REAL-TIME DETECTION OF AIRBORNE PATHOGENS IN THE PIGGERY

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

By

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Executive Summary

In virtually all intensive farming systems, key parameters (ambient temperatures, CO_2 levels, body weights, fertility rates…) are regularly measured to control the performance of production. Many of these factors can be easily recorded and analyzed by farm managers. However, it is more difficult to monitor the impact of micro-organisms on the herd. This is partly due to the lack of simple, rapid and quantifiable procedures for sampling and detecting infectious agents at the group level. To address this need, we have tested an innovative methodology to capture bacteria from the air of the piggery, coupled with rapid diagnostic assays for the detection of swine pathogens. The rationale for monitoring airborne microorganisms in the farm includes the following considerations: 1- air sampling is rapid, mobile, unbiased, and creates no or minimal disturbance to the animals, 2- the concentration of bacteria in the air is a quantifiable, objective parameter which can represent the global air quality in almost any building type, age group, etc., and can be used to analyze variations in the general performance of herds, and 3- many pathogens shed by the infected animals can be detected directly in captured aerosols by molecular techniques, without the need for costly and time consuming bacteriological analysis.

This approach allowed us to quantify the total concentration of cultivable microorganisms per volume of air, both in a standardized laboratory setting and in a pig farm, giving us an easily measurable approximation of the air quality in different conditions. The titers of bacteria increased with the age and the density of animals, demonstrating the potential of the methodology as a general infectious risk management tool. Moreover, we developed a series of proof of concept molecular assays to detect and quantify two pathogens of importance, Entero-Toxinogenic *Escherichia coli* (ETEC) and *Actinobacillus pleuropneumomiae* (APP) in air samples. These organisms were chosen for complementary reasons: *E. coli* can be easily identified by conventional microbiology using selective media but the significance of a positive culture requires further molecular characterization of the isolate because most strains are non pathogenic; conversely, APP is a significant swine pathogen (although some serovar-dependent variation in virulence is noted) but the organism is difficult to isolate from polymicrobial specimens because proper selective media are not available. In this project, we were able to detect both organisms at low levels in the pig farm, by direct molecular assays, even in the absence of positive cultures. The methodology could be easily expanded to the quantitative detection of other significant swine pathogens.

We believe that regular air capture programs could be implemented in piggeries as part of an active surveillance plan, targeting at the same time the general air quality parameters of the farm and the early detection of specific infectious risks relevant for the industry.

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1. Introduction

The management of infectious diseases in production animals requires the collection of clinical or post-mortem specimens for laboratory isolation and identification. Conventional diagnostic microbiology relies on individual case histories and clinical findings to determine the significance of the organism(s) isolated from infected animals. Alternatively, the farm environment and its effluents can also be used to detect specific pathogens and identify risks factors. The advantages of monitoring the farm environment reside in the simplicity and rapidity of the approach, the absence of sampling bias and the possibility of assessing health risks at the group level, before clinical problems become widespread.

Air sampling is a useful methodology for the detection of diverse airborne materials in intensive animal production systems. Monitoring air quality in the farm can improve livestock health status, prevent or control infectious diseases, and help to manage public health issues. While some studies have confirmed the positive link between good air quality and livestock health and welfare in the piggery, there is little information on the usefulness of air sampling methodologies for the detection of specific pig pathogens, the diagnosis of infectious diseases and the efficacy of control measures such as fogging or nebulisation of disinfectants in pig farms.

Pathogenic bacteria commonly found in pig farms can be detected by standard microbiological culture and identification techniques. This approach is time and labour intensive, as it usually requires 3-5 days to report most microorganisms. Moreover, fastidious organisms are difficult to isolate, particularly when the specimens are contaminated by non-significant fast growing species. Alternatively, specific detection of DNA sequences by Polymerase Chain Reaction (PCR) can be used to find pathogens in mixed populations. Quantitative PCR assays with melt curve analysis are increasingly adopted by diagnostic laboratories due to their excellent specificity, sensitivity and ease of use.

It is important to be able to differentiate the subtypes of truly pathogenic microbial species from the normal flora of the host. For example, only certain strains of *E. coli* can cause diseases in pigs, while the rest are part of the normal digestive flora. Only specific molecular assays can differentiate potentially virulent *E. coli* strains from the non-pathogenic ones by demonstrating the presence of a small number of genes encoding toxins or adhesins.

Quantitative real time PCR and melting curve analysis on air samples collected in the piggery is a method of choice for developing and streamlining a “toolbox” of pathogen-specific detection and identification protocols. Once a proof-of-concept for the value of air sampling is established on a simple model organism such as *E. coli*, the methodology could be expanded to simultaneously detect major porcine pathogens in farms and correlate these data with the performance of the pigs and the influence of their genotype on their resistance to infection. Ultimately, this approach could be integrated in a real time, semi automated system for air quality monitoring on the piggery. Finally, air sampling could be used to assess the diffusion and efficacy of live attenuated vaccines in the livestock.

2. Methodology

For proof-of-concept purposes *Escherichia coli* was used as a model organism in laboratory conditions; *Actinobacillus pleuropneumoniae* (APP) was selected as a

pathogen of high diagnostics significance for the industry. The methodology was then tested for detecting various airborne microorganisms in the farm environment.

Reference bacterial strains and genotypes:

Reference strains (table 1) were obtained from Kate Hodgson, School of Pharmacy & Medical Sciences at the University of South Australia, Adelaide, IFM Quality Services (Ingleburn, NSW Australia) and Dr Pat Blackall, University of Queensland, to be used as positive controls.

Pathogen-specific PCR primers were designed and validated for the detection of pathogenic *E. coli* and APP. Partial or complete genomic sequences of these organisms have been published and several detection assays have been proposed.

The toxin genes STa, STb, LT, VT, the Adhesin F4 and the type III secretion system effector EaeA were selected for the characterisation of pathogenic *E. coli*. The RTX toxin genes *apxI*, *apxII*, *apxIII* and *apxIV* were selected for the characterization of APP. These gene targets were used in PCR assays to confirm the phenotypes of the reference strains which were later used as control templates (see below).

2.1. PCR assays:

Table 1 - Reference strains used in this study

Species	Strain	Key features
E. coli	AMR-472	Stx2e (Stx: Shiga-like-Toxin = VT: Verotoxin)
	EMA.1	O:157 (serogroup), EaeA (effacing and attaching), HhxA (Enterohaemolysin)
	R08	LT, STa, STb, F4
	EC136	LT, STa, STb, F4, EAST1 (enteroaggregative toxin), SepA (enteroaggregative protease)
	EC166	LT, STa, STb, F4, EAST1 (enteroaggregative toxin), SepA (enteroaggregative protease), O:157rfb (LPS)
	EC-IFM2007	(NTCT 12900): O157:H7, Stx (VT) negative
Actinobacillus pleuropneumoniae	HS 3617	serotype 1; Apx I, Apx II, Apx IV
	HS 3064	serotype 1;
	HS 2794	serotype 5; Apx I, Apx II, Apx IV
	HS 3659	serotype 7; Apx II, Apx IV
	HS 3718	serotype 15; Apx II, Apx III, Apx IV

Table 2 - PCR assays for detection of swine pathogens.

Organism	Target sequence	Forward Primer name	Reverse Primer name	Size (bp) of amplicon	Remarks	Reference
Streptococcus suis	Capsule	cps2J-s	cps2J-as	459		(Marois et al., 2004)
Haemophilus parasuis	16S RNA	HPS-forward	HPS-reverse	821		(Oliveira et al., 2001)
Actinobacillus pleuropneumoniae	RTX toxin Apx IV A	APXIVA-1L	APXIVA-1R	442	primary PCR	(Frey, 2003)
		APXIVANEST-1L	APXIVANEST-1R	377	nested PCR	
		ApxIV for	ApxIV rev	116	qPCR	Phil Markham, this study.
Mycoplasma hyopneumoniae	16S RNA	MhyopFor	MhyopRev	649	primary PCR	(Mattsson et al., 1995)
		MhyopForNested	MhyopRevNested	352	nested PCR	(Calsamiglia et al., 1999)
ETEC	ST_a toxin	STa1	STa2	166	qPCR	(Do et al., 2005)
	ST_b toxin	STb-1	STb-2	172	qPCR	
	LT toxin	LTA-1	LTA-2	696		
	F4 adhesin	F4-Fw	F4-Rv	764	qPCR	
	F5 adhesin	F5-Fw	F5-Rv	450		
	F6 adhesin	F6-Fw	F6-Rv	333		
	F41 adhesin	F41-Fw	F41-Rv	431		
	F18 adhesin	FedA1	FedA2	510		
EPEC	eae	eae1	eae2	917	tested only in simplex PCR.	(Aranda et al., 2007)
EPEC	bfpA	BFP1	BFP2	326	not tested	
EHEC/VTEC	stx1/stx2	VTcom-u	VTcom-d	518	tested only in simplex PCR.	
EIEC	ipaH	IpaH1	IpaH2	600	not tested	
EAgEC	aggR	aggRks1	aggRksa2	254	not tested	
ETEC (alt.)	est	STf	STr	190	tested only in simplex PCR.	
	elt	LTf	LTr	450	tested only in simplex PCR.	

