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Contents

1 Acknowledgments 5
2 Executive summary 6
3 Background 7
4 Objectives 9
5 Methodology 10
5.1 Objective 1. Identify technical gaps in field outbreak investigation and surveillance, service and reference laboratory systems and technologies 10
Objective 2. Develop cost-effective laboratory tests for bacteria and viruses associated with porcine respiratory diseases in both countries. 11
Objective 3. Implement systems that apply current technology and new tests developed in this project (Objective 2) to determine causes of respiratory disease outbreaks in the Philippines and Australia. 15
Objective 4. Establish novel mechanisms for communication among industry, government and research institutions in the Philippines on pig health 18
6 Achievements against activities and outputs/milestones 19
Objective 1: To identify technical gaps in field outbreak investigation and surveillance, service and reference laboratory systems and technologies. 19
Objective 2: To develop cost-effective laboratory tests for bacteria and viruses relevant to both countries 21
Objective 3: To implement systems that apply current technology and new tests developed in this project (Objective 2) to determine causes of respiratory disease outbreaks in the Philippines and Australia. 23
Objective 4: To establish novel mechanisms for communication among industry, government and research institutions in the Philippines on pig health 25
7 Key results and discussion 27
Objective 1. Identify technical gaps in field outbreak investigation and surveillance, service and reference laboratory systems and technologies. 27
Objective 2. Develop cost-effective laboratory tests for bacteria and viruses associated with porcine respiratory diseases in both countries. 30
Objective 3. Implement systems that apply current technology and new tests developed in this project (Objective 2) to determine causes of respiratory disease outbreaks in the Philippines and Australia. 42
Objective 4: To establish novel mechanisms for communication among industry, government and research institutions in the Philippines on pig health 64
8 Impacts 68
Scientific impacts – now and in 5 years 68
Capacity impacts – now and in 5 years 69
Community impacts – now and in 5 years 70
Communication and dissemination activities 73
9 Conclusions and recommendations 75
Conclusions 75
Recommendations 76
10 References 77
References cited in report 77
List of publications produced by project 82

11 Appendixes 84
11.1 Appendix 1: Detailed laboratory protocol for the quantification of Porcine Circo-Virus Type 2 84
11.2 Appendix 2: Multiplex polymerase chain reaction assay to identify and serotype isolates of *Actinobacillus pleuropneumoniae* of serovars 1, 5, 7, 12 and 15 89
Appendix 11.3: Details of the five virulence-associated polymerase chain reaction assays for *Haemophilus parasuis* 91
Appendix 11.4: Details of the multi-locus sequence typing technique for *H. parasuis* 93
Appendix 11.5: Details of design, questionnaire, data management and analysis for the cross-sectional study 95
Appendix 11.6: Details of study protocol for the validation study 98
Appendix 11.7: Details of two typical standard operating procedures – Spot indole test and the complete growth medium (BA/SN) 108
Appendix 11.8: Typical identification table for certain pathogenic bacteria associated with pig respiratory diseases. 111
Appendix 11.9: List for Reference Quality Control Strains 112
Appendix 11.10: Examples of New Protocols and Procedures for Disease Investigations 114
Appendix 11.11: Summary of histological lesions of bronchopneumonia and peribronchial cuffing for dorsocaudal and cranioventral lobes for each category of pig (healthy, suspect *Mycoplasma hyopneumoniae* and suspect *Actinobaciullus pleuropneumoniae*) in the validation study 124

1 Acknowledgments

A number of individuals and organisations in both Australia and the Philippines provided key support and assistance to this project.

In the Philippines, the project was overseen by a Project Overview Group that included representatives from industry groups (the Pork Producers Federation of the Philippines and the Philippines National Federation of Hog Farmers Inc) as well as key government groups (National Meat Inspection Service and the Livestock Development Council) – the input of these groups was a key contribution to the project. A key focus of the project involved on-farm and slaughter-house studies in the Pampanga and Bulacan provinces. The co-operation and assistance of the farmers and management and staff of the slaughterhouses was essential in the performance of these field-based studies.

In Australia, the technical assistance of Dr Mark O'Dea of the Animal Health Laboratories in Perth for the provision of training and reagents in the immunohistochemistry detection of porcine circo-virus type 2 is gratefully acknowledged. The development of a molecular test for porcine circo-virus type 2 was done with the technical input of Mr Michael Leitner (then at University of Queensland now at Queensland University of Technology). Professor Mark Schembri (University of Queensland) was a key contributor to the technology used to design the new molecular serotyping tool for *Actinobacillus pleuropneumoniae*.

2 Executive summary

The capacity of the pig industries of the Philippines and Australia to produce food, income and livelihood support is constrained by respiratory diseases and the capacity of producers and service providers to identify, diagnose and control them. These respiratory diseases, caused by several types of virus and bacteria, are major causes of economic loss in both countries causing, in the Philippines, about one quarter of all pig mortalities and, in Australia, losses of between $30 and $100 per sow per year.

The aim of the project was to support the pig industries of the Philippines and Australia to reduce mortality and morbidity associated with respiratory diseases. Specifically, in the Philippines, the project sought to improve investigation, surveillance and diagnostic systems for respiratory diseases (linking both field and laboratory staff); to develop effective communication strategies for stakeholders associated with the pig industry and to build the competence of Philippine researchers and the capability of their institutions. In Australia, the project aimed to develop new diagnostic tests that would be provided on a user pays basis to the Australian pig industry.

In the Philippines, integrated surveillance and disease investigation systems were established including three manuals and a number of protocols and forms. In the laboratory, at both the national and the regional level, a series of novel diagnostic assays (with full validation) were established. These new systems were then used in two large studies, one of which compared and contrasted respiratory disease in commercial and smallholder piggeries using lung scoring (a surveillance methodology introduced by this project) and the second which used lungs from pigs at slaughter to confirm the capacity of the new integrated regional field and laboratory services and which also evaluated the performance of some of the newly developed diagnostic assays. Project communication strategies included publication of manuals on aspects of disease investigation and surveillance, a Swine Health Monitoring System that has been integrated into the national animal health information system and numerous workshops that provided hands-on training to government and private company technical staff.

In Australia, a series of new molecular-based assays to identify and type *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis* were established and are now provided on a user pays basis to the Australian pig industry.

The project has achieved considerable impact in both Australia and the Philippines. In both countries, new generation diagnostic assays are now available from a laboratory with a national coverage. In the Philippines, significantly improved diagnostic services (field and laboratory) have been established in Region III. Improved communication among all stakeholders has been achieved at a both a high organisation level (national producer organisations and central government offices) and a local level (between farmers and regional government offices providing both field and laboratory based services). Considerable enhancement of capacity to investigate and diagnose porcine respiratory diseases (and indirectly a range of other infectious diseases) has been achieved.

A follow up project that delivers similar improvements to two other key pig producing regions (“Scale Out”) and which builds on the gains from the current project by undertaking a “Eco-Health” driven project with a focus on pig health in Region 3 (“Scale Up”) has been developed to ensure on-going additional benefits to the smallholder pig industry of the Philippines.

3 Background

This project addresses the problems caused by respiratory diseases of pigs, which are major causes of economic losses to the pig industries of both the Philippines and Australia and currently prevent export of pork from the Philippines.

The disease agents of major importance in the two countries differ, but the underlying skills and technologies necessary for their diagnosis, the design of control programs and improved productivity are the same in both countries. There are mutual benefits to both countries through sharing of reagents, testing of new techniques in a wide range of disease and production environments and increased capacity for endemic and exotic disease control.

