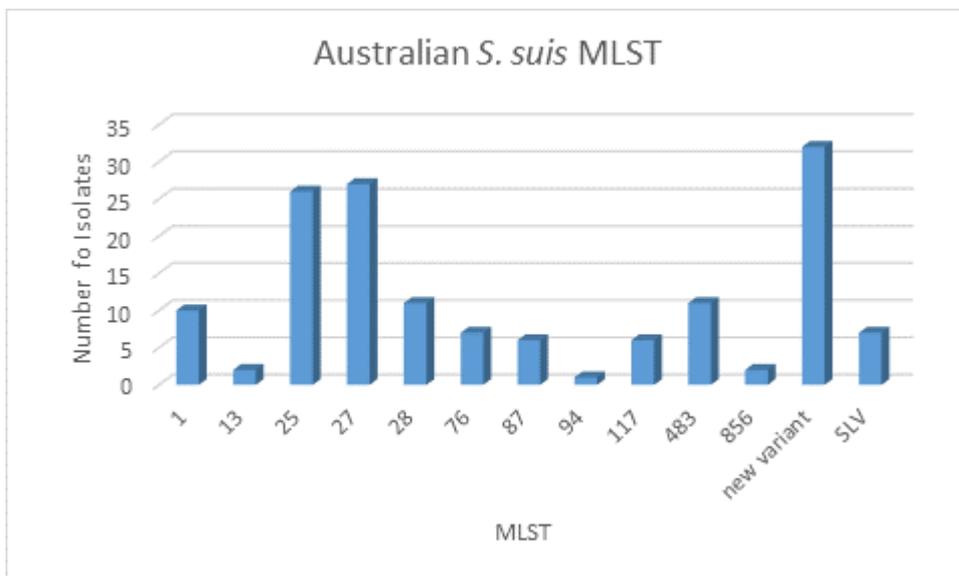


Figure 2: *S. suis* MLSTs from Australian clinical swine isolates (n=148)

The carriage of antimicrobial resistance genes was similar between the commensal and pathogenic strains with the most common resistance genes being *tetO* (tetracycline) at 91.9% and 89.9% respectively, and *ermB* (erythromycin) at 89.2% and 83.9% respectively. Other common genes in commensal and pathogenic strains included *InuB* (lincosamides) at 51.4% and 4.0% respectively, *aadE* (aminoglycosides) at 48.6% and 2.7% respectively and *fexA* at 10.8% and 0.7% (Table 4). All commensal isolates and 81.9% of pathogenic isolates carried genes against multiple antimicrobial drug classes. The most common combination of genes was *tetO* and *ermB* in 35.1% and 57.0% of commensal and pathogenic isolates respectively.

Table 5: Proportion of *S. suis* isolates carrying antimicrobial resistance genes. Note commensal strains refers to tonsillar isolates.

Gene	% present in commensal strains	% present in clinical strains
<i>aadD</i>	-	1.3
<i>aadE</i>	48.6	2.7
<i>ant(6)-Ia</i>	5.4	3.4
<i>aph(3')-III</i>	8.1	4.7
<i>ermB</i>	89.2	83.9
<i>ermA</i>	-	6.0
<i>fexA</i>	10.8	0.7
<i>lnuB</i>	51.4	4.0
<i>lnuC</i>	-	0.7
<i>lsa</i>	24.3	-
<i>mefA</i>	2.7	1.3
<i>msrD</i>	2.7	2.0
<i>optrA</i>	-	2.7
<i>Spc</i>	2.7	5.4
<i>tetO</i>	91.9	89.9
<i>tetM</i>	-	1.3
<i>tetW</i>	-	5.4

Discussion

While not directly providing the outcomes anticipated when this study was designed, the findings have produced a large amount of information regarding Australian strains of *S. suis*, and the differences between commensal tonsillar flora and isolates from diseased pigs.

Of particular interest was the degree of genetic difference between the tonsil isolates and the clinical isolates. While identified by MALDI-TOF as *S. suis*, many of the isolates did not display genetic signatures of *S. suis*, such as the presence of *gdh*, absence of putative virulence factors, inability to be genetically serotyped and non-typeable MLSTs. In addition to this, many of these isolates showed very low identities of 5-70% to *S. suis* using our in-house WGS pipeline, which returned identities of 60-95% for clinical, easily typed, isolates. Biochemical testing on a small number of isolates also did not detect *S. suis*. This indicates that that these commensal isolates may be normal tonsillar-pharyngeal bacteria, which are similar to *S. suis*, but are a separate species or subspecies which are not involved in disease, and it is our conclusion that these isolates are not representative of commensal *S. suis* causing clinical disease. It is likely the identity of these isolates could only be resolved through long read sequencing such as PacBio to obtain the complete genome. This was beyond the scope of this study.