Table 3 - PCR conditions for the selected assays

Organism	Primer F name	Primer F sequence	Primer R name	Primer R sequence	size	Cycle						
						Denaturation		Annealing		Extension		Cycles
						°C	s	°C	s	°C	s	
Streptococcus suis	cps2J-s	GTTGAGTCCTTATACACCTGTT	cps2J-as	CAGAAAATTCATATTGTCCACC	459	94	30	60	30	72	30	40
Haemophilus parasuis	HPS-forward	GTGATGAGAAGGGTGGTGT	HPS-reverse	GGCTTCGTCACCCTCTGT	821	94	30	59	30	72	120	30
Actinobacillus pleuropneumoniae	APXIVA-1L	TGGCACTGACGGTGATGA	APXIVA-1R	GGCCATCGACTCAACCAT	442							
	APXIVANEST-1L	GGGGACGTAACTCGGTGATT	APXIVANEST-1R	GCTCACCAACGTTTGCTCAT	377	94	30	54	30	72	30	35
	ApxIV for	AAAGTCACGGTTCAAAATTGG	ApxIV rev	GCCATCGACTCAACCATCTT	116	95	10	54	30	N/A; 2 step PCR		40
ETEC	STa1	TCTTTCCCCTCTTTTAGTCAG	STa2	ACAGGCAGGATTACAACAAAG	166	94	60	55	60	72	60	30
	STb-1	ATCGCATTTCTTCTTGCATC	STb-2	GGGCGCCAAAGCATGCTCC	172	94	60	55	60	72	60	30
	LTA-1	GGCGACAGATTATACCGTGC	LTA-2	CCGAATTCTGTTATATATGTC	696	94	60	55	60	72	60	30
	F4-Fw	GGTGATTTCAATGGTTCGgtc	F4-Rv	ATTGCTACGTTCAGCGGAGCG	764	94	60	55	60	72	60	30
	F5-Fw	TGGGACTACCAATGCTTCTG	F5-Rv	TATCCACCATTAGACGGAGC	450							
	F6-Fw	TCTGCTCTTAAAGCTACTGG	F6-Rv	AACTCCACCGTTTGTATCAG	333							
	F41-Fw	GAGGGACTTTCATCTTTTAG	F41-Rv	AGTCCATTCCATTTATAGGC	431							
	FedA1	GTGAAAAGACTAGTGTTTATTTC	FedA2	CTTGTAAGTAACCGCGTAAGC	510							
Mycoplasma hyopneumoniae	MhyopFor	GAGCCTTCAAGCTTCACCAAGA	MhyopRev	TGTGTTAGTGACTTTTGCCACC	649							
Mycoplasma hyopneumoniae	MhyopForNested	ACTAGATAGGAAATGCTCTAGT	MhyopRevNested	GTGGACTACCAGGGTATCT	352	94	30	62	45	72	30	30
EPEC	eae1	CTGAACGGCGATTACGCGAA	eae2	CGAGACGATACGATCCAG	917	94	20	50	30	68	60	35
EPEC	BFP1	AATGGTGCTTGCGCTTGCTGC	BFP2	GCCGCTTTATCCAACCTGGTA	326							
EHEC/VTEC	VTcom-u	GAGCGAAATAATTTATATGTG	VTcom-d	TGATGATGGCAATTCAGTAT	518	94	20	50	30	68	60	35
EIEC	IpaH1	GTTCCTTGACCGCCTTTCCGATACCGTC	IpaH2	GCCGGTCAGCCACCCTCTGAGAGTAC	600							
EAgEC	aggRks1	GTATACACAAAAGAAGGAAGC	aggRksa2	ACAGAATCGTCAGCATCAGC	254							
ETEC (alt.)	STf	ATTTTTMTTTCTGTATTRTCTT	STr	CACCCGGTACARGCAGGATT	190	94	20	50	30	68	60	35
	LTf	GGCGACAGATTATACCGTGC	LTr	CGGTCTCTATATTCCCTGTT	450	94	20	50	30	68	60	35

The gene targets for detecting a relevant panel of swine pathogens were selected from a review of the literature. The assays examined in this project are listed in the Tables 2 and 3. Real time quantitative PCR was compared to conventional culture to assess the sensitivity and specificity of the different methods.

For ETEC titration experiments, cultures of reference strains were inoculated in duplicate in LB broth and incubated at 37C in aerobic conditions for 18 hours. Serial dilutions of the cultures were prepared and plated in duplicate on MacConkey agar to determine the titres of each strain. A set volume of each strain was harvested by centrifugation and the genomic DNA was prepared from the bacterial pellet and resuspended in nuclease free water. The minimal number of organisms detected by PCR was established using real time qPCR on a Qiagen (Corbett) Rotogene system in presence of Cyto9.

2.2. Protocols for collection of air sample:

The air sampling protocol was calibrated under controlled conditions, using aerosols of pure bacterial cultures re-suspended and diluted to reach various concentrations representative of the bacterial loads that are expected to be found in piggeries. The aerosols were generated from standardised cultures of *E. coli* strain K12 by two Collison 6-jet CN25 nebulisers (BGI Instruments) connected to a compressed air supply and fitted onto a closed container chamber. Two versions of the chamber were constructed; a large one of approximately 1000L capacity and a smaller portable one of approximately 150L. The chambers were equipped with air inlets fitted with low voltage electric fans to maintain a constant inward airflow, and several ports for air sampling. The main outlet of the large container was connected to an aspiration system with a HEPA filter while the small one was placed in a class II biosafety cabinet. The working diagram of a calibration chamber is given in the Figure 1.

The microorganisms present in a set volume of air were captured either by direct

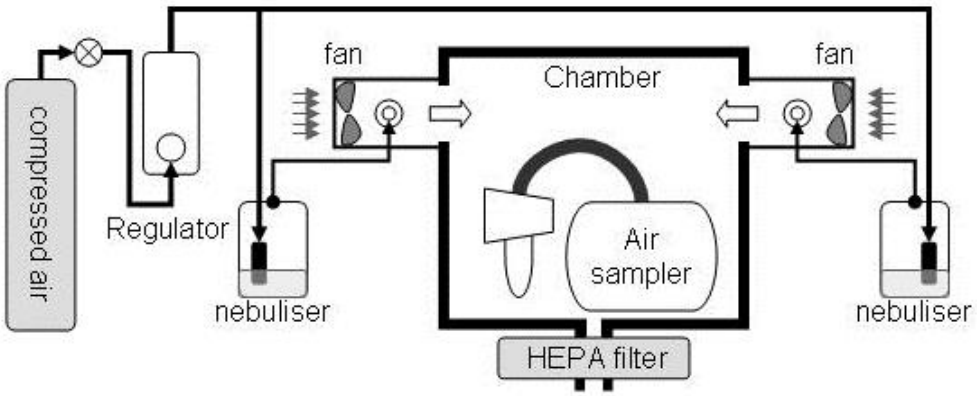

Figure 1: working diagram of a calibration chamber for air sampling.

impact onto the surface of selective or non selective agar plates using a commercial portable air sampler (Millipore M Air T instrument), by gentle bubbling of a preset volume of air into a liquid trap such as sterile buffer or broth, using an air pump (Buck Air-sampler), or by placing a commercial cyclonic sampler (Bertin Coriolis Micro) into the chamber. The cyclonic air sampler was connected to a sterile, single use screw cap tube containing a set volume of Phosphate Buffered Saline (PBS) to act as a liquid trap. The PBS-filled tubes were weighted to measure accurately the initial quantity of PBS used. Following an air sampling run, the tube weighted again and 0.1 mL of the PBS containing the captured airborne organisms

was plated in duplicate on selective or non-selective media, either directly, or after serial dilutions with sterile PBS or after concentration by centrifugating the entire residual PBS volume and re-suspending the pellet into a smaller volume. After each nebulisation, the chamber was purged by running the fans for 3 minutes. The concentration of organisms in the air was determined by counting the cfu per plate after incubation at 37C for 18 hours.

2.3. Farm studies:

A pig farm was investigated at regular intervals to evaluate the performance of the methodology in the field. Air samples were collected using the Coriolis instrument with 10 ml sterile PBS as a capture buffer. For proof-of-concept studies, total flora, coliform and *Staphylococcus* sp. counts were investigated by conventional bacteriology using Sheep Blood Agar (SBA), MacConkey Agar (MAC) and Mannitol Salt Agar (MSA), respectively. The presence of ETEC and APP was assessed by real time PCR. After incubation, colonies of various organisms were picked from the plates for diagnostics confirmation and phenotypic characterization. Different time points and production types (farrowing, weaner and finisher) were compared.

3. Outcomes

3.1. Air sampling methodology: choice and development of an operating protocol for pig farm monitoring

A commercial air sampler, specifically developed for the assessment of bio-contamination in various environments (Coriolis Micro, Bertin technologies) imported in Australia by Sapphire Bioscience (Waterloo, NSW), was evaluated and compared to a conventional battery operated plate impactor (Millipore M air T instrument). Plate impactors work by blowing a pre-set volume of air through a micro-perforated grid onto the surface of a microbiological agar plate. They are easy to operate but can only detect cultivable organisms. If the concentration of airborne bacteria is unpredictable and/or strongly variable, as expected in a piggery, the plate can become too crowded to count. For this reason it is necessary to successively collect a range of air volumes on series of plates which must be incubated in order to find the accurate cfu count. Moreover the method is not suitable for the direct detection of microorganisms by molecular assays such as PCRs as the plates must be incubated first. By contrast, the Coriolis sampler uses a cyclonic capture inflow that collects and concentrates airborne live organisms into a tube containing a sterile liquid trap. The air flow and sampling time can be adjusted to collect a wide range of volumes of air. The machine is battery operated and a programmable delayed start can also be used. The system is designed to avoid cross-sample contamination and to allow the testing of recovered microbial suspensions by microbiological cultivation or by molecular methods such as PCR assays, either directly or after diluting or concentrating by centrfugation. Therefore the cyclonic air capture methodology was thought to offer a better flexibility and practicality compared to a standard impactor. However the procedure had not been tested by us in field conditions and required some validation experiments before transferring the methodology into piggeries.

To optimise the protocol of air collection, the Coriolis system was first tested in controlled conditions at the University of Melbourne Faculty of Veterinary Science animal and laboratory facilities. The sampling procedure and general performance of the machine were tested in two nebulisation chambers (approx. 1000L and 150L), that were filled with an aerosol of a standard non-pathogenic culture of *E. coli* strain BL21 as the model airborne microorganism. Different concentrations and various collected air volumes were tested. A pilot experiment indicated that the small chamber was more reliable than the large one and was therefore

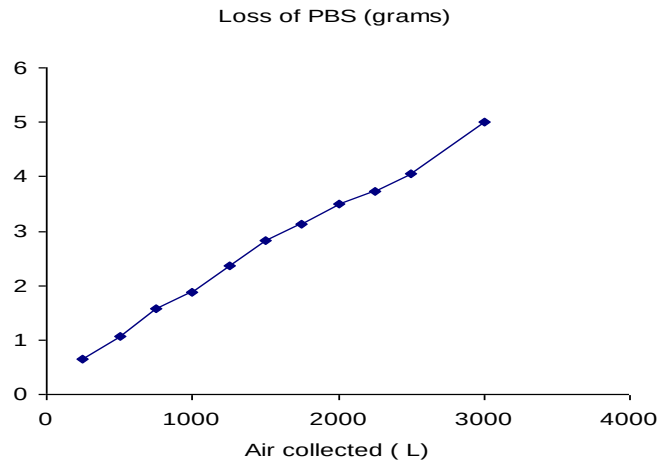

Figure 2: loss of collecting buffer during air sampling. The quantity of buffer present before and after sampling various volumes of air was determined by measuring the initial and final weights of the tubes. The weight difference is plotted against the total volume of air collected during each run.

selected for all subsequent validation experiments in the laboratory.

The volume of liquid trap recommended by the Coriolis manufacturer ranges between 2.5 and 15 mL. The airflow and sampling time can be adjusted to collect a wide range of volumes of air. However, in our hands, increasing the total volume of sampled air decreased signifcantly the residual volume of buffer left in the sampling tube at the end of the collection process (Figure 2). This phenomenon was reproducible and most likely due to the rapid evaporation of the buffer during the collection step, as a large quantity of air is aspirated and vortexed into a relatively small volume of liquid. In pig farms, the temperature and relative humidity of the air is expected to vary significantly from day to day. This could affect the accuracy of quantitative monitoring of airborne organisms over time if an insufficient quantity of buffer is used. Conversely, using a higher volume of buffer (superior to 15mL) was shown to dramatically decrease the organism recovery rate and the accuracy of the measurement. Therefore, we used a set volume of 10 mL sterile PBS as a standard liquid trap for all our subsequent experiments. Furthermore, in order to facilitate comparative observations in the farm conditions, all samples were collected using the same airflow and sampling time during the field studies. Using these conditions we observed very little variation of the residual volume of liquid trap between each farm visit (see below).