The project contributes to the strategic goal of the ACIAR-Philippines cooperation agreement to 'assist in increasing productivity, marketability and international competitiveness for Philippines agricultural products, taking into account the impacts of trade liberalisation'. Pork accounts for 60% of meat produced and consumed in the Philippines and in 2009 the first exports of pork from Philippines to international markets were blocked because of an outbreak of porcine reproductive and respiratory syndrome (PRRS) virus. This setback came almost at the same time as the Philippines was being granted FMD-free status, which would have provided access to many international markets.

The ACIAR-Philippines cooperation agreement also aims 'to address food security by supporting research that would provide smallholder farmers and traders with increased cash income, supporting the purchase of staple foods." Between 60 and 80% of all pork production in the Philippines remains in the hands of smallholders who are increasingly being sought by the larger commercial companies as contract growers and fatteners. There is therefore good alignment between the interests of the industry as a whole (providing economic growth and potential exports) and the smallholder sector (providing food security). The majority of pig production occurs on the islands of Luzon and Mindanao.

It is against this background that the Philippines government requested Australian collaboration for new research embracing field investigation and laboratory diagnosis for the support of control programs for respiratory diseases in the pig industry. The export issue was the catalyst but it is also recognised that respiratory diseases have disrupted pig production in the Philippines for many years, reducing sector competitiveness, smallholder incomes, domestic market availability and stability of the price of pork products.

In Pampanga province, for example, one of the provinces where the project had its field operations, there were heavy losses between 2007 and 2009, associated with epidemics of typical PRRS virus in 2007 and then a more virulent highly pathogenic PRRS virus in 2008-2009 which is believed to have entered the Philippines with illegal imports of pig products. In May 2009, PRRS affected 1,900 farms in 20 of the 21 municipalities, with 31% of 9,930 cases dying.

The Australian pig industry (2.4m pigs, $1.24 bn annual production) fully recognises the importance of all respiratory diseases as a major cost of production and disease control is a key strategy of Australian Pork Limited (APL) and the Pork Collaborative Research Centre (CRC), the major funding bodies for the industry in Australia.

The Australian Pork Industry National Research, Development and Extension Strategy, released shortly before the start of this project, recognised the need to enhance investment of research, development and extension funds from sources other than the traditional APL and Pork CRC. This project aligned closely with this national strategy as it provided complementary funds to support two organisations – Queensland Department of

Employment, Economic Development and Innovation (DEEDI) (now Department of Agriculture and Fisheries) and UQ - that already had a strong ongoing support from both APL and the Pork CRC and a long-term commitment to pig research.

The project was integrated into the industry and government priority programs for the pig industry in both countries. The synergies of working on respiratory disease in both countries in a single project included diagnostic testing in different disease backgrounds and the training of Philippines and Australian veterinarians in endemic and exotic disease detection and control.

The project complements the ongoing FAO regional project, "Environmental Animal Health Management Initiative", which was extended into Laos and Cambodia, then to Myanmar and Vietnam. This project is also based at the Philippine Animal Health Centre (PAHC). During the earlier years of this project, the US Department of Agriculture was also collaborating with the Bureau of Animal Industries in a Biosecurity Engagement Program to enhance the capability of PAHC in molecular diagnostics for swine infections, notably PRRS, and to investigate the immunogenicity of current vaccines against virulent PRRS (Chinese strain). The Bureau was also currently conducting sero-surveillance for Ebola - Reston, PRRS and other porcine viruses at slaughterhouses in Regions 1, 3 and 4. The project complements these FAO and USDA initiatives and at the time of proposal development it was hoped that it would be possible to work closely with them.

The Australian component of the project involves the national reference laboratory (the Microbiology Research Group of QDEEDI at the time of the project initiation, now of the University of Queensland) for the major respiratory pathogens of pigs in Australia (*Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, *Pasteurella multocida, Actinobacillus suis* and *Streptococcus suis*). Thus the Australian pig industry will have direct and immediate access to the new diagnostic technologies developed in this project. These technologies are essential to underpin sustainable prevention and control programs (e.g. vaccination, targeted antibiotic treatment programs, regional eradication programs). The central role of the Microbiology Research Group in providing the existing specialised identification and typing services to the Australian pig industry means that the new technologies will be immediately available to the entire Australian industry.

At the time of proposal development, ACIAR was supporting one project on pig production and health in Laos (AH/2004/046) and two in Indonesia (AH/2007/106; AH/2006/156). It was intended that this proposed project interacted with these ACIAR projects, and with AusAID, CARD and Centre de Coopération Internationale en Recherche Agronomique pour le Développement projects in Vietnam, through workshops and cross-visits.

A concluded ACIAR-funded project in the Philippines (AS2/1994/121) was based in the Visayas and focussed on smallholder systems and cooperative arrangements for feed and health supplies. ACIAR also supported a project (AS2/2000/098) on the diagnosis of tick-borne disease at PAHC. The project team was aware of the successes and failures of these projects and built on research relationships created by them.

4 Objectives

The aim of the project was to support the pig industries of the Philippines and Australia to reduce mortality and morbidity associated with endemic respiratory disease, and the risks from exotic disease, through the following objectives:

1. Identify technical gaps in field outbreak investigation and surveillance, service and reference laboratory systems and technologies.

2. Develop cost-effective laboratory tests for bacteria and viruses associated with porcine respiratory diseases in both countries.

3. Implement systems that apply current technology and the new tests developed in this project (Objective 2) to determine the causes of respiratory disease outbreaks in the Philippines and Australia.

4. Establish novel mechanisms for communication among industry, government and research institutions in the Philippines on pig health.

5 Methodology

Objective 1. Identify technical gaps in field outbreak investigation and surveillance, service and reference laboratory systems and technologies

This objective was addressed in a collaborative manner using a panel of technical experts assembled from within the full Australian and Philippines project research team. The work occurred at two sites that had been identified as part of an initial scoping visit to prepare for this project. This scoping activity had identified that there was a need for the national laboratory, the Philippines Animal Health Center (PAHC), to be a centre of activity as PAHC has a role to support all Regional Animal Disease Diagnostic Laboratories (RADDLs) and this ensures that benefits from this project spill over to all regions. The involvement of PAHC was also driven by the fact that PAHC has a capacity to undertake high-technology based diagnostic tests (for example using real time polymerase chain reaction assays) and that capacity is not available in the RADDLs. The scoping visit clearly identified the need to involve a front-line diagnostic laboratory (i.e. an RADDL) as well as regional field staff. Several different options of regional centres of activity were considered with the final decision being that Region 3 would be optimal. This decision reflected the fact that two of the Provinces (Bulacan and Pampanga) had a significant pig industry (both commercial and smallholder operations), had energetic and active field staff based in respective Provincial Veterinary Offices (PVOs) and had a well-resourced RADDL that had a capacity for polymerase chain reaction (PCR) work and was staffed by highly motivated individuals keen to improve diagnostic services.

The Australia-based research team members actively working on this Objective were Drs. Blackall and Turni (Bacteriology), Dr Meers (Virology), Dr Parke (Pig health) and Drs Barnes and Alawneh (Epidemiology). The team included Philippines-based project members with similar skill sets. Over the time period of the project, a number of Philippines based team members contributed to this Objective - Drs Morales, Dazo, and Lopez (Bureau of Animal Industry), Drs Cruz, Azul, Legaspi and Retes (PAHC), Drs Lapuz and Mananggit (RADDL Region 3), Drs Baluyut, Basinang, Francisco, and Principe, and Ms Ignacio (Provincial Veterinary Offices) and Drs David and Lola (Project Staff – Dr Lola was a pathologist/molecular biologist based at PAHC and Dr David was a field veterinarian based at RADDL Region 3).

