2C-121: A novel and safe fogging sanitiser for MRSA decolonisation and reduction of *Actinobacillus pleuropneumoniae* aerosol transmission between pigs.

Report prepared for the
Co-operative Research Centre for High Integrity Australian Pork

By

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Executive Summary

Environmental decontamination is a key management strategy to maintaining good respiratory herd health in piggeries. *Actinobacillus pleuropneumoniae* (*A. pp*) is one of the most important endemic respiratory pathogens of Australian pigs currently controlled by in-feed antibiotic medication and other bacterial species associated with porcine respiratory disease complex (PRDC) are important secondary pathogens. Livestock-associated MRSA has been identified as a major public health issue for intensive animal production, particularly for in-contact workers and their families. Disinfection of the farm environment to eliminate these pathogenic organisms remains a key component of disease control and production efficiency. Of the many disinfectants used in farm decontamination, quaternary ammonium compounds (QAC) have been shown to prevent aerosol transmission of *A. pp* in a previous CRC project. However, QAC and many other currently used disinfectants have significant drawbacks such as being hazardous to the environment and potentially toxic to animals and piggery workers, whilst also being responsible for co-selection of resistance to antibiotics (class 1 integrons, which are major drivers of the evolution of multidrug-resistance, always contain a QAC resistance gene in their structure).

Therefore, in this project, an electrochemically activated solution (ECAS), an anolyte generated by electrolysing a dilute sodium chloride solution in a four-chamber electrolytic cell (Ecas4 Australia), was tested as an aerosol sanitiser. The Ecas4 anolyte has a neutral pH and is 100% safe; it is currently used for sanitisation of *Legionella* contamination in hospital water supplies, disinfection of dairy manufacturing equipment, and in seafood and fresh food produce as both a wash and dry fog. *In vitro* antimicrobial susceptibility testing of ECAS against field strains of *A. pp* representing the main serotypes and MRSA isolated from Australian pigs, confirmed that at very low concentrations of the anolyte in water (0.19-3.13% v/v or 0.48-7.8 ppm of active chlorine) ECAS was equally effective at killing both *A. pp* and MRSA within 30 s of exposure.

SYBR green-dye-based real-time quantitative PCR was optimised to detect and quantify low levels of total bacteria and *A. pp* targeting the 16S rRNA and *apxIVA* genes, respectively, using previously published primer sets. Using the Coriolis air sampler device, the sample collection protocol to capture *A. pp.* from the farm environment was optimised and very low levels of *A. pp* (5.1×10^5 genomic units) were detected in both weaner rooms and grower/finisher sheds at a continuous flow farm with endemic pleuropneumonia. Furthermore, a novel step was

introduced into the qPCR by treating samples with 50 µm propidium monoazide to differentially quantify live and dead bacterial cells in the sample, an ideal rapid quantitative assay for determining the effectiveness of aerosol disinfection methods.

To examine the effectiveness of Ecas4 dry fogging in eliminating *A. pp* and other aerosolised bacteria, a proof-of-concept trial was conducted in a recently vacated weaner room at the same continuous flow piggery using a protocol adapted from Dr Peter McKenzie's Pork CRC project 2C-117. A 1-$\log_{10}$ reduction in total bacterial count was observed after the first hour of fogging, a 2-$\log_{10}$ reduction was observed after fogging for two and three hours, while 99.9% (3.7-$\log_{10}$) of total bacteria were effectively inactivated by Ecas4 dry fogging after five hours of discontinuous treatment. However, since we were not able to conduct fogging trials with pigs in the weaner room, due to the potential for creating a large number of immunologically naïve animals entering the high-risk environment of the grower shed, we could not capture *A. pp*. from the weaner room environmental air samples. Hence, disinfection efficacy on farm against *A. pp* could not be determined. Nevertheless, the pilot results with total bacteria are encouraging enough to upscale to a much larger trial in the grower shed in a future project.

The outcomes of this innovative project will support application of Ecas4 technology specifically in disinfection of respiratory pathogens in the farm environment and fulfil the aims of Sub-Program 2C: Replacement of Antibiotics with Effective Integrated Health Strategies. An added bonus of this project is that we have optimised a diagnostic assay to detect low levels of *A. pp* (as little as 0.1 pg of DNA) from the air of the farm environment and a novel qPCR method to discern live bacteria from dead bacteria, addressing another Pork CRC Sub Program 2A: Novel Disease Diagnostics.

Contents

1. Introduction

Respiratory diseases are the substantial inhibitors of economic returns from intensive swine production. Significant economic losses in swine production worldwide are attributed to the costs of veterinary services, poor feed-to-body conversion ratio, carcass disposal and management expenses. The degree of production losses vary with various causative agents involved in respiratory infection of pigs. Swine respiratory infections are caused by bacterial (and some viral, though less significant in Australia) etiological agents individually or in tandem and is collectively referred to as porcine respiratory disease complex (PRDC) (Brockmeier 2003). The most common causes of chronic forms of PRDC are primary and secondary bacterial pathogens such as *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, *Streptococcus suis*, *Haemophilus parasuis*, *Bordetella bronchiseptica*, *Actinobacillus pleuropneumoniae*, *Actinobacillus suis*, and *Salmonella choleraesuis*. A new species of pathogenic *Actinobacillus* has recently been identified in Australian pigs. The clinical manifestations of PRDC depends upon the farm production management system and the infective organism causing the disease.

Prevalence of respiratory disease in swine farms is related to respiratory health, farm air quality and herd density of pigs (Gardner, Willeberg et al. 2007). For instance, increasing the pigs from 400 to 1,200 per airspace increases the risk of respiratory diseases nine-fold (CRC 2014). Similarly, a production system consisting of 1,000 pigs per airspace compounded by multi-age pigs, and presence of *A. pleuropneuominae* (*A. pp*), *M. hyopneumoniae*, and porcine circovirus type 2 (PCV2) causes outbreaks of pleuropneumonia with high mortality and morbidity rates with persistent ill threat. Globally, pleuropneumonia caused by *A. pp* is one of the leading causes of PRDC (Thacker and Thanawongnuwech 2002, Bochev 2007), and within the Australian pig industry, *A. pp* remains one of the most important endemic respiratory disease pathogens, with both virulent and mild strains regularly identified (serovars 1, 5, 7, 12 and 15 predominate) (Stephens, Gibson et al. 1990, Blackall, Bowles et al. 1999, Blackall, Klaasen et al. 2002). Changes in confounding factors such as co-infection with other respiratory pathogens, management strategies and environmental factors may cause regular outbreaks, even in herds harbouring mild serovars of *A. pp* such as serovar 7 (Peter McKenzie, personal communication).