The air sampling protocol was also tested in clean rooms and in various procedure rooms at the Veterinary Hospital of the Faculty of Veterinay Science at Werribee. Up to six cubic meters of air were sampled in two consecutive runs and the lowest

limit for reliable detection of airborne bacterial load was estimated at 100 organisms per cubic meter of air, a concentration that is orders of magniture lower than normal values in a pig farm, where much higher concentrations of airborne organisms are expected to be present.

To determine the accuracy of the sampling protocol, a series of *E. coli* BL21 suspensions with concentrations ranging from 1.5 10^4 to 2.4 10^5 cfu/mL were prepared by serially diluting an overnight stock culture at a 1:2 ratio with sterile broth, and each suspension was then sequentially nebulised into the chamber, starting with the lowest titre. A pre-set volume of air (1500L, 6 min sampling time at 250L/min) was collected for each concentration of nebulised *E. coli* suspension. After each run the chamber was purged to eliminate residual aerosolised suspensions. The number of bacteria in the volume of air collected was determined by inoculating 0.1 mL of liquid sample on duplicate MacConkey agar plates and the average cfu counts per cubic meter of air was calculated (Figure 3).

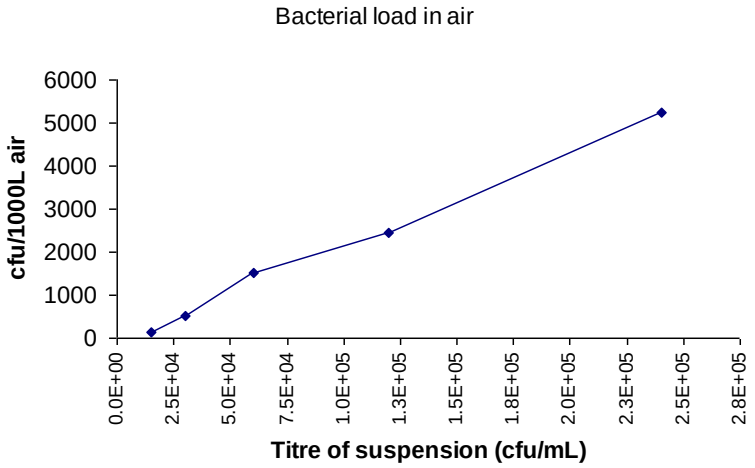

Figure 3 - Relationship between bacterial concentrations released in the chamber and plate counts from collected air samples.

An excellent corelation between the concentration of nebulised bacteria and the colony counts was observed. The number of cfu collected in the air was proportional to the titre of the bacterial suspension aerosolised in the chamber, indicating that plate counts numbers from field samples are likely to quantify the level of air contamination in the pig farm reliably, across a range of conditions.

Next, the relationship between the volume of air collected and the cfu plate counts was investigated by nebulizing an *E. coli* BL21 suspension at a unique titre (2. 10^5 cfu/mL) and collecting various volumes of air, ranging from 250 to 2500L (incremental sampling times set from 1 to 10 minutes, at constant airflow of 250L/min). The number of bacteria per volume of air collected was determined by duplicate plate counts on MacConkey agar and the results of a representative experiment are presented in Figure 4. The bacterial load in the air could be determined consistently and accurately across a large range of air volumes collected. The plate counts increased linearly with the volume of air captured, while the calculated bacterial concentrations (log cfu/cubic meter) reached similar values at all tested conditions, indicating that the procedure was reliable and that a standardized protocol could be adapted to the various conditions encountered in a pig farm by simply extending or reducing the collection time.

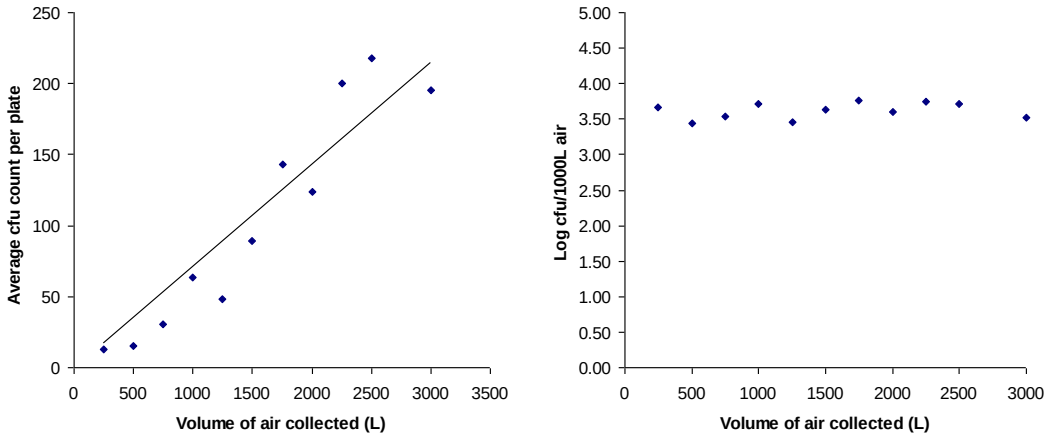

Figure 4 - Relationship between volume of air collected per run and plate counts from air samples. Left: linear relation between plate counts and sampling time. Right: log-transformed plate counts values per cubic meter of air.

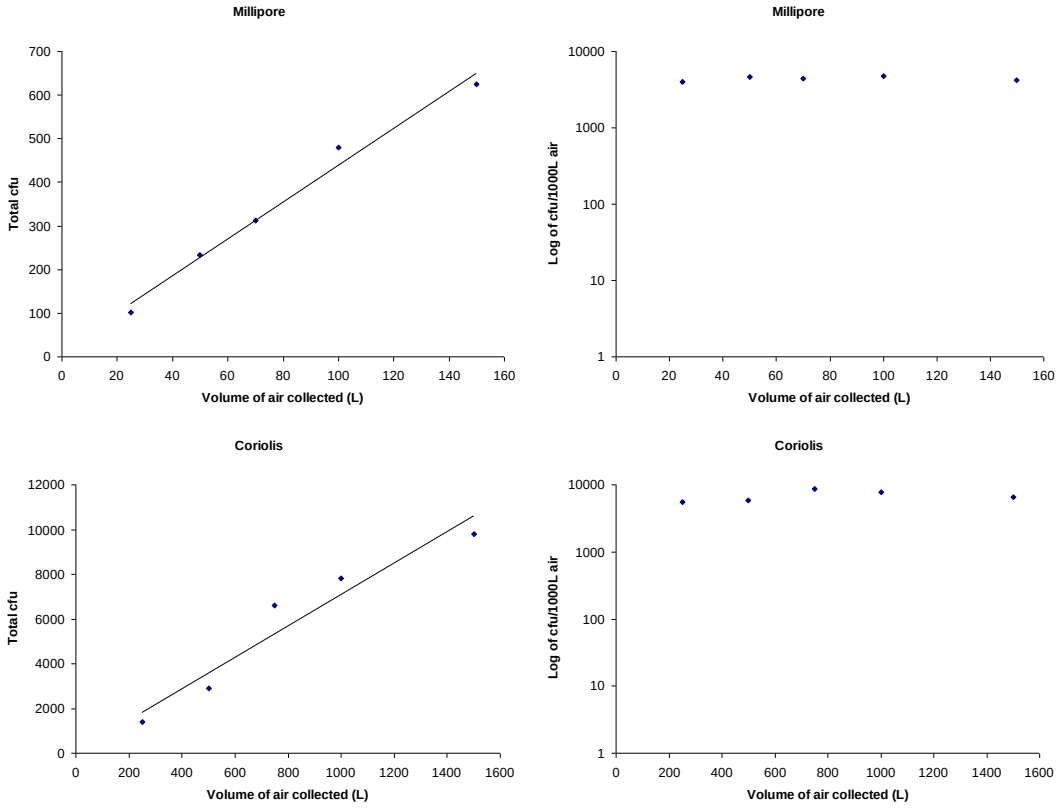

Figure 5 - comparison of a plate impactor (top, Millipore) and a cyclonic air capture (bottom, Coriolis). Left: total number of cfu collected in a run. Right: bacterial concentrations per cubic meter of air (Logarithmic scale).

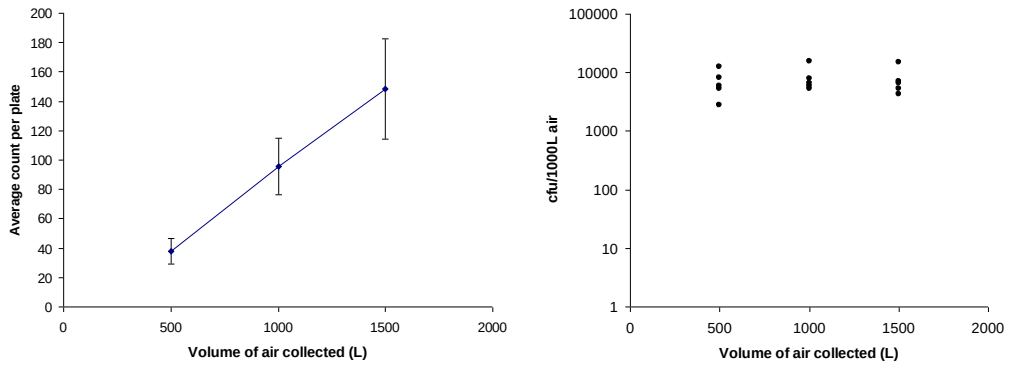

Figure 6 -Repeatability of the air sampling protocol. Values from 5 independent experiments. Error bars: Standard Errors.

The cyclonic air sampler (Coriolis) methodology was compared to a conventional plate impactor system (Millipore M air T) by placing both instruments in the nebulisation chamber which was then filled with aerosolized *E. coli* generated from a suspension containing 3.10^5 cfu/mL and operating the samplers in parallel. Both systems gave similar results over a range of sampled air volumes (Figure 5) and the repeatability of the procedure over five independent experiments for three air collection volumes, 500L, 1000L and 1500L (Figure 6) was found satisfactory.

Overall, an excellent correlation was observed between the volume of air sampled and the total plate counts and the measured titers of airborne bacterial load were

not significantly altered by the volume of air sampled. These results confirmed that cyclonic air sampling in liquid trap is an adequate and practical methodology with the best potential to be applied to air sampling in the farm.