The Philippines team assembled a comprehensive set of documents that covered the protocols relevant for disease investigations and disease surveillance activities undertaken by the relevant Region 3 field staff. A similar set of protocols was assembled for the diagnostic tests provided by RADDL Region 3 and PAHC.

The joint research team then critically reviewed these protocols and documents in the light of the need for relevant sustainable systems that provided cost effective disease diagnosis services to the Philippines pig industry. This review looked for gaps in the capabilities of disease diagnosis. As well, a review of the current research activities (relevant to diagnostic assay development underway) at PAHC (and funded by other projects) was performed in order to ensure that duplication of activities across different projects was avoided.

Objective 2. Develop cost-effective laboratory tests for bacteria and viruses associated with porcine respiratory diseases in both countries.

This Objective had two very different geographical regions of activity – the Philippines and Australia. This dual focus was a key feature of the project – research activities relevant (and sustainable) for both countries were undertaken in parallel in both countries.

Philippines Component

With the Philippines component, Objective 2 was a follow-on from Objective 1. Hence, essentially the same research team members and the same locations were involved in Objective 2 as in Objective 1. On the basis of the activities undertaken under Objective 1, gaps were identified in both the laboratory-based protocols, the range of laboratory assays available for the diagnosis of respiratory diseases and the protocols available for field investigations.

A series of steps were taken to address these technical gaps. Intensive hands-on training of relevant Philippines staff during visits to partner Australian organisations was undertaken. The intensive training involved both laboratory and field staff, specifically Drs Lola, Mananggit and Retes (laboratory aspects) and Drs David and Lola (field investigation aspects). In a similar manner, multiple trips were undertaken by all Australian team members to both Philippines sites (PAHC and RADDL Region 3/PVOs Bulacan and Pampanga, Region 3) to deliver hands-on training to the Philippines team. As well as individual-to-individual training, key Australian team members delivered focussed workshops that targeted both Philippines research team members as well as the broader professional community that services the Philippines pig industry.

In addition to training, there was a need for known reference strains to be used as quality control (QC) strains or reagents in the various assays and tests to be developed in the Philippines. The laboratory of Drs Blackall and Turni provided or sourced a range of materials that were then shipped to the collaborating institutes in the Philippines – with all shipments having the prior approval of the Bureau of Animal Industry and a relevant Import Permit.

An additional centre of activity was involved in this objective – Central Luzon State University (CLSU) where Dr C. Domingo led a research program to develop a series of rapid, low cost molecular diagnostic assays. These assays were based on the Loop-mediated Isothermal Amplification (LAMP) technology. This technology is similar to PCR but differs in that the reaction occurs at single temperature, removing the need for the complicated equipment required to undertake PCR (which typically involves multiple stages at two or three different temperatures). As well, the results of a LAMP test can be read with visually by the naked eye or with simple UV lighting. The LAMP assays developed in this work targeted the following organisms:-

- *A. pleuropneumoniae* - based on an existing LAMP assay that targets the *apxIV* gene (Yang et al., 2010)
- *H. parasuis* - based on an existing LAMP that targets the *infB* gene (Zhang et al., 2012)
- *Mycoplasma hyopneumoniae* - based on an existing LAMP that targets the *mhp165* gene (Li et al., 2013).

In addition, the CLSU research group also established conventional PCR assays for *A. pleuropneumoniae* and *M. hyopneumoniae* to allow a comparison of the new LAMP technologies with existing accepted PCR tests. The *A. pleuropneumoniae* PCR was a published assay that targeted the *apxIV* gene (Schaller et al., 2001) while the *M. hyopneumoniae* PCR was a published assay that targets the *p36* gene (Caron et al., 2000).

Another assay development involved collaboration of the PAHC staff (Drs Azul, Legaspi, Lola and Retes) with Australian team members (Dr Meers and Mr Leitner). Additional key collaboration was provided by Dr O'Dea from the Animal Health Laboratories in Perth, Western Australia. This work focussed on the development of a suitable diagnostic assay for porcine circo-virus type 2 (PCV2). In the initial work, Dr Lola was given hands on training in the use of immunohistochemistry (IHC) to detect (PCV2) in the laboratory of Dr O'Dea. The key reagents – a processed lung sample that was positive for PCV2 as well as a monoclonal antibody specific for PCV2 – were provided by Dr O'Dea and were shipped to PAHC as part of the QC reagents mentioned above. The IHC methodology was the formal Australian and New Zealand Standard Procedure (O'Dea, 2010).

As well, a published quantitative real-time PCR assay for PCV2 (Olvera et al., 2004) was initially established at the UQ in the laboratory of Dr Meers (with the technical input of Mr Leitner). After the successful establishment of the assay at UQ, the key reagents were shipped to PAHC and Mr Leitner travelled to PAHC to provide initial hands on-training to Drs Azul, Legaspi and Retes. The protocol for the quantitative PCV2 assay is provided as Appendix 11.1. The Philippines team then continued to work on the establishment and validation of the assay.

While not laboratory-based activities, as originally envisaged in the proposal, considerable activity was undertaken in training the relevant Philippines project team members in field investigation techniques and protocols. In general, the training was delivered by Dr Parke (UQ) to the regional-based research team – Drs Baluyut, Basinang, David, Francisco and Principe and Ms Ignacio. The training focussed on two main areas – A) performing an on-farm investigation and B) quantitatively assessing the lesions present in the lungs of pigs at slaughter (termed lung scoring). The on-farm protocols were essentially based on the practices and techniques developed by Dr Parke in his years as a practicing pig veterinarian. The lung scoring system was based on the system originally developed by Straw et al. (Straw et al., 1986).

Australian component

This activity took place within the Microbiology Research Group (University of Queensland) at the EcoSciences Precinct in Brisbane. The research was led by Dr Turni and was performed by Ms Singh (project staff). The work consisted of two main themes – development of a new diagnostic assay for *A. pleuropneumoniae* and another new diagnostic assay for *H parasuis.* A collaboration with Prof Schembri (School of Molecular BioSciences, University of Queensland) was a critical component of the *A. pleuropneumoniae* work.

The focus of the *A. pleuropneumoniae* work was to develop a molecular-based assay to replace conventional serotyping for the specific recognition of serovar 15 – a very common *A. pleuropneumoniae* serovar in Australian pigs. The development of a diagnostic assay requires the availability of a representative range of strains and field isolates that reflect the organisms that should be positive in the assay as well as the organisms that should be negative in the assay. As the Microbiology Research Group is a national and international reference centre for *A. pleuropneumoniae*, a comprehensive collection of strains and isolates was available for this work. The collection consisted of the reference strain for each of 15 currently recognised serovars of *A. pleuropneumoniae*, 411 field isolates of *A. pleuropneumoniae* (394 from Australian pigs, 11 from Indonesian pigs, five from Mexican pigs and one from a New Zealand pig), which had been submitted for routine identification and serotyping, and a total of 26 non-target species that represented species commonly found in pigs. The non-target species were organisms that had to give a negative reaction in any diagnostic assay for *A. pleuropneumoniae*.

The 411 field isolates had been confirmed as *A. pleuropneumoniae* by phenotypic methods as previously described (Blackall and Pahoff, 1995) or by a published PCR (Gram and Ahrens, 1998) in prior work. In addition, the field isolates had already been

serotyped by classical methods using antisera to the reference strains for serovars 1 to 12 and 15 (Blackall et al., 1999; Blackall et al., 2002; Eaves and Blackall, 1988).