Besides timely vaccination with efficacious bacterins and antibiotic usage in drinking water or feed, successful prevention of PRDC requires proper management of environmental stressors.

In particular, reduction of environmental bacterial load through fogging has resulted in reduced antibiotic usage, delayed the onset of *A. pp* pleuropneumonia (APP) and dropped morbidity and case fatality (CRC 2014). However, decontamination of the farm environment, including aerosols is currently achieved through use of disinfectants that can be toxic or irritant, environmental contaminants or co-select antimicrobial resistance in bacteria. Examples of common chemicals used as disinfectants include sodium hypochlorite, hydrogen peroxide, chlorine dioxide, quaternary ammonium compounds (QAC), peroxyacetic acid and iodophors (Fotheringham 1995, Kim, Hung et al. 2000, Al-Haq, Seo et al. 2002, Thorn, Lee et al. 2012, Thorn, Robinson et al. 2013, CRC 2014). While all these chemicals are very effective at reducing microbial load, they have disadvantages including differential effectiveness against a variety of bacteria (Meakin, Bowman et al. 2012), co-selection of antibiotic resistance (Loughlin, Jones et al. 2002), corrosiveness (Nicklas, Böhm et al. 1981), concerns with skin irritation and cross resistance to other disinfectants and antibiotics. Furthermore, for aerosol disinfection schemes to be successful at preventing transmission of *A. pp* between carrier and naïve pigs on farm, exposures must occur continuously for a frequency of 2 min every 30 min (CRC 2014).

The Ecas4 anolyte is an electrochemically activated solution (ECAS), i.e. an environmentally friendly broad-spectrum biocidal agent manufactured by Ecas4 Australia Pty Ltd, which has a rapid disinfection potential against a broad range of microorganisms (Thorn, Lee et al. 2012, Khazandi, Deo et al. 2017). Generally, the most sought after anolyte is of near neutral pH having high oxidation-reduction potential (ORP) (> 850 mV). To obtain optimum anolyte with the above-stated solution features, Ecas4 have developed and patented a novel anolyte generation technology that has a reactor with four chambers (Bohnstedt 2006) consisting of two cathode compartments and two anode compartments. Unlike its 2-chamber predecessors, which have been used overseas for many years with limited success, the Ecas4 technology (to which two international patents apply) allows producing hypochlorous acid (350–400 mg/L of active chlorine) at neutral pH from a dilute brine solution of 0.5% NaCl (Figure 1) (Migliarina and Ferro 2014). Direct current triggers electrochemical activity at the electrode interfaces and, within the anodic chamber, transforms the dilute saline solution into an activated "metastable' state containing a mixture of active chlorine compounds (oxidising moieties) such as HOCl and ClO^- (Prilutsky and Bakhir 1997). The amount of active chlorine in the resulting Ecas4 anolyte is dependent on the characteristics of the electrochemical cell and its operating parameters, which also determines the physicochemical properties of the anolyte.

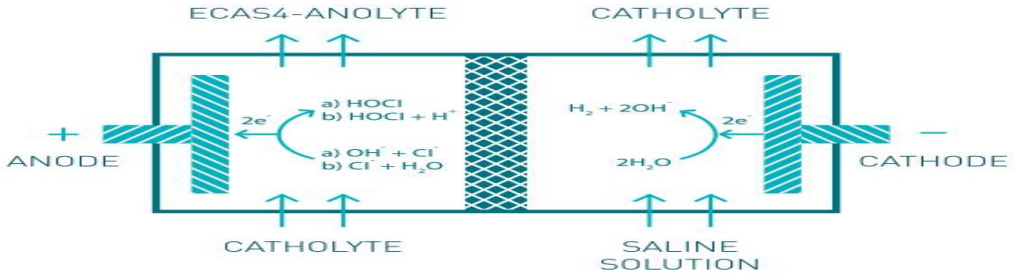

Figure 1. Schematic representation of Ecas4 anolyte production

In broader terms, ECAS has antimicrobial activity against both Gram-positive and Gram-negative bacteria, yeasts and fungi, with no reported toxicity, and is produced from low cost easily available raw material, being both environmentally compatible and easily generated in situ. The ECAS has disinfection activity equivalent to 80% ethanol and is superior to 0.1% chlorhexidine or 0.02% povidone iodine (Tanaka, Fujisawa et al. 1999). However, previous applications of ECAS produced under strongly acidic conditions have been limited due to corrosion of processing equipment and phytotoxic effect on plants (Abadias, Usall et al. 2008, Guentzel, Lam et al. 2008, Cao, Zhu et al. 2009). Due to recent advancement in the technique for generating ECAS with slightly acidic pH (5-6.5), it has been widely used in sanitisation of vegetables with excellent efficacy (Koide, Takeda et al. 2009, Rahman, Ding et al. 2010, Hao, Liu et al. 2011).

Novel developments in the technique of anolyte production at neutral pH with excellent oxidative properties and drastically reduced material corrosiveness have also boosted the application of ECAS in both medical settings and food sanitization. ECAS has recently been used in small-scale trials in medical, food and veterinary applications and decontamination processes, and is being increasing employed in hospital water disinfection (Barrott et al. 1990) and as a sanitiser in the food industry (Al-Haq et al. 2002, Ozer and Demirci 2006, Huang et al. 2008, Khazandi et al. 2017). A neutralised ECAS has been employed specifically for the treatment of wounds as a dressing product (Luca Dalla Paola, Brocco et al. 2006) in place of general broad-spectrum antibiotics that are restricted in use because of development of antibiotic resistance. It also has been successfully trialled in preventing or reducing rates of infection with MRSA in postoperative infections (Ohno, Higashidate et al. 2000, Ichihara, Fujii et al. 2004). In addition, it has been successfully employed widely in disinfection and eradication of *Legionella* biofilms in hospital water systems in European countries (Migliarina

and Ferro 2014) and recently in Australia (https://www.ecas4.com.au/adelaide-hospital-introduces-revolutionary-water-technology-set-to-radically-improve-patient-safety-and-recovery/). Our research group has investigated its application in the sanitisation of seafood, resulting in delayed onset of spoilage and preserve its palatability longer (Khazandi, Deo et al. 2017), and its efficacy in the sanitation of salad leaves (Ogunniyi et al., manuscript in preparation).