3.2. Molecular techniques: detection of swine pathogens by PCR assays and Real Time Quantitative PCR assays.

E. coli

A conventional multiplex PCR assay previously developed by Aranda et al. (2007) for the detection of Pathogenic *E. coli* (ETEC, EPEC, EHEC, and VTEC) was evaluated for its diagnostic ability and used to confirm the phenotypes of the reference strains obtained from various sources at the beginning of the project. However in our hands the multiplex reaction gave ambiguous results and the PCRs were used instead as independent (simplex) assays. When the reaction was set up as a normal PCR, each strain genotype was confirmed as expected. Although the simplex PCRs were prone to non-specific amplification, it was possible to correctly identify the pathotypes based on the band intensity on agarose gel (Figure 7). The strains R08, EC136 and EC166 were confirmed as being ETEC isolates and were selected for further characterization as positive reference materials. Because of the ambiguous results obtained with the multiplex approach, we selected a different set of primers designed by Do et al. (2005) to develop and test a real time quantitative PCR (RT qPCR). Pathotype-specific RT qPCR assays for the F4 adhesin gene and the thermostable toxins STa and STb were developped for the quantitative detection of ETECs.

The limit of detection of the RT qPCR was established using the *E. coli* 16S RNA gene as a species-specific sequence and the genes encoding the toxins STa, STb and the adhesin F4 as ETEC-specific sequences. Portions of these genes were

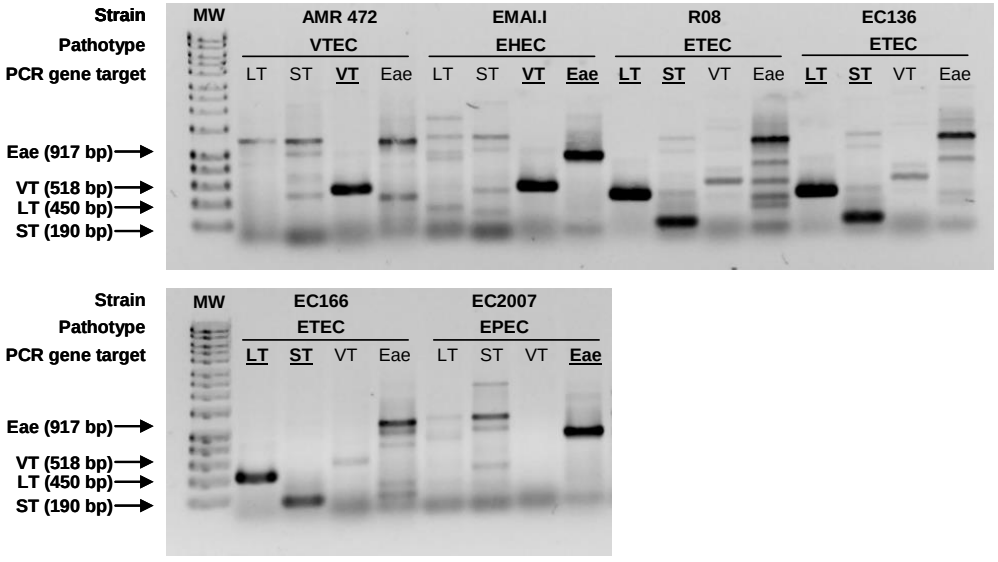

Figure 5 -Pathotype-specific PCRs for ETEC, EPEC, EHEC and VTEC. The primers originally designed by Aranda et al. (2007) for a multiplex assay were used in separate reactions (simplex PCRs) for LT, ST, VT and EaeA coding sequences. Positive PCR amplicons are indicated in bold underlined.

cloned separately into the pGEM-T vector and propagated in the *E. coli* K-12 laboratory strain. The plasmid vectors carrying each target sequences were purified and used to generate qPCR standard curves. The assays were validated using DNA extracts from the non pathogenic *E. coli* K12 or from the ETEC isolate

R08. The lower limit of detection per sample was approximately 2.10^3 K-12 target DNA copies (based on 16sRNA assay) and 800 to 90 target DNA copies of ETEC based on the F4, and STa and STb assays (Table 4).

To confirm these results, duplicate independent cultures of three ETEC reference strains, R08, EC136 and EC166 were harvested, titrated and used to prepare total genomic DNAs that served as templates for the PCR assays after serial dilutions. The range of genome copies per PCR and the approximate level of detection of the assays is detailed in Table 5, confirming that the F4 and STa/STb qPCR assays for ETEC detection are robust and sensitive.

Table 4 - Characterization of ETEC qPCRs using purified plasmid preparations of cloned target genes

Assay	Template	ng/ul	size (bp)	Target molecules/ul	Limit of detection per assay	melt temp
16S RNA	E coli R08	4.6	>1Mbp	4.E+06	2.E+03 copies	-
LT	plasmid LT	46	3510	1.E+10	6.E+05 copies	83.8
STa	plasmid STa	68	3144	2.E+10	1.E+02 copies	79
STb	plasmid STb	68	3172	2.E+10	1.E+02 copies	79
F4	plasmid F4	64	3696	2.E+10	8.E+02 copies	88.7

Table 5 - Validation of real time Q-PCR assays for STa, STb and F4 using independent ETEC cultures.

PCR assay	Strain	Number of genome copies per PCR assay		
		First dilution tested	Last dilution tested	Limit of detection
STa	R08 – A	$7\ 10^6$	$7\ 10^{-3}$	$7\ 10^3$
	R08 - B	$1\ 10^7$	$1\ 10^{-2}$	$1\ 10^3$
STb	EC166 - A	$6\ 10^6$	$6\ 10^{-3}$	$6\ 10^2$
	EC166 - B	$7\ 10^6$	$7\ 10^{-3}$	$7\ 10^2$
F4	EC136 - A	$2\ 10^6$	$2\ 10^{-3}$	$2\ 10^2$
	EC136 - B	$4\ 10^6$	$4\ 10^{-3}$	$4\ 10^2$

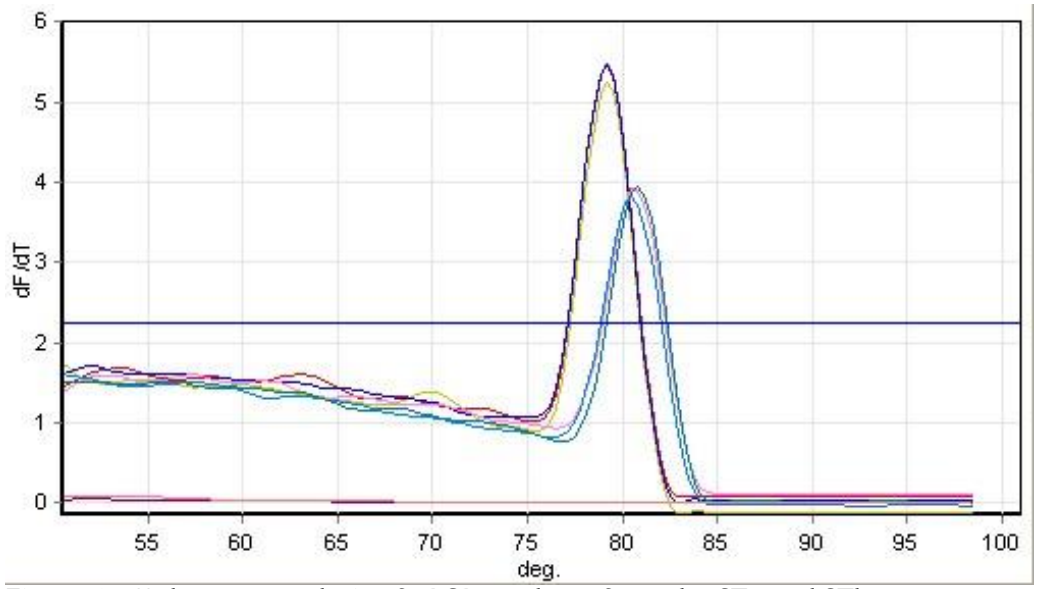

Figure 6 - Melt curve analysis of qPCR products from the STa and STb assays

No.	Colour	Name	Peak 1	No.	Colour	Name	Peak 1
1		STa EC136	79.2	5		STb EC136	80.7
2		STa EC166	79.2	6		STb EC166	80.5
3		STa R08	79.2	7		STb R08	80.8
4		STa Water		8		STb Water	

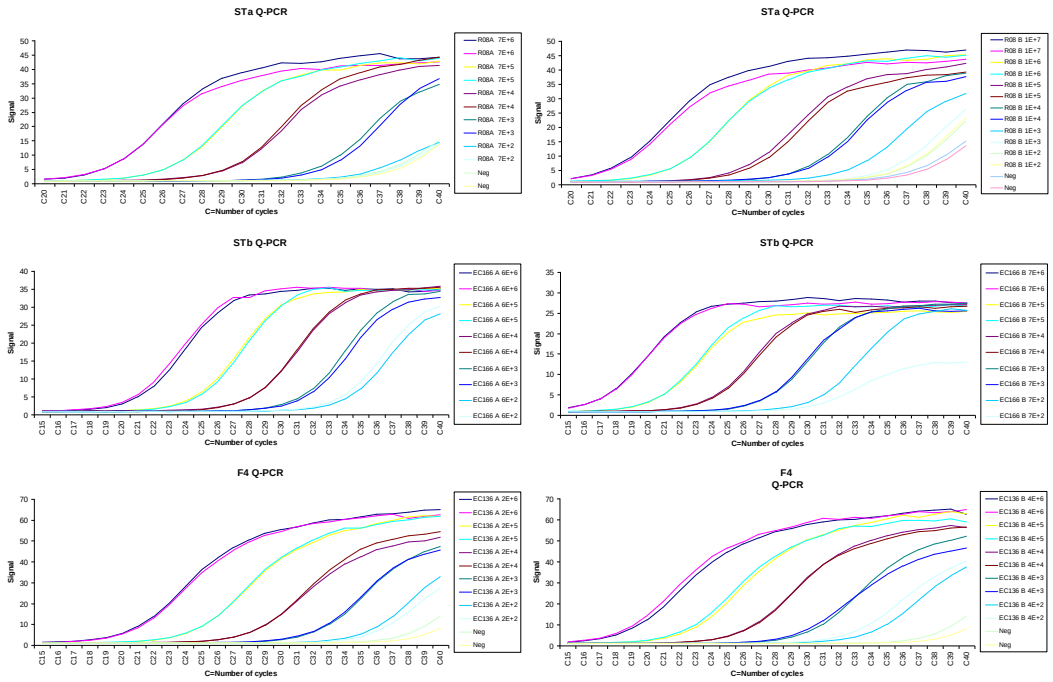

Figure 7 - Real Time qPCR analysis of serial dilution of ETEC genomic DNA prepared from known cultures. The PCR targets are indicated above each graph. Each assay was run in duplicate.