The main antigen involved in determining the serovar in *A. pleuropneumoniae* is the capsular polysaccharide (Blackall et al., 2002). Hence, knowledge of the gene sequences associated with the capsular polysaccharide should allow the development of a molecular assay to specifically recognize serovar 15. The DNA region involved in the export of capsule polysaccharide to the required location on the surface of the organism is known as the *cpx* region with the region consisting of four genes designated *cpxDCBA* (Ward and Inzana, 1997). Unfortunately, there is no public knowledge of the *cpx* region of serovar 15. However, the *cpxA* gene is a conserved region for all serovars (Schuchert et al., 2004). Using this knowledge, a product from the *cpxA* gene of serovar 15 was obtained using conserved primers used on other serovars of *A. pleuropneumoniae* (Schuchert et al., 2004). An arbitrary primer PCR approach was then used to step down the *cpx* region till the *cpxD* gene was reached. The PCR product from the *cpxD* region of serovar 15 was then sequenced and the resultant sequence used to design primers that were, in theory, specific for serovar 15.

The final format of the PCR that was developed was a multiplex format that confirmed an isolate as being *A. pleuropneumoniae* and, at the same time, determined if the isolate was serovar 1, 5, 7, 12 or 15. The primers used in this PCR were the species-specific primers reported by Gram and Ahrens (1998), the serovar specific primers for serovars 1, 7 and 12 reported by Angen *et al.* (2008) and the serovar 5 specific primers reported by Lo *et al.* (1998). The serovar 15 primers were developed in this study as described above. Full details of the multiplex PCR including primer sequences and reaction conditions are provided in Appendix 11.2.

The *H. parasuis* component of the work involved an evaluation of two proposed molecular approaches for sub-typing *H. parasuis*. One set of assays was purported to identify if an isolate of *H. parasuis* was virulent. This is an important issue as some isolates of *H. parasuis* are fully pathogenic and cause Glässers disease while other isolates are non-pathogenic and are part of the normal flora of the upper respiratory tract of the pig (Aragon et al., 2012). The second set of assays was developed by others to allow typing of *H. parasuis* below the species level (Mullins et al., 2013). This typing scheme was based on multi-locus sequence typing and is accepted “gold standard” method for strain typing to follow disease outbreaks (Maiden et al., 1998).

As with the *A. pleuropneumoniae* work, the *H. parasuis* work required a set of well characterised strains and field isolates in order to evaluate the proposed assays. The collection used in this work consisted of the reference strain for each of the recognised 15 serovars as well as 76 field isolates obtained from Australian pigs. These field isolates came from nasal and internal organs and covered all the different serovars known to be present in Australia. All the field isolates had been confirmed as *H. parasuis* by the PCR of Oliveira *et al*. (2001) and had been serotyped according to the method by Turni and Blackall (2005).

The potential virulence assays involved PCR assays for the following genes – a virulence-associated trimeric autotransporter gene (*vtaA*), a putative hemolysin gene (Assavacheep et al.; Zhou et al.), a lipopolysaccaride sialytransferase gene (*IsgB*), a polysaccharide biosynthesis gene (*capD*) and a predicted truncated outer membrane ferric hydrocamate receptor gene (Zhou et al.). Appendix 11.3 contains the full technical details of all five PCR assays and the relevant references.

The multi-locus sequence typing (MLST) work involved determining the partial sequences of seven housekeeping genes which were the β chain of ATP synthase (*atpD*), malate dehydrogenase (*mdh*), translation initiation factor IF-2 (*infB*), ribosomal protein b subunit (*rpoB*), 6-phosphogluconate dehydrogenase (*6pgd*), glyceraldehyde-3-phosphate dehydrogenase (*g3pd*) and fumarate reductase B (*frdB*). Full technical details of the PCR amplification and sequencing of the seven genes are contained in Appendix 11.4.

The sequences of each gene for each isolate were then compared with the existing database (see full details in Appendix 11.4). This then resulted in each isolate being given a seven digit number that corresponds to alleles recorded in the database. This seven digit number (effectively an allelic profile) is then identified as a Sequence Type (ST). As an example, isolate HS2857 has *atpD* allele 7, *infB* allele 31, *mdh* allele 4, *rpoB* allele 2, *6pgd* allele 4, *g3pd* allele 3 and *frdB* allele 6 – a seven digit code of 7, 31, 4, 2, 4, 3, 6. This then is recorded as ST 122 at the MLST database.

Objective 3. Implement systems that apply current technology and new tests developed in this project (Objective 2) to determine causes of respiratory disease outbreaks in the Philippines and Australia.

This Objective, like Objective 2, had two very different geographical regions of activity – the Philippines and Australia.

Philippines Component

With the Philippines component, Objective 3 was a follow-on from Objectives 1 and 2. Hence, the same research team members and the same locations were involved in Objective 3 as in Objectives 1 and 2. Two large-scale studies were undertaken to implement aspects of the new technologies and new protocols developed under Objective 2. These new technologies and protocols include the laboratory based aspects described in detail in Objective 2 as well as the new field protocols (including lung scoring). The entire Australian and Philippines research teams had input into the design and/or conduct of one or both of the two studies. Critically important inputs in terms of design were provided by the epidemiologists associated with the project - Drs Alawneh and Barnes (Australia) and Dr R. Domingo (Philippines). The laboratory aspects of this Objective involved Drs Manangitt, Baccay and Ongtangco (RADDL Region 3), Dr C. Domingo (CLSU) and Drs Azul, Lola and Retes (PAHC). The field, abattoir and data entry work involved Drs Baluyut, Basinang, Francisco and Cuevas, Mss Ignacio and Manio, and Messrs Cunanan, Sto. Domingo, de Guzman and Almaro (PVOs), Dr David (Project Veterinarian based at RADDL Region 3) and Ms Corales (Project Communication Officer based at PCAARRD).

Part of Objective 3 is capacity building of Filipino field and laboratory personnel. As such the Project Field Veterinarian (Dr David) and Veterinarian and Technical Staff of RADDL Region 3 and Provincial Veterinary Offices of Bulacan and Pampanga were intensively trained in lung scoring and necropsy procedures. From that, the trained Filipino Veterinarians trained the meat inspectors of Bulacan and Pampanga and the Technical Staff of CATMON Livestock Multi-Purpose Cooperative (Bulacan) in the lung scoring technique. Moreover, laboratory researchers from BAI and RADDLs Region 2, 3, 4, 5, 7, 8, 9, 10, 11, 12 and 13 were trained on bacterial isolation and identification technique, lung scoring and sample collection protocols by the Philippine Project Team (Drs Mananggit, Legaspi, Azul, Retes and David).

Cross-sectional study

The first study was a large abattoir-based cross-sectional study that aimed to estimate prevalence of and risk factors associated with respiratory disease lesions in finisher pigs at slaughter in the Bulacan and Pampanga provinces. Full details of the descriptive component of the study are available in Alawneh et al. (2014)

The source population for this study was farms slaughtering finisher pigs on randomly selected calendar dates at 29 government operated abattoirs in Bulacan and Pampanga provinces. Systematic random sampling was used at the time of slaughter to select the study population (i.e. the pig and its farm of origin, see Appendix 11.5 for details). The selected pig was then subjected to lung scoring using a methodology based on that of Straw et al (1986) (see Appendix 11.5 for more details of the methodology).

A questionnaire was designed that consisted of three main sections. The first section collected general information about the farmer and farm profiles, such as the farmer demographics and level of education, farm location, type of production system (small-holder or commercial), who provided health advice to the farm, animal husbandry and reproduction management.

The second section requested information on the following: whether pigs on the farm come in contact with wild animals or pigs from neighbouring farms, number of workers living on the farm, swill feeding and 'feedback' practice (feeding pig manure, viscera or aborted material to pigs to increase herd immunity), farm biosecurity measures, ability of farm workers to recognise sick and healthy pigs, animal health monitoring and management, and farmer awareness of pig density within a 500 metre radius relative to the farm.