The isolation of these bacteria indicates that tonsil sampling in the live pig is unlikely to be suitable as a diagnostic tool, or as a tool to predict circulating clones from which to develop autogenous vaccines. While isolation of *S. suis* from tonsils is used in studies, it is generally from postmortem samples [6, 7], enabling deep sampling of the tonsillar crypts, potentially allowing access to *S. suis* which are not present on the tonsil surfaces. In this study, it is likely that swabs of the tonsil-pharyngeal region were dominated by recovery of indigenous oropharyngeal flora, which masked recovery of *S. suis*.

In contrast to the results from tonsil swabs, WGS of clinical isolates obtained from postmortem samples demonstrated that circulating clones associated with disease in Australia are predominantly serotypes 2, 3 and 1/2, and MLSTs 1, 25, 27 and 483. The WGS pipeline used here has been demonstrated to rapidly determine clonal identity, including virulence potential and carriage of antimicrobial resistance genes, from clinical samples, and can be applied for future autogenous vaccine production.

As part of this study, and incorporating results from concurrent work being undertaken, the development of a whole genome sequencing pipeline for evaluating multi-locus sequence types, virulence genes, antimicrobial resistance genes and phylogenetic relationships of *S. suis*, has been a particularly positive outcome. This pipeline has recently been used in field situations for selection of autogenous vaccine candidates for producers, and identification of pathogenic *S. suis* strains responsible for causing outbreaks on farms. This genomic pipeline will be of great benefit in the future for any further genomic studies on Australian *S. suis* strains, and rapid outbreak typing.

We have also demonstrated that using conventional methods, the recovery of potential *S. suis* isolates was quite low. The robotics methods used in Farm A demonstrated that in a carriage study separating individual bacteria on selective agar is very important for identifying the carriage of non-disease causing indicative bacteria. The robotics method also allows much larger numbers of isolates to be picked and identified in a short timeframe. Standard manual streaking methods may

mask the target organism due to limited separation obtained by traditional 16 streak method.

Conclusions

Isolation of commensal *Streptococcus suis* from the tonsils of pigs was ineffective in isolating pathogenic *S. suis* responsible for causing clinical disease in Australian pigs, and instead resulted in isolation of a potential *S. suis* subspecies.

Automatic plating and picking methods resulted in a vastly higher frequency of *Streptococcus suis* being isolated compared to the manual direct plating method currently implemented with 326 and 4 isolates retrieved respectively.

The major capsular serotypes of *S. suis* isolated from Australian clinical cases of disease include serotypes 2, 3 and 1/2.

Phylogenetic analysis based on core genome polymorphisms, grouped isolates according to MLSTs, with ST1, ST25 and ST27 being the main types identified in the pathogenic clones.

Australian isolates belonging to ST1 (serotypes 1/2, 2 and 14) had a significantly higher number of potential virulence genes than other pathogenic clones.

Recommendations

Based on the outcomes from this study the following recommendations are

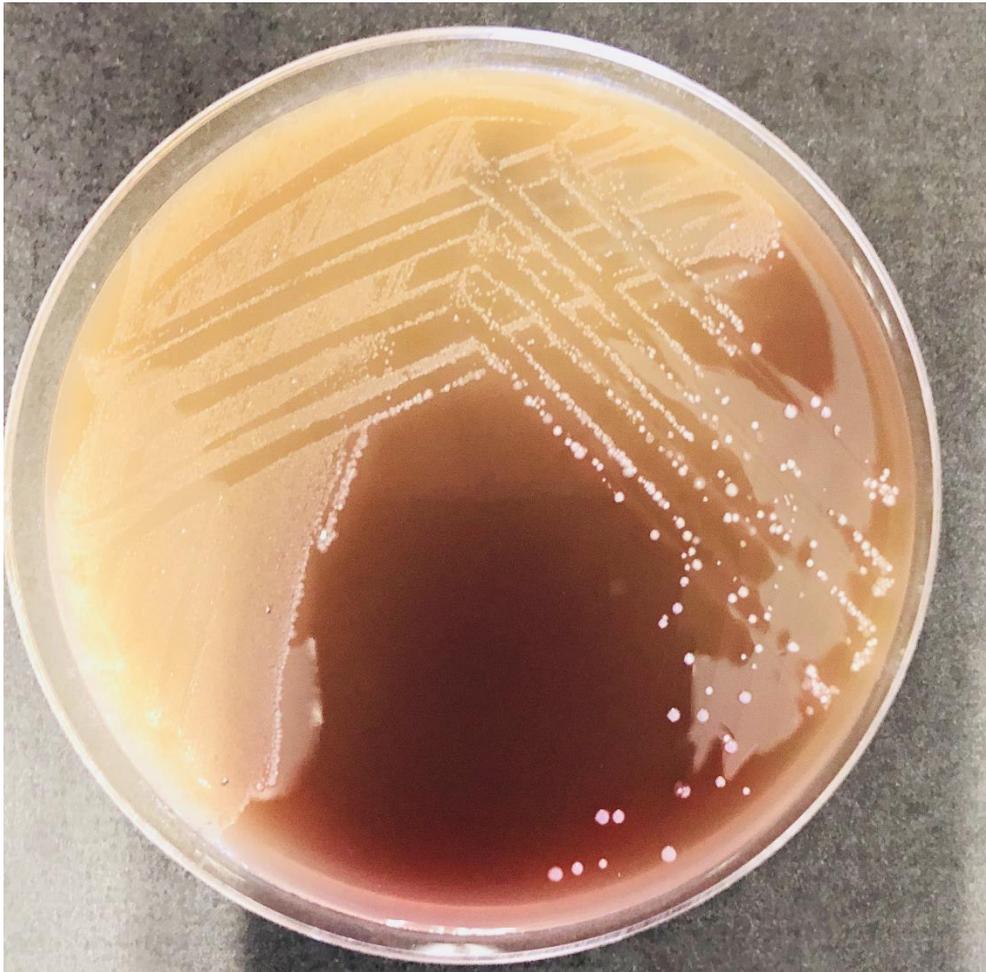
- A trivalent autogenous vaccine targeting cps 2, 3, 1/2 and ST1 25 and 27 may be effective in controlling highly pathogenic *S. suis* infections.
- In order to determine vaccine efficacy and optimum vaccine timing, development of a serological assay for determination of *S. suis* seroconversion (potentially Australian strain specific) is required