The detection, identification and quantification of microorganisms in the farm environment is an essential component of assessing farm management and biosecurity systems. Conventional culture and identification methods of pathogenic bacteria need isolation in culture media, followed by biochemical and/or serological identification, and in some cases characterisation of sub-species. Molecular- and antibody-based detection and quantification methods are faster, have many fold higher sensitivity and are more specific. Particularly, real time Polymerase Chain Reaction (PCR) methods are currently widely used in quantification and identification of bacteria because of their convenience and advantages over conventional methods. Advances in fluorescence dye-based quantitative PCR (qPCR) to measure DNA amplification as it occurs during PCR cycles using sequence specific primers have made quantification and identification of bacteria efficient, rapid and cost-effective, and the fluorescence detected at each cycle of qPCR is proportional to the amount of dsDNA target sequence present at that cycle.

In this project, SYBR green-dye-based on real-time quantitative PCR for the *A. pp apxIVA* gene developed by Schaller et al. (2001) and universal primers for amplification of bacterial 16S rDNA designed and optimised by Nadkarni et al. (2002) were utilised. These detection methods have approximately 40-fold greater sensitivity than bacterial culture, and were optimised to detect and quantify *A. pp* and total bacterial load in air samples from the farm environment. Since these qPCR methods amplify and quantify any genomic DNA specific to these primers, an innovative approach in treating samples with propidium monoazide (PMA) was included. PMA is a photoreactive membrane-impermeable dye that selectively penetrates bacteria cells with compromised membranes (considered dead) and binds covalently to dsDNA, inhibiting amplification of any dsDNA and thus allows the qPCR to differentiate between live and dead bacterial cells. Therefore, we included and optimised a PMA treatment step to evaluate the effectiveness of different aerosol disinfectants. Hence, our primary aim in developing this technology is to test ECAS as a cost effective air decontamination process when administered as a dry fog that is safe, non-toxic and does not contribute to antimicrobial resistance, with high

efficiency broad-spectrum disinfection capability and specific killing action against *A. pp* to prevent transmission between stock. Our secondary aim is to determine whether the disinfection protocol proof-of-concept pilot can be up scaled to a workable model that could be regularly implemented in a farm setting for control and prevention of transmission of aerosolised bacterial pathogens including *A. pp.* An important caveat of the model is to remain within the acceptable safety margin for free chlorine exposure to both animals and farm staff.

Thus, the overall objective is to develop a compound protocol that is safe, with no residual effect, has broad-spectrum bacterial activity and does not co-select for antimicrobial resistance. Such a compound protocol could potentially become a management norm in pig husbandry in controlling farm environment bacterial load and prevention of spread of aerosols of infectious pathogenic bacteria.

Note: In the original aims of this project, proof of efficacy studies included both *A. pp* and methicillin-resistant *Staphylococcus aureus*. However, *A. pp* was identified as the major industry concern and, after the first initial *in vitro* experiments were undertaken on both pathogens, the project then focused on test development for total bacterial load and *A. pp.*

2. Materials and Methods

2.1 In vitro time kill kinetics of Ecas4 anolyte on A. pp and Australian MRSA isolates

To obtain *in vitro* kill kinetics of ECAS on *A. pp* and MRSA, isolates representing the major *A. pp.* serovars were acquired from the ATCC collection and ACE laboratories (ATCC 27090, and clinical isolates representing serovars 1, 5 7, 12 and 15) in addition to MRSA ST30, ST93, ST131 (obtained from Murdoch University) and *S. aureus* ATCC 17055. The antimicrobial activity of various concentrations of Ecas4 anolyte at 2, 5, 10, 15, 30, 60 min and 24 h contact time on these representative isolates of *A. pp* and MRSA was determined using the CLSI method. Ampicillin was included as an antimicrobial control which has MIC breakpoint range of 0.5 – 2 µg/mL when tested against the reference strains *S. aureus* ATCC 29213 and *A. pp* ATCC 27090. Briefly, a 100 µg/mL stock solution of ampicillin was prepared in phosphate buffer (pH 8.0) and a serial dilution range from 0.125 to 16 µg/ml was prepared in phosphate buffer (pH 6.0) leaving first and second wells as positive and negative controls, respectively. Similarly, solutions containing 0.049%, 0.098%, 0.195%, 0.39 %, 0.78%, 1.56%, 3.13%,

6.25%, 12.5% and 25% (v/v) of Ecas4 anolyte were dispensed into wells 2-12 of the microtiter plate (100% ECAS produced by Ecas4 has approx. 300 ppm of free available chlorine). *A. pp* and MRSA were grown in VFM and LB broth, respectively for 12 h in CO_2-enriched and normal aerobic incubators at 37 °C. OD_{600} for *A. pp* was approximately 0.37 and for MRSA 0.57 (equivalent to 1–1.5 $\times 10^8$ CFU/mL). Aliquots of each culture were resuspended in 4-5 mL of PBS to obtain OD_{600} values of 0.07 to 0.1 (10^4-10^5 CFU/ml). A 5 µL aliquot of the *A. pp* suspension was then added to wells 2 to 12 of rows A to D. 20 µL from wells 1 to 12 were plated onto chocolate agar after 30 s, 1 min, 2 min and 4 min of contact time and incubated for 18-24 h. MIC results were interpreted as the lowest concentration that had 80% reduction in bacterial growth in comparison to the growth in the control well. Test validity was determined as per acceptable growth in the positive control well and no growth in the negative control well.

2.2 Optimisation of quantitative real time PCR for A. pp apxIVA gene primers and universal bacterial primers

2.2.1 A. pp SYBR green real time qPCR optimisation

An established real-time quantitative PCR primer set for the *apxIVA* gene developed by Schaller et al. (2001) was optimised using *A. pp* ATCC 27090 and the KAPPA fast qPCR SYBR green kit. Optimum concentration of primers and reaction conditions were determined to be able to detect *A. pp* DNA concentrations as low as 0.05 ng/µL. The following master mix volume, primer concentrations and real time PCR run parameters represents the final optimised PCR protocol.