The third section asked for information regarding pig management comprising: type of pig housing, source of water supply for the farm and herd size details at the time of the interview, details of pig feed, vaccination routine, and history of any respiratory disease outbreak or outbreak investigation carried on the farm in the last 12 months. In total, the questionnaire consisted of 71 questions, of which 10 (14.0%) were open, 13 (18.3%) were semi-open and 48 (67.7%) were closed questions with mostly dichotomous answers. Questions (n = 6) with distinct subordinate parts had at least five possible options.

The responses were collected through face-to-face interviews conducted by four experienced interviewers (two teams each comprised of two interviewers) between October 2011 and March 2012. Farmer participation was on a voluntary basis. Details of the techniques used to ensure that the interviews were as free of bias and error as possible are provided in Appendix 11.5.

Cluster, social network and spatial analyses and logistic regression models were used to analyse the lung score and farm data. Details of these analyses are provided in Appendix 11.5.

Validation Study

The laboratory tests and identification protocols introduced in this project (both culture and molecular based assays) have never been evaluated or validated under the conditions that prevail in the Philippines. Therefore the validity of their results to end users remains questionable. To determine whether the introduced tests and protocols can be run under the Philippines conditions and to compare the diagnostic performance of culture versus molecular tests as well as the different molecular assays (PCR and LAMP), this study aimed to:

a) use four of the project developed laboratory diagnostic tests - culture and PCR for *A. pleuropneumoniae* and PCR and LAMP for *M. hyopneumoniae* under Philippine conditions.
b) compare the diagnostic test performance of culture and PCR to confirm the presence of *A. pleuropneumoniae* in 13-17 weeks old grower pigs.
c) compare the diagnostic test performance of PCR and LAMP to confirm the presence of *M. hyopneumoniae* in 13-17 weeks old grower pigs.

In each province, two farms known to be high lung score farms and two known to be low lung score farms (based on preliminary lung scoring of slaughter pigs) were selected to participate in the study. Case definitions of a pig that was likely to be *A. pleuropneumoniae* infected, likely to be *M. hyopneumoniae* infected and apparently healthy were established (see Appendix 11.6). The resources available to this study meant that the total number of pigs to be sampled was set at 150 (50 possibly infected *A. pleuropneumoniae*; 50 possibly infected *M. hyopneumoniae*; 50 healthy).

Four on-farm disease investigators (ODIs) were appointed, each responsible for one farm in each province. Visits to farms were conducted on a weekly basis, with the four farms in Pampanga visited one day in one week, and those in Bulacan visited on one day the following week. Using the established case definitions, one 13-17 week old pig of each type (possibly infected with *A. pleuropneumoniae*, possibly infected with *M. hyopneumoniae* and healthy) was identified on each farm, marked and transported to the Philippine Abattoir Development Corporation (PADC) facility in San Fernando,

Pampanga. The pigs were humanely euthanased and the required samples collected. Full details of the case definitions, pig identification and sampling methods are provided in Appendix 11.6.

The samples were then processed and the various laboratory tests performed at the three respective laboratories (RADDL Region 3, CLSU and PAHC). The culture work was done at RADDL Region 3, the LAMP and PCR tests for *A. pleuropneumoniae* and *M. hyopneumoniae* were performed at CLSU. All work was done using the methods and protocols developed in Objective 2.

At PAHC, the species specific PCR assays for *H. parasuis* and *P. multocida* developed under Objective 2 were used to examine all *H. parasuis* and *P. multocida* identified at RADDL Region 3 by phenotypic methods. In addition, the PAHC research team used a commercially available enzyme-linked immunosorbent assay for antibodies to the ApxIV toxin of *A. pleuropneumoniae* (IDEXX APP-ApxIV Ab Test) on all 156 serum samples.

The results of pairs of test in the validation study (e.g. the PCR and LAMP assay for *M. hyopneumoniae* were cross-tabulated and observed agreement, expected agreement and the kappa statistic determined using Stata® version 13. Two-by-two tables were then inputted to Pairs etc® version 3.37, Winpepi® version 11.41 to obtain percent positive agreement, percent negative agreement, bias index, prevalence index, the bias adjusted kappa statistic (BAK), the prevalence and bias adjusted kappa statistic (PABAK) and p value from McNemar’s test.

Pairs of tests were cross-tabulated and observed agreement, expected agreement and the kappa statistic determined using Stata® version 13. Two-by-two tables were then inputted to Pairs etc® version 3.37, Winpepi® version 11.41 to obtain percent positive agreement, percent negative agreement, bias index, prevalence index, BAK, PABAK and p value from McNemar’s test.

Australian Component

As detailed in the Results section, the Australian component of Objective 2 yielded one new diagnostic assay that was shown to be useful and relevant in routine respiratory disease investigations. The assay was moved to the user pays reference service provided to the Australian pig industry and is now in routine and regular use in that specialised reference service.

Objective 4. Establish novel mechanisms for communication among industry, government and research institutions in the Philippines on pig health.

The activities under this Objective were driven by the Philippines team with key contributions by Dr Villar (Philippines Project Leader), Ms Corales (the project employed communications officer) and Mr de Castro (Swine Commodity Specialist, PCAARRD).

A Project Advisory Committee was formed to oversee the project.

One of the key activities undertaken was a stakeholder analysis of the pig sector and an evaluation of stakeholder views on health issues in the industry. This activity focussed on the pig industry in Region 3. The study was conducted from September-October 2011 and aimed to profile the knowledge, attitudes and practices of swine industry stakeholders using the farm-market route. A total of 356 respondents from 29 cities and municipalities in Bulacan (13/24) and Pampanga 16/22) were involved in the survey.

A range of communication mechanisms were developed under this Objective (with many links back to the other Objectives of the project):-

Project Fact Sheets and Flyers

Project HomePage – located on PCAARRD website

Hard Copy Manuals (Lung Scoring; Field Sample Collection; Disease Outbreak Investigation)

Training modules for lung scoring and field sample collection

Swine Respiratory Health Monitoring System – a database that is now integrated into the Philippines Animal Health Information System (PhilAHIS)

A number of traditional communication activities (Presentation of Project Progress to Industry meetings; Seminars by visiting Australian research team members to veterinary students in several Universities; Workshops on Lung Scoring and Sample Collection) were also undertaken. In addition, presentation of papers and posters in local and international scientific meetings and industry congresses was also undertaken by the Filipino research team members. Likewise, project output and updates were also released in Philippine-based institutional and industry journals and newsletters.

6 Achievements against activities and outputs/milestones

Objective 1: To identify technical gaps in field outbreak investigation and surveillance, service and reference laboratory systems and technologies.

no.	Activity	outputs/ milestones	completion date	Comments
1.1	Optimise field investigation, sampling and surveillance (e.g. syndromic) methodologies	Review of current approaches	April 2011	This milestone was achieved. Australian and Philippines team members have assembled a comprehensive overview of typical approaches currently used in field investigations in the Philippines. This activity was a focus of the initial Australian team visit in September 2010 and the second Australian visit in February 2011.
		Develop standards and enhance capabilities for sample collection, sample submission and cost-effective use and interpretation of laboratory tests	August 2011	This milestone was achieved. Manuals for field sample collection and lung scoring at the abattoir have been produced. Protocols for disease/herd health investigations have been developed. Laboratory protocols suitable for front-line diagnostic activities as well as central reference laboratory activities have been produced.
		Train veterinarians in application of the new standards (PC)	November 2011	This milestone was achieved. Training was provided to both project-linked veterinarians as well as other veterinarians in farm outbreak investigations, post-mortem methods and lung scoring at abattoirs.
		Review and re-design on-farm disease control and bio-security programs in light of current knowledge (A,PC)	November 2011	This milestone was achieved. Formal biosecurity protocols to ensure that on-farm disease investigations are performed with negligible risk of disease transmission between farms have been developed.
1.2	Optimise regional laboratory capacities in terms of diagnosis of porcine respiratory diseases	Audit current methods in bacteriology, virology and pathology and their use in diagnosis of porcine respiratory diseases by RADDL Region 3 (PC)	August 2011	This milestone was achieved. The relevant Australian and Philippines research team members undertook a full audit of the current methods in use in the diagnosis of porcine respiratory diseases by RADDL Region 3 (PC). This audit has allowed the development of a set of standard operating protocols (SOPs) described in the next milestone.