Melt curve analysis was performed on the PCR products amplified from three

independent ETEC strains with the STa and STb assays (Figure 8), showing a good ability of the test to differentiate each toxin gene and contribute to the determination of the genotype of a ETEC strain. Examples of qPCR standard curve data are presented in Figure 9, showing the excellent quantitative detection of the ETEC genomic DNA over a range of template dilutions. All PCRs were highly reproducible and corellated strongly with the titres of the cultures used to prepare the genomic DNA templates. Melting curve analyses were performed for each PCR product and in each case generated a single peak, indicative of a single specific PCR amplicon. Overall, the F4 adhesin qPCR was found to be the most sensitive with a limit of detection close to 100 organisms per assay

The F4 adhesin Q-PCR was also used to screen 120 fecal samples collected by Dr Alison Collins (DPI, NSW) to confirm observations obtained with other quantifying assays based on 16S RNA Q PCR and conventional microbiology diring investigation of ratios of commensal to pathogenic bacteria in sucker and weaner pig faeces. The analysis of these results is coordinated by Dr Collins.

APP

A real time quantitative two step PCR assay was developed in this study for the rapid detection of APP from air samples collected from a commercial farming environment. The primers ApxIV-for and ApxIV-rev (see Tables 2 and 3) were used. These primers were designed to anneal within a DNA fragment of the RX toxin Apx-IV that can also be amplified by a previously published conventional nested PCR assay (Figure 10).

The APP qPCR amplifies a 116 bp fragment of the *apxIV* gene present in all serotypes of APP and highly specific for the organism. A positive control for the

Figure 8 - APP-specific qPCR primers used in this study (asterisk) and relative position of published primers in the apxIV gene sequence.

quantification of APP was made by cloning the 116 bp target PCR product amplified from the isolate HS 3718 serotype 15 (Dr Pat Blackall, The University of Queensland) into the E. coli vector PGEMT (Promega). The plasmid was purified, quantified and used to create standard titration curves. The amplicon was confirmed by sequencing. The limit of detection of the qPCR assay was estimated at 10 genome copies. The assay was validated with pure cultures of APP strains 3617, 3064 (serotype 1), 2794 (serotype 5), 3659 (serotype 7), and 3718 (serotype 15). All strains gave an amplicon of the expected size (Figure 11). This assay was also able to detect airborne APP in two consecutive series of pig farm samples (see below).

3.3. Analysis of air quality in a pig farm.

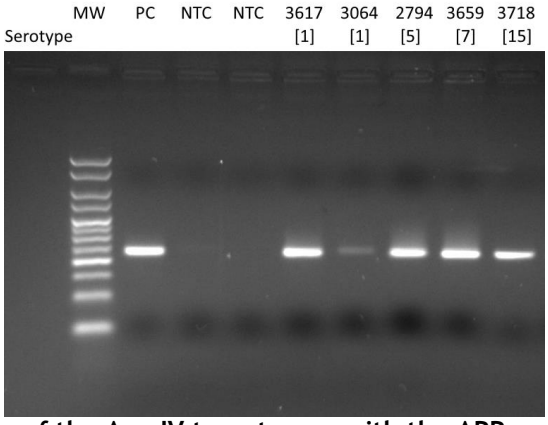

Figure 9 - Amplification of the Apx IV target gene with the APP-specific pimers ApxIV-for and ApxIV-rev. MW: DNA standard; PC: positive (cloned plasmid) control; NTC: negative (no template) control. Serotypes are indicated in brackets.

To test the methodology in field conditions, a first round of air sampling was performed in a selected pig farm near Bendigo, Victoria in December 2013, and several follow up sampling were performed in 2014. The detailed reports are provided in the appendix section. Two sites (farrowing and weaner sheds), six age groups (1 and 21 days, 3, 6, 8 and 12 weeks) and three sampling points in the room (entrance, central area, extraction fans) were tested. Selective and non selective solid media were inoculated and colonies were counted on duplicate plates by visual inspection and with a dedicated open source image treatment software (open CFU). The automatic enumeration method by image analysis was found satisfactory and practical. The log-transformed values of the bacterial colony counts were in the order of 4 Log10 cfu per cubic meter of air in both the farrowing facilities and the weaner sheds, confirming that high levels of air contamination are expected for this type of environment.

Selective plates indicated the presence of numerous colonies of Gram positives organisms (including *Staphylococcus* species on MSA) and some Gram negative organisms (mainly *Escherichia coli* and other coliforms on MAC). Antimicrobial resistance profiles were also assessed on randomly picked colonies, showing the presence of complex multiple resistance profiles (see appendixes).

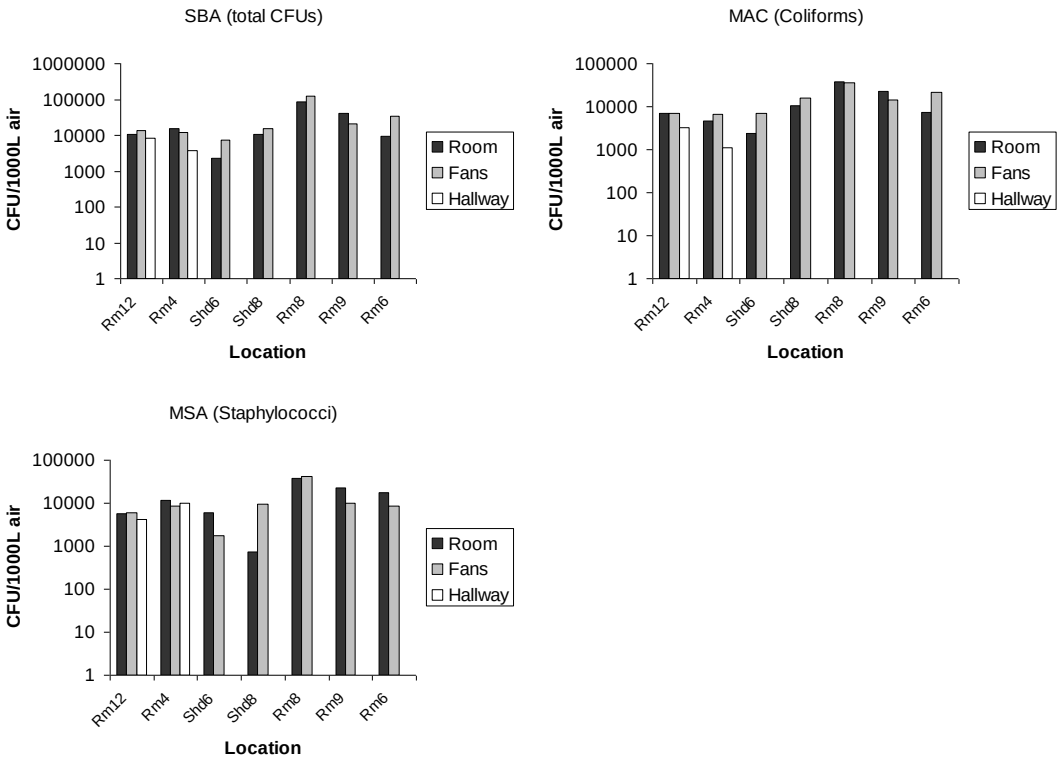

Figure 10 - Comparative influence of the air sampler position in a room on the measured concentration of microorganisms in the air.

Positioning the air sampler within a room, either next to the extraction fans or at the centre of the room gave similar numbers of airborne microorganisms, while air collected outside a room in the entrance hallway gave slightly lower titers (Figure 12). Sampling the air by the air fans or from the middle point of a room appears therefore to be a reliable and practical collection method during a farm visit

The concentration of airborne bacteria in the farrowing room did not vary significantly with the age of the suckling piglets (1 or 21 days old); however the titers increased noticeably with the age of the pigs in weaner sheds (Figure 13) and with the number of sows present in a farrowing room (Figure 14). Interestingly, a room tested few hours after being emptied and cleaned (Figure 14, point 0) still showed relatively high concentrations of microorganisms, albeit

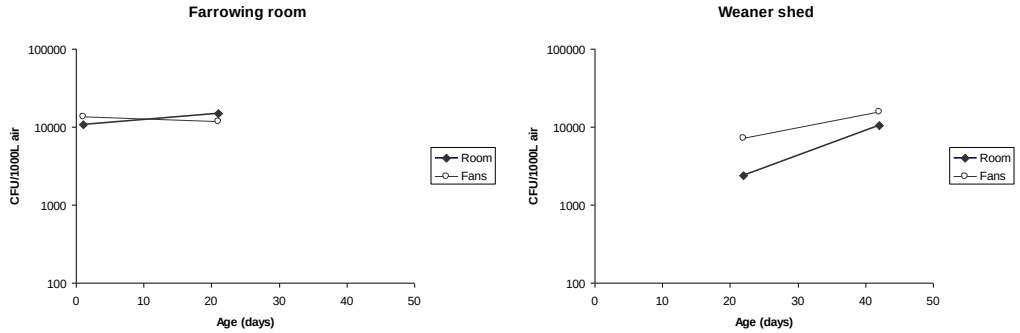

Figure 11 - relationship between the age groups and the concentration of airborne bacteria

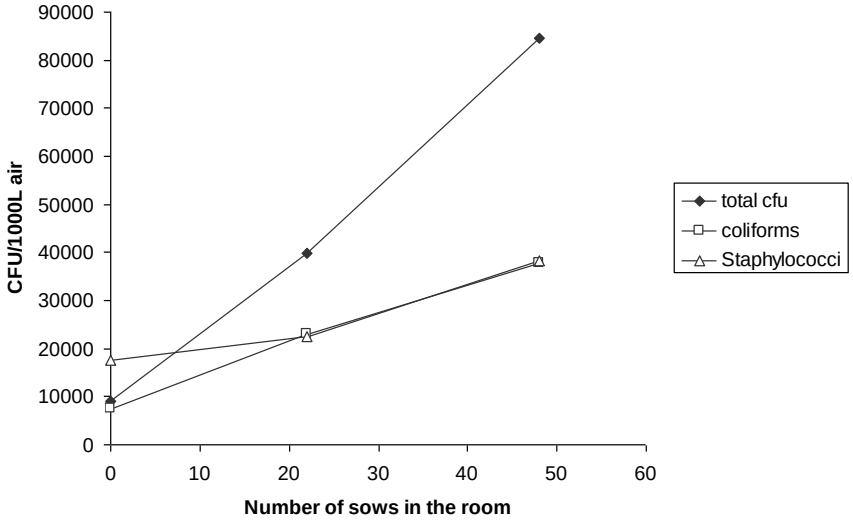

Figure 12 - relationship between the number of sows in a room and the concentration of airborne bacteria.

lower than a similar room where animals are present.

At the first visit, end-point PCR assays were performed on selected air samples, for the detection of *Mycoplasma hyopneumoniae*, *Haemophilus parasuis* and *Streptococcus suis*. All results were negative. Real time quantitative PCRs were performed for the detection of *Actinobacillus pleuropneumoniae* (APP) and Enterotoxinogenic *Escherichia coli* (ETEC). All air samples were PCR-negative for APP but one air sample was positive for two ETEC-specific PCRs, namely the F4 adhesin and the toxin STa. Colonies of *E. coli* purified from the selective plates were directly tested for the F4 adhesin by PCR and were also positive, confirming the presence of ETEC in the air of the piggery.