Reaction Super Mix	**1 Reaction**
Enzyme reaction super Mix	6 µL
Primer (F) (10 µM)	1 µL
Primer (R) (10 µM)	1 µL
H_2O	1 µL
Template DNA (*App* ATCC)	1 µL
Total	10 µL

PCR Thermocycler conditions

1. **Hold Steps**: Hold at 95 °C for 5 min

2. **Cycling** (40 cycles)
    1) 95 °C for 15 s
    2) 60 °C for 30 s acquiring on Green
3. **After Cycling:** Hold at 40 °C for 60 s
4. **Melt on Green:** Melt from 72 °C to 95 °C at 0.3 °C/s

The specificity of the *A. pp* optimised primers was tested against DNA from the following bacterial strains: *Actinobacillus lignieressi*, *Actinobacillus suis*, *Mannheimia haemolytica*, *Pasteurella multocida*, *Streptococcus suis*, *Bordetella bronchiseptica* and *Haemophillus parasuis*.

2.2.2 Universal bacterial SYBR green real time qPCR optimisation

A set of universal primers for amplification of bacterial 16S rDNA was designed by Nadkarni et al. (2002). This assay was optimised using *E. coli* DNA serial dilutions for analysis of total bacterial load from the farm environment. Optimum concentration of primers and template was determined in order to have a similar sensitivity to the *A. pp* PCR test. The reaction master mix and qPCR thermocycling conditions were identical to the *A. pp* qPCR except for primers and water volumes, which were adjusted to obtain a final volume of 9 µL of master mix per sample.

2.3 Optimisation of farm environment air sample collection protocol to detect A. pp and total bacterial load

The Coriolis air sampler from Bertin Technologies was selected as the most appropriate device for collecting and testing air samples on piggeries (Figure 2). A number of different collection volumes (5, 7.5, 10 and 12.5 mL), collection liquids (1× PBS and veterinary fastidious media (VFM)), and collection times (2, 3, 4, 5 & 6 min) were tested, with collection parameters preset at airflow rates of 300 L/min and 250 L/min. The ideal collection time, collection liquid and collection volume were determined as the combination of parameters that could successfully detect *A. pp* genomic DNA concentrations of >0.1 pg/µL from the farm environment. Culture based studies were also undertaken on the same samples.

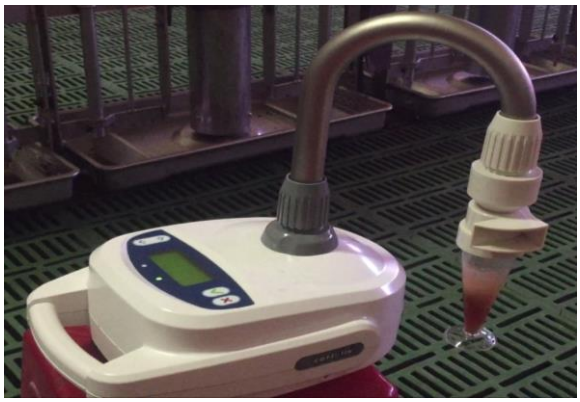

Figure 2. Coriolis air sampler from Bertin Technologies

The optimised parameters were used to quantify both *A. pp* and total bacterial load concentrations from weaner rooms (approximately 120 pigs per room) and grower/finisher sheds of a continuous flow farm with a major pleuropneumonia problem in finisher pigs. This farm was ideal for the pilot proof of concept experiments, as small scale fogging experiments could be conducted with a portable humidifier in the weaner rooms. We initially proposed to conduct continuous fogging experiments with a batch of new weaners, however, concern was raised by the consulting veterinarian that if the fogging worked too well, there may be the risk of introducing a large number of immunologically naïve pigs into the grower/finisher unit. These pigs could rapidly break with pleuropneumonia at the same time, excreting large amounts of aerosolised bacteria into the piggery environment and upsetting the delicate disease epidemiology, which was under control with medication at the time the experiments were being undertaken. The sample treatment protocol described in 2.4 was also developed to discern live and dead bacteria before and after fogging the farm environment with ECAS. It was therefore decided that the proof-of-concept fogging experiment would be undertaken in the weaner room immediately after moving the weaners into the grow-out facility.

2.4 PMA treatment of samples to differentiate live/dead bacteria by qPCR

Since qPCR detects all dsDNA sequence specific to the primers present, an additional step in sample treatment was developed. Propidium monoazide (PMA), a photoreactive membrane-impermeable dye that selectively penetrates bacteria cells with compromised membranes (considered dead) and binds covalently to dsDNA was used to optimise the qPCR protocols and differentiate between live and dead bacteria.

The following *in vitro* experiment was developed to optimise the detection and differentiation of live and dead *A. pp* and *E. coli.* Cultures were grown overnight and resuspended in phosphate buffered saline to an OD_{600} of 0.58. Samples of 5% (in ppm) Eacs4 anolite in milliQ water and milliQ water only controls were prepared and 50 µL of each bacterial suspension was added. Samples were incubated at room temperature for 5 min; 20 µL aliquots were then plated onto chocolate agar and SBA and incubated for bacterial growth. Additional samples were heated at 85 °C for 10 min to compromise the bacterial membrane integrity. Except for the viable controls not treated with PMA, all samples were then added with 12.5 µL of 2 mM PMA to obtain a final concentration of 50 µM per reaction, vortexed briefly, incubated at room temperature in the dark for 15 min, and exposed to LED light from the PMA LED LITE device for 15 min.

DNA was extracted following the genome extraction kit protocols (Zymogen whole genome extraction kit). An aliquot of 280 µL of lysis buffer was added and samples incubated for 10 min after mixing with a vortex. Silicon membrane filter tubes were labelled as above and samples were pipetted into the tubes and centrifuged at 12, 400 *x* g for 60 s. Collection tubes with eluted liquid were discarded, changed and 200 µL of pre-wash buffer added to all the membrane tubes and spun for 1 min at 12, 400 *x* g, discarding the eluted wash buffer. 500 µL of wash buffer was added and centrifuged at 12, 400 *x* g for 60 s. Membrane tubes were transferred into 1.5 mL collection tubes and added with 35 µL of sample elution buffer, incubated at room temperature for 3 min and centrifuged at 12, 400 *x* g for 30 s. DNA concentrations were measured using the DeNovix DS-11+ spectrophotometer and used for performing qPCR as described previously.