no.	Activity	outputs/ milestones	completion date	Comments
		Develop appropriate QC processes and revise standard operating protocols in relevant laboratory diagnostic methods (PC)	November 2011	This milestone was achieved. A set of SOPs for the conventional bacteriological techniques used in the diagnosis of porcine respiratory diseases have been produced. These SOPS were developed to sustainable within the conditions that prevail at the RADDL into the future. These SOPs have been further supported by the provision of a set of QC/reference strains that will allow on-going QC activities by RADDL Region 3.
		Assess capability of laboratory to apply any necessary additional tests (PC)	November 2011	This milestone was achieved. The capacity of the RADDL Region 3 to perform new diagnostic tests has been confirmed. These new tests include expansion of the capacity of the RADDL in conventional bacteriology. Specifically, the RADDL has been confirmed as having the capacity to identify two major bacterial pathogens (*A. pleuropneumoniae* and *H. parasuis*) by conventional bacteriology. As well, the RADDL has developed the capacity to apply matrix gene detection of Swine Influenza by Reverse Transcriptase-Polymerase Chain Reaction technology (RT-PCR).
1.3	Optimise central reference facilities at PAHC relevant to diagnosis of porcine respiratory diseases	Audit current methods, capacity and use of PCR, RT-PCR and virus isolation relevant to the diagnosis of porcine respiratory diseases (PC)	August 2011	This milestone was achieved. The Philippines and Australian teams have audited the capacity and use of molecular diagnostic tests to support the diagnosis of porcine respiratory disease.
		Develop appropriate QC processes and revise standard operating protocols in relevant methods (PC)	November 2011	This milestone was achieved. The research team reviewed the existing protocols. Additional documentation and forms have been produced and their use adopted by the PAHC-based research team.
		Assess capability of laboratory to apply any necessary additional tests (PC)	November 2011	This milestone has been achieved. The project team identified that tests for PCV2 are required and that PAHC has the capacity to develop such tests. A project team member from PAHC was sent to an Australian laboratory specialising in the diagnosis of PCV2 to receive hands-on training in two assays for PCV2.

PC = partner country, A = Australia

Objective 2: To develop cost-effective laboratory tests for bacteria and viruses relevant to both countries.

no.	Activity	outputs/ milestones	completion date	Comments
2.1	Development of new relevant diagnostic technologies in Philippine laboratories	Establishment of LAMP Assays (PC)	March 2013	This objective was achieved. The CLSU research team developed LAMP assays for *A. pleuropneumoniae, H. parasuis* and *M. hyopneumoniae*. Using the reference standards for target and non-target bacteria provided by the Australian research team, analytical sensitivity and specificity has been established for all three assays.
		PCR test for porcine circovirus 2 developed and validated at PAHC (PC, A)	December 2014	This objective was achieved. There were considerable delays and a number of difficulties encountered in this work. A conventional PCR was initially developed and validated. However, as the diagnostic need is for an assay that quantifies the amount of virus and not just the presence of the virus, alternative tests were considered. An immunohistochemistry test that visualises the virus and the lesions in the relevant lymphoid material was developed and validated. However, this assay requires an experienced operator and is not easy to sustain. Hence, the final assay that was developed was a quantitative real-time PCR that provides a direct estimate of the amount of virus present. The real-time PCR was initially developed and validated in Australia and then transferred to PAHC where it was established and validated in an analytical setting.
		PCR tests for serotyping of *A. pleuropneumoniae* and *P. multocida* established at PAHC (PC, A)	December 2014	The PCR for the recognition of *A. pleuropneumoniae* serovars 1, 5, 7 and 12 was established and validated at PAHC by August 2013. The PCR to identify *P. multocida* was established and validated at PAHC by August 2013. However, the multiplex PCR to recognise the lipopolysaccharide-based serovars could not be validated by the end of the project.
		Establishment of conventional methods for isolation and identification of *A. pleuropneumoniae*, *H. parasuis* and *P. multocida* at RADDL (PC, A)	June 2012	This milestone was achieved. Hands-on training, provision of QC strains from Australia, and the formalisation of isolation and identification protocols all ensured that these sustainable and effective methods are now embedded at RADDL Region 3.

no.	Activity	outputs/ milestones	completion date	Comments
		Development and validation of LAMP assay for *A. pleuropneumoniae*, *H. parasuis* and *M. hyopneumoniae* at RADDL (via CLSU student) (PC, A)	December 14	This milestone was achieved. Dr Domingo and the CLSU team developed LAMP assays for *A. pleuropneumoniae*, *H. parasuis* and *M. hyopneumoniae*. These assays have been transferred to RADDL Region 3. However, as detailed in later Milestones, the field validation study raised questions about the diagnostic suitability of these assays. Hence, the assays are not in routine use at RADDL Region 3.
2.2	Development of new, relevant diagnostic technologies in Australian laboratories	Development of a PCR-based test to replace conventional serotyping by antisera of *A. pleuropneumoniae* serovar 15 (A)	November 2013	This milestone was achieved. There were considerable delays caused by the difficulty of the work but a PCR that specifically recognised *A. pleuropneumoniae* serovar 15 was developed and validated.
		Establishment of a multiplex PCR to serotype the three dominant serovars of *A. pleuropneumoniae* (serovars 1, 7 and 15) found in Australian pigs (A)	December 2013	This milestone was achieved. In fact, the final version of the assay was a multiplex that confirmed identification of a suspect isolate as *A. pleuropneumoniae* and then identified if the serovar was serovar 1, 5, 7, 12 or 15.
		Development and evaluation of a PCR to differentiate virulent and non-virulent strains of *H. parasuis* based on trimeric autotransporter genes (A)	December 2014	This milestone was achieved. The PCR for the trimeric autotransporter genes was established. However, evaluation of the assay indicated that the assay did not reliably differentiate virulent and non-virulent strains of *H. parasuis*. A range of other potential virulence gene assays were established. However, none proved capable of correctly recognising virulent and non-virulent isolates.
		Establishment and evaluation of MLST scheme for typing of *H. parasuis* (A)	December 2014	This milestone was achieved. The MLST scheme was established and shown to be capable of typing *H. parasuis* isolates.