The farm site was revisited in 2014 for further validation of the sampling protocols. During two consecutive visits, the APP qPCR gave low level positive signals in a total of 11 air samples collected in triplicate per room (or in duplicate for one room) from two groups of pigs. The qPCR signals were above the limit of detection of approximately 10 genome copies per assay (i.e. in the order of 10^3 cfu/ cubic meter of air) for all samples. No increase in APP titers was noticed at the second visit four weeks later, when the same groups of pigs were re-tested. This suggests that although no outbreak of APP infection occurred on this occasion, a background circulation of a low-pathogenic strain was present. The PCR product was sequenced and confirmed by sequence alignment to match the expected portion of the apxIV gene targeted by the assay. Unfortunately it was not possible to reisolate a live APP culture from the plates, due to the high level of airborne contaminants overgrowing on the plates and the lack of selective media for this organism. In the absence of pure culture it is not possible to ascertain the serotype of the strain(s) found in the farm. (NB: the PCR reaction used on the air samples was developed for APP detection only and does not differentiate between serovars, as the ApxIV coding sequence is expected to be present in all APP strains). Nevertheless these encouraging results demonstrate the usefulness of the methodology for APP detection.

4. Application of Research

The air sampling methodology was shown to be a simple, robust and versatile approach to monitor infectious risks in pig farms. It is possible to determine the level of airborne microbial contamination in air samples with a good accuracy and repeatability. It is also possible to interpolate or extrapolate the measured cfu counts, either by varying the volume of air sampled at the collection time or by adjusting the dilution of the capture buffer when plated onto solid media, in order to obtain statistically significant numbers of CFUs counts on solid media. If low levels of airborne organisms are expected, the samples can be concentrated by centrifugation to maximize the recovery of organisms. The same samples can be analyzed by conventional culture using a range of bacteriological media with various selection properties, or directly by PCR assays specific for a species or

Table 6 - tool kit for detection of swine pathogens by PCR assays: summary of the available protocols tested in our laboratory.

Organism	Target sequence	type of assay	Positive control	Validated	Limit of detect.	tested in pig farm	remarks
Streptococcus suis	Capsule	PCR	Not available	no	ND	yes	
Haemophilus parasuis	16S RNA	PCR	Not available	no	ND	yes	
Actinobacillus pleuropneumoniae	RTX toxin Apx IV A	PCR	HS 3617	no	N/A	no	nested PCR
Actinobacillus pleuropneumoniae	RTX toxin Apx IV A	qPCR	HS 3617	yes	10 cfu	yes	direct detection in air
Mycoplasma hyopneumoniae	16S RNA	PCR	LKR	yes	ND	yes	nested PCR
ETEC	ST_a toxin	qPCR	R08 (LT Stab F4)	yes	100 cfu	yes	qPCR with melting curve analysis
ETEC	ST_b toxin	qPCR	R08 (LT Stab F4)	yes	100 cfu	yes	qPCR with melting curve analysis
ETEC	LT toxin	qPCR	R08 (LT Stab F4)	yes	ND	yes	
ETEC	F4 adhesin	qPCR	R08 (LT Stab F4)	yes	100 cfu	yes	qPCR with melting curve analysis. Best limit of detection.
ETEC	F5 adhesin	PCR	Not available	no	ND	no	
ETEC	F6 adhesin	PCR	Not available	no	ND	no	
ETEC	F41 adhesin	PCR	Not available	no	ND	no	
ETEC	F18 adhesin	PCR	Not available	no	ND	no	
EPEC	eae	Multiplex PCR	EMA1.1 (O157 stx eaeA)	yes	ND	no	tested only in simplex PCR.
EPEC	bfpA	Multiplex PCR		no	ND	no	not tested
EHEC/VTEC	stx1/stx2	Multiplex PCR	AMR472 (stx2e)	yes	ND	no	tested only in simplex PCR.
EIEC	ipaH	Multiplex PCR	?	no	ND	no	not tested
EAgEC	aggR	Multiplex PCR	?	no	ND	no	not tested
ETEC (alt.)	est	Multiplex PCR	R08 (LT Stab F4), EC136, EC166	yes	ND	no	tested only in simplex PCR.
ETEC (alt.)	elt	Multiplex PCR	R08 (LT Stab F4), EC136, EC167	yes	ND	no	tested only in simplex PCR.

even a pathotype of micro-organism. The molecular assays validated in this study allow the quantitative detection of the target micro-organisms in heavily contaminated samples, without the need for bacterial culture.

Our results provide a preliminary proof of concept for the detection of airborne ETEC and APP. We have also developed PCR detection assays for three other bacterial pathogens of significant importance for the Pork industry (*M. hyopneumoniae*, *H. parasuis*, *S. suis*) that could complete a molecular "toolkit" for the monitoring of major infectious risks in the piggery. The list of PCR assays included in this toolkit is summarized in Table 6. When coupled with specific PCR assays, air sampling can become a powerful and rapid detection system to help detect or quantify pathogens of interest for the pig industry without the need for time consuming bacteriological culture.

Finally the air sampling methodology can be applied to research projects aiming at evaluating the performance of pigs in genetic selection programs by providing regularly some background quantitative data on the air hygiene of the testing facility over time.

5. Conclusion

This project represents a proof-of-concept study on the feasibility of air sampling in farm for the quantitative detection of microorganisms. At this stage we did not identified any major issue with the methodology. The range of applications for air sampling is quite extensive, from the general management of air hygiene, identification of ventilation defects in buildings, to the surveillance of specific pathogens. The assays co-developed with the air sampling protocol can be tailored to the specific epidemiological situation of a pig farm, where one pathogen might be causing most of the problems, or to a general purpose surveillance program for the early detection of infectious agents relevant for the pork industry. Finally the methodology can contribute to various research and development projects to evaluate the impact of air hygiene on the performance of pigs in pork genetics selection schemes or to improve animal welfare.

6. Limitations/Risks

The main limitation of the methodology is the difficulty, or even the impossibility of reisolating specific microorganisms in the absence of selective media, in particular when the air samples are heavily contaminated by ambient airborne non significant microorganisms. This can be alleviated by using PCR or other molecular assays directly on the sample. Unfortunately at the moment some specific applications such as serotyping still require the isolation of pure cultures, which can be obtained more readily from clinically infected animals or post-mortem specimens. However it is reasonable to expect that serotype-specific PCR assays adapted to air samples could be developed to address this problem in the future.

7. Recommendations

We recommend the use of regularly scheduled air sampling programs in piggeries, coupled with (1) specific qPCRs for the detection of pathogens and (2) concurrent conventional microbiology analysis, to establish baseline data on infectious risk and air hygiene in farm conditions. Air samples can be collected next to the extraction fans in ventilated rooms, or in the middle of the room in open sheds with minimal time and effort. Samples kept at 4C can be sent to the laboratory by courier or frozen if PCR assays are only to be used. The analyses developed in this project could be performed as a fee for service in specialized diagnostics laboratories under GLP at a reasonable cost.

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9. Appendices: Complete reports on air analysis in a pig farm

9.1. Appendix 1: Air analysis report 16/12/2013

Air analysis report

16th December 2013

Prepared by
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Procedure:

Girgarre and Bears Lagoon pig farms were visited by Anne Watt and Phil Markham in the presence of Ole Jensen on the 18th of November 2013.
Air samples were collected using a coriolis® air sampler with the following settings:

- Flow: 250L/min.
- Collection time: 10 min.
- Total volume of air collected: 2500L
- Recovery medium: 10 ml sterile Phosphate Buffered Saline (PBS), pH 7.4.

Air samples were collected from two rooms with in the farrowing facility at Girgarre and two weaner sheds at the Bears lagoon facility.
At Girgarre, room 12 contained piglets at one day of age while room 4 contained piglets of approximately 21 days old. Air samples were collected from approximately 120cm above the ground. For each room, air samples were collected:

- from the hallway outside the farrowing room,
- at the entrance of the room,
- at the extraction fans within the room.

At the Bears lagoon facility, shed 6 and shed 8 contained pigs aged 3 weeks and 6 weeks, respectively. Air samples were collected from approximately 60 cm above the ground. From each shed, air samples were collected:

- in the centre of the room,
- at the extraction fans within the room.

Samples were plated immediately after collection in the farm and after being transported at 4°C to the laboratory for 2.5 hours. For each sample, 0.1 ml of PBS sample were aseptically plated, in duplicate:

- on Sheep Blood Agar (SBA) for total bacterial count,
- on Mannitol Salt Agar (MSA) for detection of *Staphylococcus* species, and
- on Mac Conkey Agar (MCA) for detection of *Enterobaceriaceae*.

The plates were incubated at 37°C overnight and colony forming unit (CFU) were counted manually or with the software OpenCFU. The average CFU per sample was calculated for each sample. Bacterial titres in the air were expressed as average CFU per 25L of air, for each sample area.
Individual colonies were picked and purified for identification and for antimicrobial susceptibility testing by the Calibrated Dichotomous Susceptibility (CDS) method.
For each sample, 2 ml of collection medium was centrifugated at 12000g for 10 minutes to collect bacterial cells. The pellets were resuspended in 0.1 ml of sterile molecular grade water and incubated at 100C for 5 minutes to extract DNA.
Polymerase Chain Reaction assays were performed to detect the following pathogens*: Actinobacillus pleuropneumoniae, Mycoplasma hyopneumoniae,*

Haemophilus parasuis, *Streptococcus suis*, and Enterotoxinogenic *Escherichia coli*, using 5 microliters of DNA extracts as a template. The protocols for these PCR assays are currently under validation in the APCAH laboratory.

Results:

Total bacterial counts on SBA

No significant difference was observed between the plates inoculated directly in the farm and the plates inoculated after transport to the laboratory.
Growth on SBA indicated the presence of various organisms, with a majority of Gram positive bacteria, including suspect *Staphylococcus*, *Micrococcus*, *Streptococcus* and *Enterococcus* species. Representative organisms were picked and purified, based on colony morphology, Gram staining and biochemical tests. Species identification was performed by carbohydrate fermentation tests and API rapid ID kits. Final identification results are pending.
Results are summarised in Tables 1 and 2.