2.5 Ecas4 fogging experiment of pig shed

To disinfect the farm environment, an Ecas4 anolyte concentration of 150 ppm was used to generate a dry fog containing 0.25 ppm (0.75 mg/m^3) of active chlorine. The environment was fogged for 3 min every 30 min, during 5 h, using an ultrasonic humidifiers that produces a mist with droplets having a diameter in between 1 and 5 µm (Figure 3). Initial environmental air samples were collected before fogging and then samples were collected every hour during fogging using the Coriolis air sampler. Air samples were collected at the rate of 250 L/min for 3 min in 10 mL of sterile veterinary fastidious medium. After each sampling, the sample collection chamber was purged with 0.1% H_2O_2 to wash away the residual sample and disinfect, in preparation for next sample collection. Samples were chilled immediately on ice. 1 mL

aliquots of sample were centrifuged at 12, 400 x g for 7 min and supernatants were discarded. DNA from samples was then extracted and purified following the Purelink genomic DNA purification kit protocol. DNA concentration was measure by DeNovix DS-11+ spectro-photometer and qPCR was set up for total bacteria and *A. pp* quantification. A 2 mL aliquot of each sample in VFM was also incubated overnight in 5% CO_2 at 37 °C overnight to further confirm presence or absence of *A. pp* in the farm environment.

Figure 3. Ultrasonic portable dry fog generator

Sample aliquots of 100 µL were also obtained for preparation of serial dilutions in peptone water, inoculated onto plate count agar and incubated overnight at 37 °C under aerobic conditions, to determine the efficacy of Ecas4 dry fogging on disinfection of bacteria in the farm environment.

3. Research results

3.1 In vitro kill kinetics of Ecas4 anolyte on A. pp serovars and MRSA

In vitro antimicrobial susceptibility testing (time kill kinetics and minimum inhibitory and bactericidal concentration testing) of ECAS against field strains of *A. pp* and livestock-associated MRSA as well as the important human-disease-associated clone ST93, recently identified in Australian pigs, showed that very low concentrations of Ecas4 anolyte in water (0.48-7.8 ppm) were equally effective at killing both *A. pp* and MRSA within 30 s of exposure.

3.2 Optimisation of qPCR primers for A. pp and total bacteria detection and quantification

Reverse and forward primer concentrations of 10 µM for *A. pp apxIVA*, in a reaction volume of 10 µL per reaction, could detect and quantify as little as 1.0 pg of *A. pp* ATCC 27090 and other *A. pp* serovars. For the 16S rDNA universal bacterial gene primers, 750 nM of each of forward and reverse primer in a final volume of 10 µL was able to detect *E. coli* DNA concentrations as low as 1.2 pg per reaction.

Quantification curves of serial dilutions of *A. pp* ATCC 27090 and serovars 1, 5, 7, 12, 15 from 500 ng to 0.05 pg per reaction were optimised. Quantification curves and their corresponding Cq values for *A. pp* ATCC 27090 DNA serial dilutions are shown in Figure 4 and Table 1. The calibration curve is shown in Figure 5.

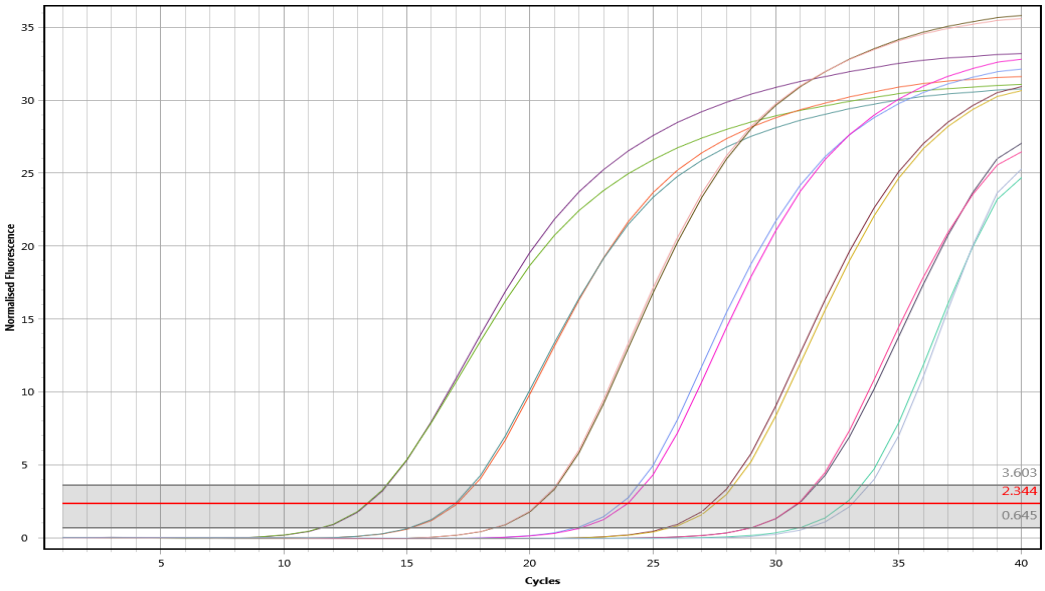

Figure 4. Amplification curves of 10-fold serial dilutions of *A. pp* ATCC DNA and NTC

Table 1. Cq values of *A. pp* ATCC 20790 DNA serial dilutions and NTC

Well	Sample	DNA conc. (pg/reaction)	Cq	R^2
1	*A. pp* ATCC 1:1	5,000	13.41	0.99
2	*A. pp* ATCC 1:1	5,000	13.37	0.99
3	*A. pp* ATCC 1:10	500	17.04	0.99
4	*A. pp* ATCC 1:10	500	16.96	0.99
5	*A. pp* ATCC 1:100	50	20.37	0.99
6	*A. pp* ATCC 1:100	50	20.33	0.99
7	*A. pp* ATCC 1:1,000	5.0	23.69	0.99
8	*A. pp* ATCC 1:1,000	5.0	23.97	0.99
9	*A. pp* ATCC 1:10,000	0.5	27.35	0.99
10	*A. pp* ATCC 1:10,000	0.5	27.54	0.99
11	*A. pp* ATCC 1:100,000	0.05	30.92	0.99
12	*A. pp* ATCC 1:100,000	0.05	30.88	0.99
13	NTC	0	32.79	0.99
14	NTC	0	33.11	0.99