PC = partner country, A = Australia

Objective 3: To implement systems that apply current technology and new tests developed in this project (Objective 2) to determine causes of respiratory disease outbreaks in the Philippines and Australia.

no.	Activity	outputs/ milestones	completion date	Comments
3.1	Utilise the existing and new field and laboratory systems to investigate the causes of porcine respiratory disease outbreaks in the Philippines	Use of PCR to identify serovars of *A. pleuropneumoniae* found in diagnostic samples referred to PAHC (PC)	December 2014	This objective was not achieved. The serotyping PCR was successfully achieved but no cultures of *A. pleuropneumoniae* were obtained in either of the large field studies so the assay could not be applied to determine the serovars of *A. pleuropneumoniae* from pigs in the Philippines.
		Use of PCR to identify serovars of *P. multocida* found in diagnostic samples referred to PAHC (PC)	December 2014	This objective was partially achieved. The typing PCR was not successfully established at PAHC. However, the assay was used in Australia on DNA extracts from *P. multocida* sent by PAHC. The work indicated that 15 isolates were genetic serovar L6 and 7 isolates were genetic serovar L3.
		Structured survey designed (PC, A)	December 2014	This objective was achieved. In fact, two large field studies were designed – one a lung scoring study and the second a validation study looking at the performance of some of the diagnostic assays developed in the project.
		Use of new field and laboratory methodologies at front-line diagnostic level to identify causes of porcine respiratory disease in Philippines (PC,A)	December 2014	This objective was achieved. The lung scoring study established the prevalence of gross pathological lesions in pigs and provided insight into the risk factors associated with high lung scores and the occurrence of pleurisy. The Project Field Veterinarian, and Veterinarians and Technical Staff of Provincial Veterinary Offices of Bulacan and Pampanga were intensively trained on lung scoring and necropsy procedures. The validation study demonstrated that the front line diagnostic assays developed for use at RADDL Region 3 were functioning well. *P. multocida* and *H. parasuis* were confirmed as being present in pigs.
3.2	Utilise the relevant new diagnostic tests to aid in the investigation and control of porcine respiratory disease outbreaks in Australia	All new bacterial diagnostic tests developed within the project are formally documented as a Standard Operating Protocol and available to the Australian pig industry via the DEEDI reference laboratory services (A)	December 2014	This objective was achieved. Over the term of the project, the Australian-based research group became a fully integrated UQ and Queensland Government laboratory.

no.	Activity	outputs/ milestones	completion date	Comments
		All new bacterial diagnostic tests developed within the project are formally offered to the DEEDI and UQ diagnostic laboratories that serve the Queensland pig industry (A)	December 2014	This objective was achieved. As noted above, the main Australian host laboratory became an integrated UQ/Queensland Government laboratory. The integrated laboratory offers a user pays service that includes the new PCR based serotyping of *A. pleuropneumoniae* and the MLST typing of *H. parasuis* to the Australian pig industry. As the assay that was planned to distinguish virulent and non-virulent *H. parasuis* was shown to be un-reliable, this assay is not offered to the Australian pig industry.

PC = partner country, A = Australia

Objective 4: To establish novel mechanisms for communication among industry, government and research institutions in the Philippines on pig health.

no.	Activity	outputs/ milestones	completion date	Comments
4.1	Establish Project Overview Group	Identify all relevant stakeholders involved in the broad area of pig health (PC)	March 2011	This milestone was achieved.
		Establishment of a project overview group (PC)	June 2011	This milestone was achieved.
		First annual meeting of project overview group (PC)	March 2011	This milestone was achieved.
		Second annual meeting of project overview group (PC)	April 2012	This milestone was achieved.
		Third annual meeting of project overview group (PC)	April 2013	This milestone was achieved.
4.2	Review current mechanisms for communication used by stakeholders	Stakeholder analysis undertaken to identify key issues that impact on communication (PC)	June 2011	This milestone was achieved. A Stakeholder Matrix was developed.
		Monitor and evaluate current communication mechanisms used in industry (PC)	October 2011	This milestone was achieved. A Communication Research Study was conducted that involved a total of 428 respondents who covered commercial farms, backyard farms, livestock and meat inspectors, field veterinarians, laboratory veterinarians, pharmaceutical representatives, slaughterhouse operators and livestock traders.
4.3	Establish novel mechanisms for communication	On basis of communication theory and on the outcomes of Activity 4.2, novel mechanisms for communication identified and proposed (PC)	March 2012	This milestone was achieved. A suite of communication mechanisms (brochures/flyers, formal documents, Webpage, training/teaching modules, project database) were identified based on the outcomes of the Stakeholder Analysis.
		Novel mechanisms for communication established (PC)	December 2014	This milestone was achieved – with the communication activities using all of the mechanisms identified as key communication means in the Research Study under Objective 4.2

no.	Activity	outputs/ milestones	completion date	Comments
4.4	Review of efficacy of novel mechanisms for communication	Stakeholder analysis undertaken and completed to aid in evaluation of efficacy of novel communication mechanisms	December 2014	This milestone was not achieved. While communication mechanisms were developed and used (see Objective 4.3), no formal review based on Stakeholder Analysis was able to be undertaken. Evaluation of developed communication mechanism is appropriately done after the project to effectively determine its usefulness.
4.5	Collation of available industry statistics	Collate and summarise available industry statistics and other data.	December 2014	This milestone was achieved.

7 Key results and discussion

Objective 1. Identify technical gaps in field outbreak investigation and surveillance, service and reference laboratory systems and technologies.

The audit addressing this Objective sought to document the capacities of the two government laboratories associated with this project in terms of diagnostic tests relevant to porcine respiratory diseases. An example of the type of analysis undertaken is provided in Table 7.1 for the central PAHC laboratory. A similar analysis was performed for RADDL Region 3 as well as the field services provided in Bulacan and Pamapanga provinces by the relevant PVOs.

The audit also reviewed the research capacity, expertise and technical support level of the laboratory of Dr C. Domingo at CLSU.

The audit provided clear evidence that the basis of an effective diagnostic testing service was available via the key government provided services - regional field staff from PVOs in Bulacan and Pampanga, RADDL Region 3, and PAHC. The audit identified a number of areas where improvements in methodologies and/or documentation would significantly improve diagnostic services and these are presented in the relevant sections below.

RADDL Region 3

At this frontline diagnostic laboratory, the key needs were identified as follows:-

- For improved isolation and identification of growth factor dependent pathogens – *A. pleuropneumoniae* and *H. parasuis*
- For abbreviated, sustainable, validated, document identification schemes for major porcine respiratory pathogens
- Access to internationally recognised QC strains to ensure on-going quality of laboratory tests and methodologies
- Tto adopt internationally approved guidelines for the performance and interpretation of antimicrobial sensitivity tests (including use of internationally recognised QC strains)
- For a rapid molecular based assay for swine influenza
- To evaluate if LAMP technology, widely regarded as an effective, can be a sustainable, low cost alternative to PCR assays

Region 3 Field Services

The audit indicated that there was a need for formal documented protocols to cover the key activities associated with routine disease investigations on pig farms. Specific needs were as follows:-

- Farm investigation biosecurity protocol (biosecurity checklist and clearance, exit itinerary)
- Farm disease investigation form
- Necropsy form
- Laboratory examination request form

As well, the audit identified a need to develop the skills (and associated documentation) associated with lung scoring – a process of formally evaluating the gross signs of respiratory disease in slaughtered pigs.

PAHC

As PAHC has a front-line diagnostic role as well as a reference, centralised testing role, the audit identified two different sets of needs. The first set of needs was linked with the front-line diagnostic service role of PAHC and was very similar to those needs identified for RADDL Region 3:-

- For improved isolation and identification of growth factor dependent pathogens – *A. pleuropneumoniae* and *H. parasuis*
- For abbreviated, sustainable, validated, document identification schemes for major porcine respiratory pathogens
- Access to internationally recognised QC strains to ensure on-going quality of laboratory tests and methodologies
- To adopt internationally approved guidelines for the performance and interpretation of antimicrobial sensitivity tests (including use of internationally recognised QC strains)

In the role of a centralised, specialised reference centre, the audit identified additional needs for PAHC:-

- For rapid specific molecular based assays for the identification of key pathogens - *A. pleuropneumoniae*, *H. parasuis* and *P. multocida*
- For molecular based assays for the serotyping/genotyping of *A. pleuropneumoniae* and *P. multocida*
- For molecular assay for quantification of PCV2

The audit noted the need for a rapid and specific assay for PRRS but also noted that other research projects at PAHC were working on these assays. Hence, to best use available resources, the recommendation was to focus the ACIAR activities on the PCV2 test development.