Girgarre: Table 1: Colony forming unit growth on sheep blood agar (SBA) per 25 L of air

Location	Age Group	Sample area	Average CFU/25L air
Room 12	**1 Day old**	Hall way	213
	1 Day old	Within room	267
	1 Day old	By Extraction fans	343
Room 4	**21 Days old**	Hall way	94
	21 Days old	Within room	375
	21 Days old	By Extraction fans	295

Bears Lagoon: Table 2: Colony forming unit growth on sheep blood agar (SBA) per 25 L of air

Location	Age Group	Sample area	Average CFU/25L air
Shed 6	**3 Weeks**	Centre of room	59
	3 Weeks	By extraction fans	179
Shed 8	**6 Weeks**	Centre of room	265
	6 Weeks	By extraction fans	395

Total bacterial counts on MAC

All samples indicated the presence of various non-*Enterobacteriaceae* organisms on MAC, including suspect *Staphylococcus* species.
Total CFU counts are summarised in Tables 3 and 4.
Few samples indicated the presence of coliforms. Five air samples (Room 12 Hall way, Room 4 hall way, within room and extraction fan, Shed 6 extraction fan) indicated the presence of suspect *E. coli* in low to moderate numbers (1-10 CFU/25L). Presumptive identification of *E. coli* was based on colony morphology and lactose fermentation. Representative suspect *E. coli* colonies were picked and purified for further identification and antibiogram.
Non-lactose fermenting *Enterobacteriaceae* (e.g. Salmonella sp.) were not detected.

Girgarre: Table 3: Total colony forming unit growth on Mac Conkey agar (MAC) per 25 L of air; ND: not detected.

Location	Age Group	Sample area	Average CFU/25L air	Suspect *E. coli*
Room 12	**1 Day old**	Hall way	80	Yes
	1 Day old	Within room	171	ND
	1 Day old	By Extraction fans	173	ND
Room 4	**21 Days old**	Hall way	28	Yes
	21 Days old	Within room	115	Yes
	21 Days old	By Extraction fans	166	Yes

Bears Lagoon: Table 4: Total colony forming unit growth on Mac Conkey agar (MAC) per 25 L of air; ND: not detected.

Location	Age Group	Sample area	Average CFU/25L air	Suspect *E. coli*
Shed 6	**3 Weeks**	Centre of room	59	ND
	3 Weeks	By extraction fans	179	Yes
Shed 8	**6 Weeks**	Centre of room	265	ND
	6 Weeks	By extraction fans	395	ND

Total *Staphylococcus* sp. counts on MSA

All samples indicated the presence of suspect *Staphylococcus aureus* as well as non *S. aureus* colonies. Presumptive identification was based on growth on the selective medium MSA, colony morphology and Mannitol fermentation.
Total CFU counts are summarised in Tables 5 and 6.

Girgarre: Table 5: Total colony forming unit growth on mannitol salt agar (MSA) per 25 L of air

Location	Age Group	Sample area	Average CFU/25L air
Room 12	**1 Day old**	Hall way	106
	1 Day old	Within room	140
	1 Day old	By Extraction fans	152
Room 4	**21 Days old**	Hall way	250
	21 Days old	Within room	287
	21 Days old	By Extraction fans	219

Bears Lagoon: Table 6: Total colony forming unit growth on mannitol salt agar (MSA) per 25 L of air

Location	Age Group	Sample area	Average CFU/25L air
Shed 6	**3 Weeks**	Centre of room	150
	3 Weeks	By extraction fans	44
Shed 8	**6 Weeks**	Centre of room	18
	6 Weeks	By extraction fans	238

Identification of *Streptococcus* species.

Representative colonies of suspect *Streptococcus* and *Enterococcus* sp. were picked from the SBA plates (extraction fan samples from 3 and 6 week weaners) and were sub-cultured on SBA. The orientation diagnostic at the genus level was based on the colony morphology, Gram staining and catalase test. No *Streptococcus suis* was detected. *Enterococcus durans* was identified. Other organisms were identified at the genus level only as *Streptococcus* sp. Further identification at the species level by 16S RNA sequencing is underway.

Identification of *Staphylococcus* species.

Suspect *Staphylococcus sp*. colonies were picked from the SBA plates (extraction fan samples from all age groups) and were sub-cultured on SBA and MSA. The orientation diagnostic for the genus *Staphylococcus* was based on the colony morphology, Gram staining and catalase test. Purified *S. aureus* isolates were identified based on colony morphology on MSA and a species-specific rapid agglutination diagnostic kit.

Antimicrobial sensitivity

E. coli: Two representative colonies (extraction fan samples from 1 day old and 3 week weaners) were picked and tested by the CDS method. All organisms displayed the same antimicrobial resistance profile.
S.aureus: Three representative colonies were picked and tested by the CDS method. Methicillin resistance was tested by sub-culture on Oxacillin-MSA plates. Each organism displayed some differences in their resistance profiles. All organisms were Methicillin sensitive.

Results are summarised in table 7A-7D.

Table 7A: CDS results for *E. coli*

Organism	Antimicrobial	Disc Potency	Result
E. coli	**Ampicillin**	25	Sensitive
	Tetracycline	30	Resistant
Shed 3	**Gentamicin**	10	Sensitive
	Trimetoprim	5	Resistant
	Sulfafurazole	300	Resistant
	Enrofloxacin	5	Sensitive
	Ceftiofur	30	Sensitive

Table 7B: CDS results for *S. aureus*

Organism	Antimicrobial	Disc Potency	Result
S. aureus	**Penicillin**	05U	Resistant
	Tetracycline	30	Sensitive
Org. # 1	**Cefoxitin**	10	Sensitive
	Trimetoprim	5	Sensitive
	Sulfafurazole	300	Sensitive
	Erythromycin	5	Resistant
	Ceftiofur	30	Sensitive

Table 7C: CDS results for *S. aureus*

Organism	Antimicrobial	Disc Potency	Result
S. aureus	**Penicillin**	05U	Resistant
	Tetracycline	30	Resistant
Org. # 8	**Cefoxitin**	10	Sensitive
	Trimetoprim	5	Resistant
	Sulfafurazole	300	Sensitive
	Erythromycin	5	Sensitive
	Ceftiofur	30	Sensitive

Table 7D: CDS results for *S. aureus*

Organism	Antimicrobial	Disc Potency	Result
S. aureus	**Penicillin**	05U	Resistant
	Tetracycline	30	Resistant
Org. # 9	**Cefoxitin**	10	Sensitive
	Trimetoprim	5	Resistant
	Sulfafurazole	300	Sensitive
	Erythromycin	5	Resistant
	Ceftiofur	30	Sensitive

PCR results

The whole air samples were tested directly for the presence of specific pathogens by PCR assays under development or validation in our laboratory. The air from the three week old weaner group (Bears Lagoon, Shed 6) was positive for the enterotoxigenic *E.coli* (ETEC) specific adhesin gene F4 and toxin genes STa and STb. Suspect *E. coli* colonies could not be detected on the corresponding MAC plates. Replating the same sample gave ambiguous results because of abundant contaminant flora. Tentative PCR results are given in Table 8. Other sample areas were negative for ETEC-specific PCRs.

Table 8: Polymerase chain reaction (PCR) results for a range of pig pathogens.

PCR assay	Positive control	Air sample result
Actinobacillus pleuropneumoniae	HS 3617	All samples negative
Mycoplasma hyopneumoniae	LKR	All samples negative
Haemophilus parasuis	Not available	All samples negative

Streptococcus suis	Underway	Inconclusive
Enterotoxinogenic *Escherichia coli*	Strain R08; Genotype F4, Lt, STa, STb	Shed 6 Bears Lagoon: ETEC (F4, STa, STb) positive

Several *E. coli* purified from the primary MAC plates are currently individually tested by the same ETEC-specific PCR assays in an attempt to reisolate the same strain.

9.2. Appendix 2: Air analysis report 31/3/2014

Air analysis report

31st March 2014

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Procedure

Girgarre pig farm was visited by Anne Watt and Brendan Kehoe on the 20th of March 2014.
Air samples were collected using a coriolis® air sampler with the following settings:

- Flow 300L/min
- Collection time 10 min
- Total volume of air collected 3000L
- Recover medium: 10 ml sterile Phosphate Buffered Saline (PBS), pH 7.4.

Air samples were collected from three rooms at the farrowing facility. Room 8 containing 48 sows with full litters between 15-20 days old, room 9 containing 22 sows with full litters 15-20 days old and room 6 a room that had been emptied cleaned and disinfected that morning. For each room samples were collected:

- At the entrance of the rooms,
- At the extraction fans within the room.

Samples were immediately stored at 4°C and transported back to the laboratory for plating. For each sample, 0.1ml of PBS sample were aseptically plated in duplicate

- On Sheep Blood Agar (SBA) for total bacterial count,
- On Mannitol Salt Agar (MSA) for detection of *Staphylococcus* species, and
- On Mac Conkey Agar (MAC) for detection of *Enterobaceriaceae*

The plates were incubated at 37°C overnight and colony forming unit (CFU) were counted manually or with the software Open CFU. The average CFU per sample was calculated. Bacterial titres in the air were then expresses as average CFU per 30L of air, for each sample area.
Individual colonies were picked and purified for identification and for antimicrobial susceptibility testing by the Calibrated Dichotomous Susceptibility (CDS) method. For each sample, 2 ml of collection medium was centrifugated at 12000g for 10 minutes to collect bacterial cells. The pellets were resuspended in 0.1 ml of sterile molecular grade water and incubated at 100°C for 5 minutes to extract DNA. Polymerase Chain Reaction (PCR) assays were performed to detect the following pathogens: *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, *Haemophilus parasuis*, *Streptococcus suis*, and Enterotoxinogenic *Escherichia coli*, using 5 microliters of DNA extracts as a template. The protocols for these PCR assays are currently under validation in the APCAH laboratory.

Results

Total bacterial counts on SBA

Growth on SBA indicated the presence of various organisms, with a majority of Gram positive bacteria, including suspect *Staphylococcus*, *Micrococcus*, *Streptococcus* and *Enterococcus* species. Representative organisms were picked and purified, based on colony morphology, Gram staining and biochemical tests. Results are summarised in Table 1.

Table 1: Colony forming unit growth on sheep blood agar (SBA) per 30 L of air

Location	No. sows	Sample area	Average CFU/30L air

Room 8	48	Within Room	2540
		By Extraction fan	3635
Room 9	22	Within Room	1195
		By Extraction fan	620
Room 6	0	Within Room	274
		By Extraction fan	1045

Total bacterial count on MAC

All samples indicated the presence of various non-*Enterobacteriaceae* organisms on MAC, including suspect *Staphylococcus* species.
Total CFU counts are summarised in table 2.
Few samples indicated the presence of coliforms 2. Samples indicated the presence of suspect *E. coli* in low numbers (1-10 CFU/30L). Presumptive identification of *E. coli* was based on colony morphology and lactose fermentation. Representative suspect *E. coli* colonies were picked and purified for further identification and entibiogram.
Non-lactose fermenting *Enterobacteriaseae* (e.g. Salmonella sp.) were not detected.

Table 2: Colony forming unit growth on Mac Conkey agar (MAC) per 30 L of air; ND not detected.