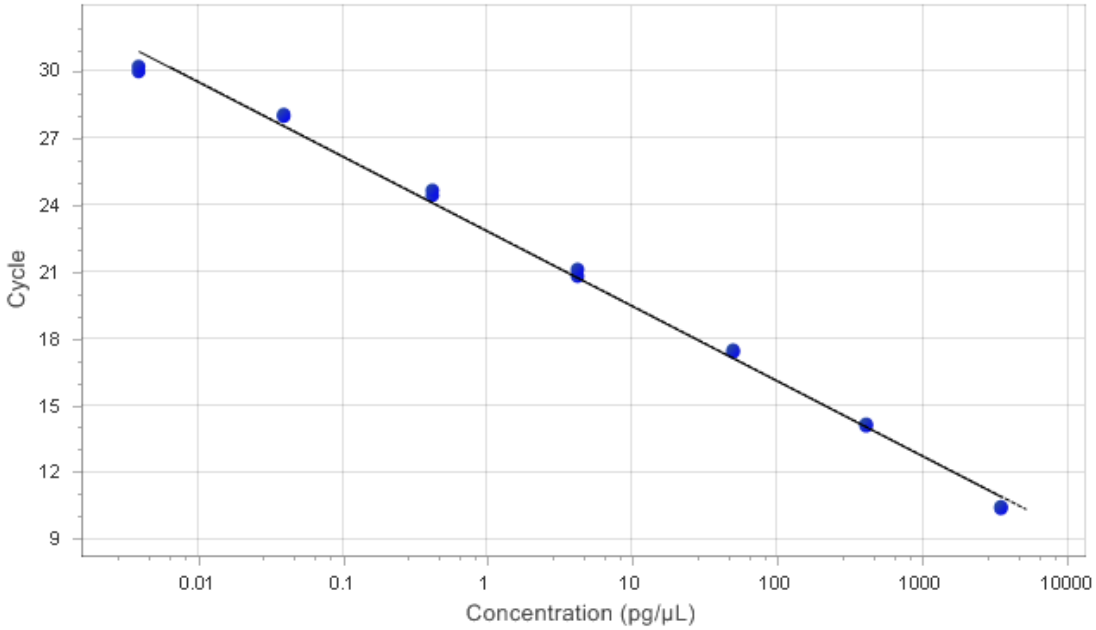

Figure 5. Calibration curve of serial dilutions of *A. pp* ATCC DNA (0.05 to 5,000 pg)

The quantification curve of 10-fold serial dilutions of *E. coli* and corresponding Cq values are presented in Table 2 and Figure 6, respectively. The sensitivity of qPCR was validated to detect total bacterial DNA at 1.5 pg per reaction. The calibration curve is shown in Figure 7.

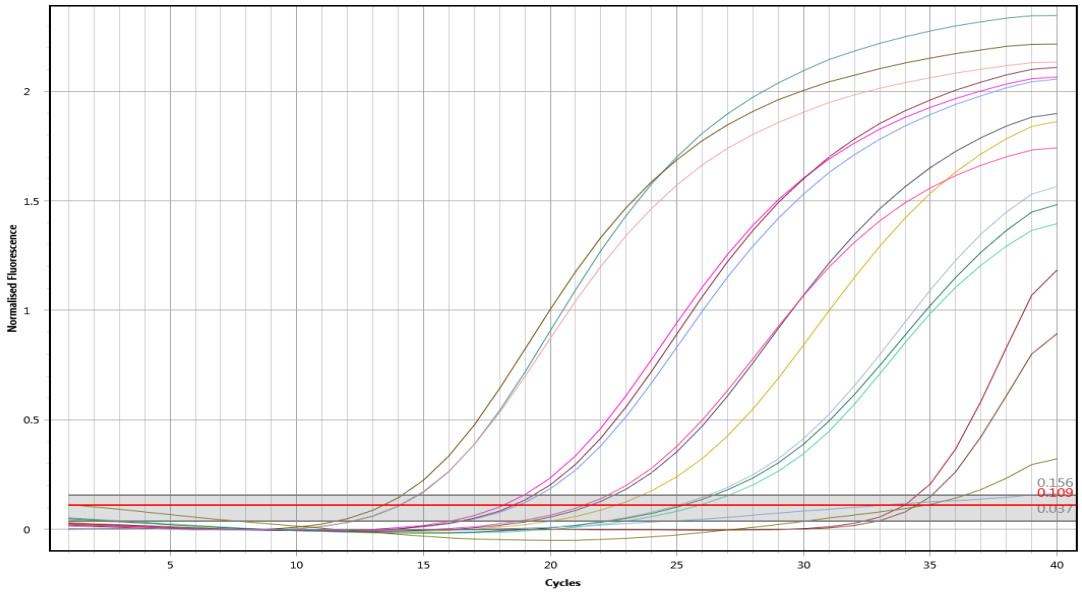

Figure 6. Amplification curves of 10-fold serial dilutions of *E. coli* DNA and NTC

Table 2. Cq values of *E. coli* DNA serial dilutions and NTC

Well	Sample	DNA conc (pg/reaction)	Cq	R^2
1	*E. coli* 1:10	4,100	14.05	0.99
2	*E. coli* 1:10	4,100	13.38	0.99
3	*E. coli* 1:10	4,100	14.02	0.99
4	*E. coli* 1;100	151	18.69	0.99
5	*E. coli* 1;100	151	18.11	0.99
6	*E. coli* 1;100	151	18.50	0.99
7	*E. coli* 1:1,000	15	22.55	0.99
8	*E. coli* 1:1,000	15	21.54	0.99
9	*E. coli* 1:1,000	15	21.28	0.99
10	*E. coli* 1:10,000	1.53	25.83	0.99
11	*E. coli* 1:10,000	1.53	25.01	0.99
12	*E. coli* 1:10,000	1.53	25.20	0.99
13	NTC	0	34.72	0.99
14	NTC	0	33.96	0.99
15	NTC	0	34.42	0.99

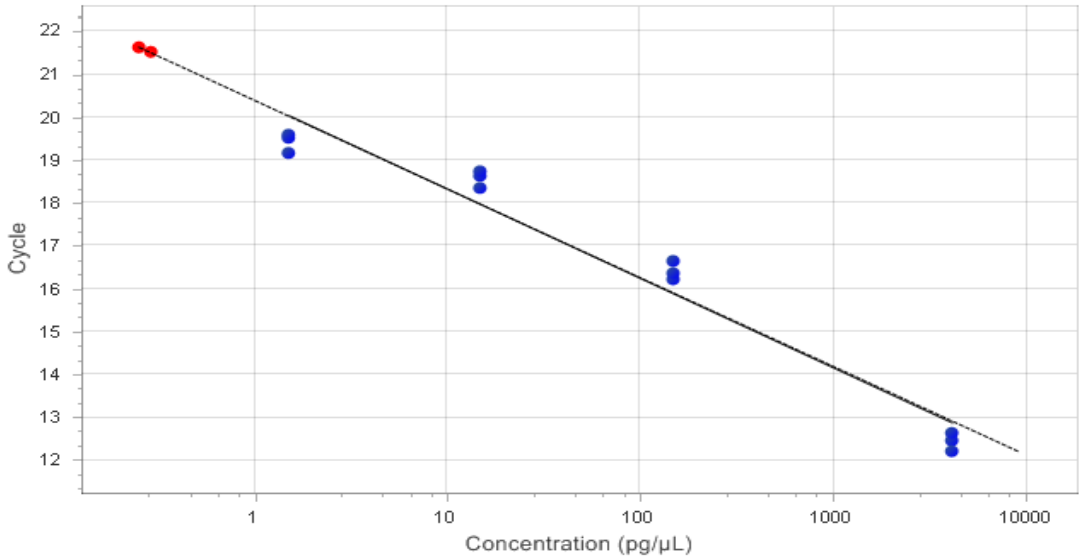

Figure 7. Calibration curve of serial dilutions of *E. coli* DNA (1.5 to 4,100 pg)