- (1) Develop a serological assay to assess immune response to *S. suis*
- (2) Perform a preliminary on-farm vaccine trial to evaluate the efficacy of cps 2, 3 and 1/2 in collaboration with Sunpork and ACE laboratories, utilising the serological assay developed as per recommendation 1 to assess immune response. Incorporate an epidemiologically relevant sample size to assess clinical disease risk and odds ratios between vaccinated and non-vaccinated cohorts.
- (3) Identify novel vaccine preparations including adjuvants, subunits and whole bacteria inactivation methods. Global research indicates autogenous vaccines based on formalin-inactivated bacterins have variable results. We envisage using the serological assay developed as per recommendation 1 to assess the immunogenicity of a number of different vaccine preparations.

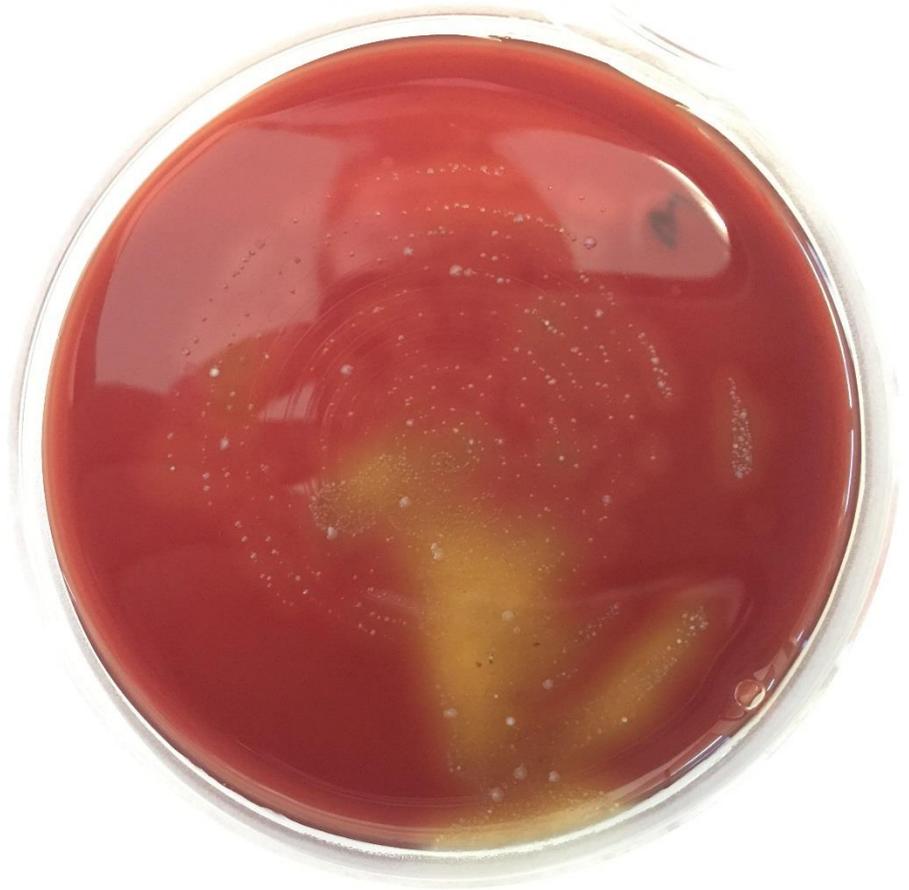
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Appendix 1



Manual 16 streak plating method. Note marked overgrowth on plate and very few of the white/grey pinpoint colonies present as individual colonies suitable for subculture.



Robotic spiral plating method. Note much larger number of pinpoint white/grey colonies discernible as separate and suitable for subculture.