Location	No. sows	Sample area	Average CFU/30L air	Suspect *E. coli*
Room 8	48	Within Room	1130	2
		By Extraction fan	1072	2
Room 9	22	Within Room	692	ND
		By Extraction fan	423	ND
Room 6	0	Within Room	225	ND
		By Extraction fan	655	ND

Total *Staphylococcus* sp. counts on MSA

All samples indicated the presence of suspect *Staphylococcus aureus* as well as non *S. aureus* colonies. Presumptive identification was based on growth on the selective medium MSA, colony morphology and Mannitol fermentation.
Total CFU counts are summarised in Table 3

Table 3: Colony forming unit growth on Mannitol Salt Agar (MSA) per 30 L of air.

Location	No. sows	Sample area	Average CFU/30L air	Suspect *S. aureus*
Room 8	48	Within Room	1151	10
		By Extraction fan	1280	12
Room 9	22	Within Room	675	5
		By Extraction fan	302	5
Room 6	0	Within Room	526	4
		By Extraction fan	260	2

Identification of *Streptococcus* species.

Representative colonies of suspect *Streptococcus* and *Enterococcus* sp. were picked from the SBA plates and sub-cultured on SBA. The orientation diagnostic at the

genus level was based on the colony morphology, Gram staining and catalase test. No *Streptococcus suis* was detected.

Identification of *Staphylococcus* species.

Suspect *Staphylococcus* sp. Colonies were picked from the SBA plates and were sub-cultured onto SBA. The orientation diagnostic for the genus *Staphylococcus* was based on the colony morphology, Gram stain and catalase testing. *Staphylococcus aureus* isolates were identified based on colony morphology on MSA and a species-specific rapid agglutination diagnostic kit.

Antimicrobial sensitivity

Staphylococcus aureus isolated from each room were tested for antimicrobial sensitivity and all showed the same pattern of resistance.

Antimicrobial	Disk Potency	Result
Penicillin	5U	Resistant
Tetracycline	30	Sensitive
Oxacillin	10	Sensitive
Trimetoprim	5	Sensitive
Sulfafurazole	300	Sensitive
Erythromycin	15	Resistant
Ceftiofur	30	Sensitive

PCR results

The whole air samples were tested directily for the presence of pathogens by PCR assays under development or validation in our laboratory. All samples were negative

PCR assay	Positive control	Air sample result
Actinobacillus pleuropneumoniae	HS 3618	All samples negative
Mycoplasma hyopneumoniae	LKR	All samples negative
Haemophilus parasuis	Not available	All samples negative
Streptococcus suis	Underway	All samples negative
Enterotoxinogenic *Escherichia coli*	Strain R08; Genotype F4, LT, STa, STb	All samples negative

9.3. Appendix 3: Air analysis report 8/4/2014

Air analysis report

8th April 2014

Prepared by
Anne Watt, Dr Phil Markham and Dr Marc Marenda
Asia Pacific Centre for Animal Health, Faculty of Veterinary Science, The University of Melbourne
250 Princes Hwy Werribee 3030 Vic.

Procedure

Bears lagoon pig farm was visited by Anne Watt and Brendan Kehoe on the 8th of April 2014.
Air samples were collected using a coriolis® air sampler with the following settings:

- Flow 300L/min
- Collection time 10 min
- Total volume of air collected 3000L
- Recover medium: 10 ml sterile Phosphate Buffered Saline (PBS), pH 7.4.

Air samples were collected from two sheds housing 8 and 12 week old pigs. Two samples were collected from a shed containing 200 8 week old at a lower than average stocking density. Closed shed with pumped in ventilation. Coughing and sneezing was observed in the pigs. Three samples were collected from a shed containing 1300 12 week old pigs. A small amount of coughing was observed, shed allowed for natural air flow.
Samples were collected from each end of the shed and from the centre of the shed for the 12 week old group.
Samples were immediately stored at 4°C and transported back to the laboratory for plating. For each sample, 0.1ml of PBS sample were aseptically plated in duplicate

- On Sheep Blood Agar (SBA) for total bacterial count,
- On Mannitol Salt Agar (MSA) for detection of *Staphylococcus* species, and
- On Mac Conkey Agar + penicillin (MAC) for detection of *Enterobaceriaceae*

The plates were incubated at 37°C overnight and colony forming unit (CFU) were counted manually or with the software Open CFU. The average CFU per sample was calculated for each sample. Bacterial titres in the air were then expresses as average CFU per 30L of air, for each sample area.
For each sample, 2 ml of collection medium was centrifugated at 12000g for 10 minutes to collect bacterial cells. The pellets were resuspended in 0.1 ml of sterile molecular grade water and incubated at 100°C for 5 minutes to extract DNA. Polymerase Chain Reaction (PCR) assays were performed to detect the following pathogens: *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, *Haemophilus parasuis*, *Streptococcus suis*, and Enterotoxinogenic *Escherichia coli*, using 5 microliters of DNA extracts as a template. The protocols for these PCR assays are currently under validation in the APCAH laboratory.

Results

Total bacterial counts on SBA

Growth on SBA indicated the presence of various organisms, with a majority of Gram positive bacteria, including suspect *Staphylococcus*, *Micrococcus*, *Streptococcus* and *Enterococcus* species. Representative organisms were picked and purified, based on colony morphology, Gram staining and biochemical tests.

Results are summarised in Table 1.

Table 1: Colony forming unit growth on sheep blood agar (SBA) per 30 L of air.

Location	No. pigs	Average CFU/30L air
8 weeks	200	4192
12 weeks	1300	6283

Total bacterial count on MAC

All samples indicated the presence of various non-*Enterobacteriaceae* organisms on MAC.
Total CFU counts are summarised in table 2.
Few samples indicated the presence of coliforms . Samples indicated the presence of suspect *E. coli* in low numbers (1-10 CFU/30L). Presumptive identification of *E. coli* was based on colony morphology and lactose fermentation. Representative suspect *E. coli* colonies were picked and purified for further identification and entibiogram. Non-lactose fermenting *Enterobacteriaseae* (e.g. Salmonella sp.) were not detected.

Table 2: Colony forming unit growth on Mac Conkey agar with penicillin (MAC) per 30 L of air.

Location	No. pigs	Average CFU/30L air	Suspect E.coli
8 weeks	200	27	7
12 weeks	1300	39	4

Total *Staphylococcus* sp. counts on MSA

All samples indicated the presence of suspect *Staphylococcus aureus* as well as non *S. aureus* colonies. Presumptive identification was based on growth on the selective medium MSA, colony morphology and Mannitol fermentation.
Total CFU counts are summarised in Table 3

Table 3: Colony forming unit growth on Mannitol Salt Agar (MSA) per 30 L of air.

Location	No. pigs	Average CFU/30L air	Suspect aureus
8 weeks	200	208	15
12 weeks	1300	1248	85

Identification of *Streptococcus* species.

Representative colonies of suspect *Streptococcus* and *Enterococcus* sp. were picked from the SBA plates and sub-cultured on SBA. The orientation diagnostic at the genus level was based on the colony morphology, Gram staining and catalase test.
No *Streptococcus suis* was detected.

Identification of *Staphylococcus* species.

Suspect *Staphylococcus* sp. Colonies were picked from the SBA plates and were sub-cultured onto SBA. The orientation diagnostic for the genus *Staphylococcus* was based on the colony morphology, Gram stain and catalase testing.

Antimicrobial sensitivity

Staphylococcus aureus isolates showed the same pattern of antimicrobial sensitivity as that detected in the previous visit.

Antimicrobial	Disk Potency	Result
Penicillin	5U	Resistant
Tetracycline	30	Sensitive
Oxacillin	10	Sensitive
Trimetoprim	5	Sensitive
Sulfafurazole	300	Sensitive
Erythromycin	15	Resistant
Ceftiofur	30	Sensitive

PCR results

The whole air samples were tested directily for the presence of pathogens by PCR assays under development or validation in our laboratory. **All samples returned positive results for *Actinobacillus pleuropneumoniae* at low levels**. All other results were negative.

PCR assay	Positive control	Air sample result
Actinobacillus pleuropneumoniae	**HS 3618**	**All samples positive**
Mycoplasma hyopneumoniae	LKR	All samples negative
Haemophilus parasuis	Not available	All samples negative
Streptococcus suis	Underway	All samples negative
Enterotoxinogenic *Escherichia coli*	Strain R08; Genotype F4, Lt, STa, STb	All samples negative

9.4. Appendix 3: Air analysis report 14/5/2014

Air analysis report

14th May 2014

Prepared by
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Procedure

Bears lagoon pig farm was visited by Anne Watt and Marc Marenda on the 14th of May 2014.
Air samples were collected using a coriolis® air sampler with the following settings:

- Flow 300L/min
- Collection time 10 min
- Total volume of air collected 3000L
- Recover medium: 10 ml sterile Phosphate Buffered Saline (PBS), pH 7.4.

Air samples were collected from two sheds containing 16 and 18 week old pigs. Three samples were collected from a shed containing 1300 16 week old pigs in an open style shed. Two samples were collected from an open style shed containing 1044 18 week old pigs. These pigs had tested positive to *Actinobacillus pleuropneumoniae* in the previous farm visit. Samples were collected from each end of the sheds and from the centre of the shed for the 16 week old group.
Samples were immediately stored at 4°C and transported back to the laboratory for plating. For each sample, 0.1ml of PBS sample were aseptically plated in duplicate

- On Sheep Blood Agar (SBA) for total bacterial count,
- On Mannitol Salt Agar (MSA) for detection of *Staphylococcus* species, and
- On Mac Conkey Agar + penicillin (MAC) for detection of *Enterobaceriaceae*

The plates were incubated at 37°C overnight and colony forming unit (CFU) were counted manually or with the software Open CFU. The average CFU per sample was calculated for each sample. Bacterial titres in the air were then expresses as average CFU per 30L of air, for each sample area.
For each sample, 2 ml of collection medium was centrifugated at 12000g for 10 minutes to collect bacterial cells. The pellets were resuspended in 0.1 ml of sterile molecular grade water and incubated at 100°C for 5 minutes to extract DNA. Polymerase Chain Reaction (PCR) assays were performed to detect the following pathogens: *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, *Haemophilus parasuis, Streptococcus suis*, and Enterotoxinogenic *Escherichia coli*, using 5 microliters of DNA extracts as a template. The protocols for these PCR assays are currently under validation in the APCAH laboratory.

Results

Total bacterial counts

Due to contamination of the collection media, bacterial counts and analysis of these samples cannot be determined.

PCR results

The whole air samples were tested directily for the presence of pathogens by PCR assays under development or validation in our laboratory. **All samples returned positive results for *Actinobacillus pleuropneumoniae* at low levels.** All other results were negative.

PCR assay	Positive control	Air sample result

Actinobacillus pleuropneumoniae	**HS 3618**	**All samples positive**
Mycoplasma hyopneumoniae	LKR	All samples negative
Haemophilus parasuis	Not available	All samples negative
Streptococcus suis	Underway	All samples negative
Enterotoxinogenic *Escherichia coli*	Strain R08; Genotype F4, Lt, STa, STb	All samples negative