3.3 Optimisation of farm environment air sample collection protocol to detect A. pp and total bacterial load

The optimised parameters for collecting air samples of farm environmental are as follows: Veterinary Fastidious Media (VFM) was the best collection liquid (as it could be used for both culture of *A. pp* and real-time PCR, with no loss of viability of organisms) in 10 mL volumes. A pre-set collection rate of 250 L/min for 3 min was able to detect *A. pp* at 200 pg (5.1×10^6 GU) in 2 mL of VFM. However, the quantity of *A. pp* increased by 8-fold to about 1,600 pg (2.1×10^8) in 2 mL samples incubated in VFM overnight in 5% CO_2. Similarly, very low levels of *A. pp* were captured from the farm air of the grower/finisher shed. The amount of total bacterial DNA detected using these collection parameters was approximately 1,200 pg (1.5×10^8 GU) in 2 mL of VFM. Total bacterial DNA also increased to 3.6×10^9 GU after incubation.

3.4 PMA treatment of samples to differentiate live/dead bacteria by qPCR

Cq values of amplified DNA of viable (live) *E. coli* and dead *E. coli* control (500 µL of aliquot killed by boiling at 85 °C for 15 min) samples after treatment with 50 µM PMA and quantified using universal bacterial primers at 700 nM are shown in Figure 8. PMA treatment of samples resulted in more than 10,000 fold reduction in DNA amplification from the killed *E. coli* sample. A similar reduction in amplification of *A. pp* ATCC strain 20790 DNA was observed between viable and killed *A. pp* following treatment with PMA and qPCR with *apcIVA* gene primers (Figure 9).

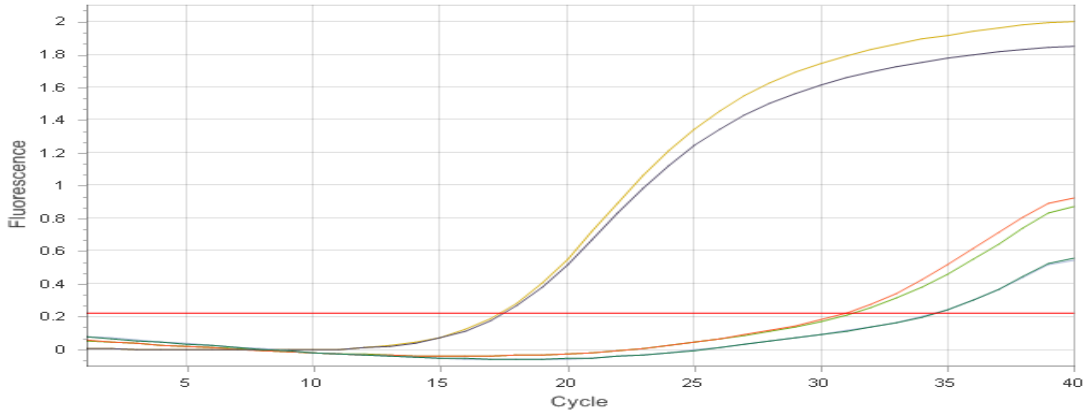

Figure 8. Cq values of DNA of live (Cq = 17) and dead (Cq = 31.5) *E. coli* control after PMA treatment.

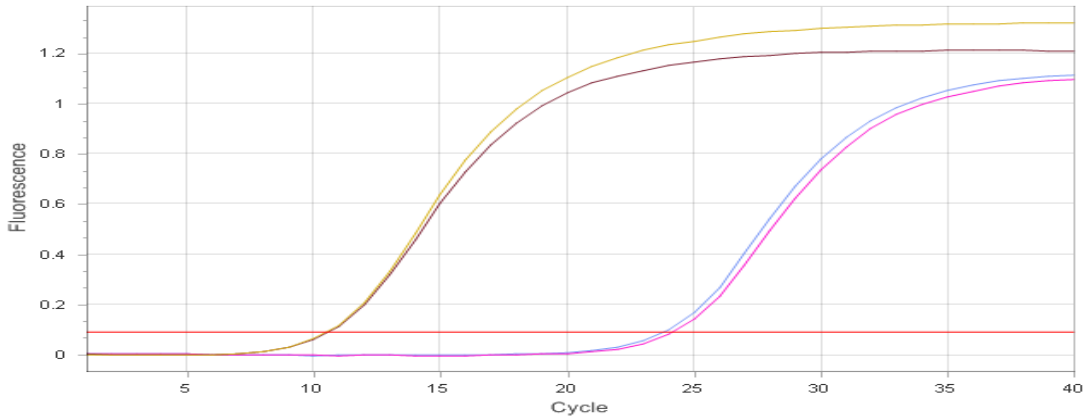

Figure 9. Cq values of DNA of live (Cq = 17) and dead (Cq = 31.5) *A. pp ATCC 27090* control after PMA treatment.

3.5 Ecas4 fogging results of pig shed

At the time of the proof-of-concept experiment, real time qPCR did not detect any *A. pp* DNA in the air from the vacated weaner room that was collected into VFM and incubated overnight at 5% CO_2 at 37 °C (Table 3). However, real time qPCR for detection and quantification of total bacterial DNA before and after Ecas4 decontamination showed a 1.4 log fold reduction in bacterial numbers in the first 2 h of fogging. This was followed by a 2 log fold reduction after 3 h, and 4.5 and 5 log fold reduction after 4 and 5 h of fogging, respectively (Table 4). We can conclude that the reduction in terms of percentage of bacterial is close to 100% after 5 f of Ecas4 fogging at 0.25 ppm of active chlorine in the air. However, the marked reduction in numbers after 1 h is likely to reduce markedly the potential transmission between animals.

Table 3. *A. pp* qPCR quantification results

Sl. No.	Sample	*A. pp* concentration (pg/µL)
1	Before fogging	0.00
2	1 h after fogging	0.00
3	2 h after fogging	0.00
4	3 h after fogging	0.00
5	4 h after fogging	0.00
6	5 h after fogging	0.00
7	Before fogging (CO_2)	0.00
8	1 h after fogging (CO_2)	0.00
9	2 h after fogging (CO_2)	0.00
10	3 h after fogging (CO_2)	0.00
11	4 h after fogging (CO_2)	0.00
12	5 h after fogging (CO_2)	0.00

Table 4. Total bacterial load (pg/µL), log fold and % reduction before and after Ecas4 fogging quantified by qPCR using universal bacterial primers

Sl. No.	Sample details	DNA conc. (pg/µL)	DNA conc. (Log_{10})	Log reduction	% reduction
1	Before fogging	3,930	3.59		
2	1 h after fogging	143.1	2.15	1.43	96.359
3	2 h after fogging	182	2.26	1.33	95.369
4	3 h after fogging	30.5	1.48	2.11	99.224
5	4 h after fogging	0.12	-0.92	4.51	99.997
6	5 h after fogging	0.03	-1.52	5.11	99.999

Similarly, bacterial numbers enumerated following plating onto plate count agar and overnight incubation are presented in Table 5; results correlated closely with the live/dead PCR assay. One log fold reduction in total bacterial counts was observed after the first hour of fogging, a 2 log reduction was observed at 2 and 3 h after fogging, while after 5 h, a 3.7 log fold reduction was found, essentially confirming that 99.9% of bacteria were effectively killed by ECAS dry fogging.

Table 5. Total bacterial count, log_{10} transformed count, log and % reduction before and after fogging with Ecas4 anolyte

Sl. No.	Sample details	Total Bacterial load (cfu/mL)	Bacterial load (Log_{10})	Log_{10} reduction	% reduction
1	Before fogging	2.04×10^4 ($2.01\text{-}2.08\times10^4$)	4.31		
2	1 h after fogging	4.4×10^3 ($3.3\text{-}5.3\times10^3$)	3.64	0.67	78.43
3	2 h after fogging	5.4×10^2 ($4.7\text{-}6.1\times10^2$)	2.73	1.58	97.35
4	3 h after fogging	1.1×10^2 ($1.0\text{-}1.17\times10^2$)	2.04	2.31	99.51
5	4 h after fogging	3.3×10^1 ($2.7\text{-}3.8\times10^1$)	1.52	2.79	99.84
6	5 h after fogging	4 ($0\text{-}8\times10^0$)	0.60	3.71	99.98

4. Application of Research

The SYBR green-based real time qPCR methodology optimised here to detect and quantify *A. pp* and 16S rDNA bacterial genomic DNA can detect extremely low levels of *A. pp* and other bacteria from the farm air environment. This robust method, in combination with the optimised air-sample collection protocol, could be applied to quantify microbial load including *A. pp* in the farm environment. 16S rDNA qPCR with universal bacterial primers is validated to accurately quantify genomic DNA with results comparable to that achieved by CFU counts using bacterial culture techniques. This method could be used as a robust tool to assess efficacy of novel and traditional farm decontamination processes without the requirement for time consuming, labour intensive and costly culture techniques. *A. pp* qPCR validated here with excellent sensitivity and specificity would be an alternative molecular method to detect *A. pp* from farm air, swabs and autopsy samples by using specific VFM to capture *A. pp* and keep cells viable during transport. In our future projects, we will aim to multiplex our *A. pp* live/dead qPCR to include *Streptococcus suis* and *Pasteurella multocida* detection.

ECAS fogging at 0.25 ppm was shown to be an effective aerosol disinfectant, effectively killing bacteria within the first hour of operation, with an almost total kill established after 5 h. This proof-of-concept project is now ready to be applied on a much larger scale in the grower/finisher shed at this continuous flow piggery with the aim of reducing bacteria load in the farm environment to prevent and control aerosol transmission of pathogenic respiratory bacteria between pigs. This has significant benefits in improving farm animal welfare and reducing the amount of antimicrobials required to control lung lesions. A cost-benefit analysis

also needs to be performed against other traditional disinfection systems. We also plan to test the system at a piggery in Australia with an endemic livestock associated MRSA problem.

5. Conclusion

Proof-of-concept on the efficacy of ECAS for aerosol decontamination of a pig farm environment has been developed in this project. The decontamination process could now be extensively trialled and used to cost-effectively disinfect agricultural industrial and livestock farm sheds to manage general air hygiene and eliminate specific pathogens from the farm environment using a non-toxic, safe disinfection protocol that also does not contribute to antimicrobial resistance development.

In addition, the qPCR quantification methods and air sampling protocol could be modified to detect additional pathogens from any livestock farm environment, epidemiological studies and for surveillance.

In conclusion, continuous ECAS dry fog decontamination of the farm environment could be easily adapted and applied in various farm settings to reduce usage of antibiotics; ECAS is an environmentally safe, non-toxic alternative that does not contribute to antimicrobial resistance development.

6. Limitations/Risks

The most prominent limitation of ECAS is the significant reduction in its antimicrobial potential in presence of high organic material loads (Selkon, Babbt et al. 1999, Oomori, Oka et al. 2000, Park, Hung et al. 2004). This has been overcome by focusing on aerosol disinfection rather than surface disinfection. Inhibition of antibacterial activity by organic materials could be effectively overcome by using a solution with greater strength, or by managing its continuous supply. Regarding strongly acidic ECAS, a drawback is its lower stability during storage and the fact it causes corrosion of equipment surfaces. Anolyte with neutral pH used in the current project does not cause corrosion of metallic surfaces and has relatively longer shelf life compared to acidic ECAS (Fabrizio, Sharma et al. 2002). Unlike other forms of active chlorine such as hypochlorite and gaseous chlorine, the hypochlorous acid (HOCl) is a ‘metastable state’ that easily reverts to NaCl solution and cannot be stored for long periods, requiring *in situ* production in order to guarantee optimal activity. Having a 4-chamber reactor on site at large

piggeries would therefore be the most cost-effective solution rather than buying ready-made product.

7. Recommendations

We recommend that further studies be undertaken with ECAS on a much larger scale, i.e. direct application in weaner rooms and grower-finisher sheds, at the same time to confirm disinfection of the farm environment at recommended dosage.

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