Capacities at CLSU

The audit identified that Dr C. Domingo and her group at CLSU had considerable expertise and experience in the development of LAMP assays. The published literature had identified that this alternative technology was of considerable potential value (if the technology can be fully validated) for front-line diagnostic laboratories such as RADDL Region 3. Hence, the recommendation was that CLSU proceed to develop LAMP assays for *A. pleuropneumoniae*, *H. parasuis* and *M. hyopneumoniae* with the intention being to evaluate these assays for their potential to be introduced as routine diagnostic assays at RADDL Region 3.

Table 7.1. Overview of diagnostic services provided by PAHC

Discipline	Assay/Service	Turn-around Time
Pathology	Gross Pathology/Necropsy	1 - 2 days
	Histopathology	2 - 3 weeks
Virology	Serology (commercial ELISA Kits) Porcine Respiratory and Reproductive Syndrome Swine Influenza Virus PCV2	3 days
	Fluorescent Antibody Tests Porcine Respiratory and Reproductive Syndrome Swine Influenza Virus PCV2	3 days
	Molecular Assays Porcine Respiratory and Reproductive Syndrome (under development)	
Bacteriology	Culture and identification	5 – 7 days
	Antibiotic sensitivity testing	5 – 7 days
	Serology (commercial ELISA Kits) *A. pleuropneumoniae* *M. hyopneumoniae*	3 days

Objective 2. Develop cost-effective laboratory tests for bacteria and viruses associated with porcine respiratory diseases in both countries.

Philippines Component

Regional Animal Disease Diagnostic Laboratory

A series of SOPs that emphasised sustainable, low cost methodologies that suited a front-line laboratory were produced. An example of such a methodology protocol is provided as part of Appendix 11.7 (indole spot test). Over a dozen of these simple, relevant identification protocols were produced.

A key set of bacterial pathogens that was identified in the audit stage as requiring attention were the growth factor dependent pathogens – *A. pleuropneumoniae* and *H. parasuis*. These bacteria are characterised by an inability to grow on the normal (default) isolation medium used almost universally in veterinary diagnostic laboratories. The RADDL staff were trained on how to grow these key pathogens on primary isolation plates by use of “nurse” colony to support the growth of *A. pleuropneumoniae* and/or *H. parasuis*. While this isolation technique is effective (the organisms are detected by their unique growth pattern of appearing as satellitic growth around the nurse colony (see Fig 7.2) further characterisation work is very difficult due to the poor growth. Hence, the RADDL staff were trained in the production of a complete growth medium (termed BA/SN) that provides luxuriant growth of either *A. pleuropneumoniae* or *H. parasuis* (see Fig 7.2.1). The technology of the preparation of the compete growth medium suitable for *A. pleuropneumoniae* and *H. parasuis* was established at RADDL Region 3 (Appendix 11.7 shows the details of the medium).

As well, a set of simplified identification tables that allowed the key pathogens likely to be encountered in a front-line diagnostic laboratory was produced (see Appendix 11.8 for examples of these Tables). These identification protocols allowed effective, low cost diagnostic services as the laboratory was able to reduce the use of expensive imported commercial identification kits.

A key support mechanism provided by the project was the provision of a set of QC strains to allow on-going active assurance programs to monitor the performance of isolation and identification protocols. These strains were carefully selected in full consultation with the relevant quarantine authorities and were imported with full approval of those authorities. These QC strains will be an asset long into the future. (Details of the QC strains are provided in Appendix 11.9).

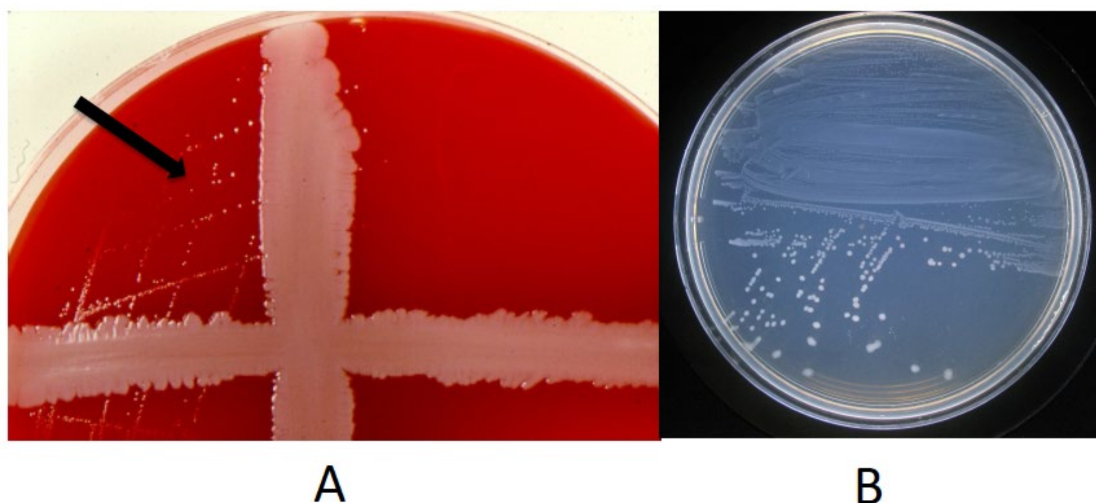


Figure 7.1. Typical growth of *H. parasuis* in the laboratory. A. The tiny dewdrop colonies of *H. parasuis* (marked by arrow) show the characteristic satellitic growth around the white nurse colony (*Staphylococcus hyicus)*. This is the typical isolation methodology. B. A pure culture of *H. parasuis* growing on the complete growth medium (BA/SN) used in this project.

Other key changes at RADDL Region 3 associated with the project were

A) Adoption of standardised protocol for antimicrobial sensitivity testing

B) Polymerase chain reaction assay for Swine Influenza Virus.

Overall, the progress in laboratory-based diagnostics at RADDL Region 3 has been very good. A range of assays and protocols are now embedded at the laboratory and key staff have been trained extensively (some staff via visits to Australia and others by hands-on training at RADDL Region 3).

Regional Field Staff

Considerable effort was spent on developing standard protocols for on-farm disease investigation processes as well as slaughterhouse-based methods to assess respiratory disease (lung scoring). A number of protocols and forms were developed and examples are provided in Appendix 11.10.

The key results achieved by the project were as follows:-

- Improved field investigation, surveillance and diagnosis system
- Standardized farm investigation biosecurity protocol (biosecurity checklist and clearance, exit itinerary)
- Standardized farm disease investigation form
- Standardized necropsy form
- Standardized laboratory examination request form
- Standardized lung scoring protocol and associated forms.

The adoption and implementation of the above protocols and procedures along with extensive hands-on training has established a core of relevant, experienced field staff who have the capacity to contribute strongly to on-going health support programs and activities for the pig industry in Region 3.

PAHC

The focus of work at PAHC covered the same sustainable, cost effective isolation and identification methodologies established in RADDL Region 3. As well, given the nature of the role of PAHC, a central facility providing specialised assays with access to a high level of technology, a series of additional molecular-based assays were targeted for establishment and validation at PAHC.

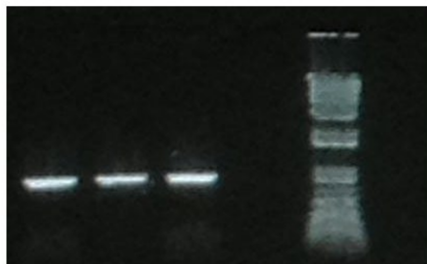
In terms of bacterial pathogens, successful implementation at PAHC of PCR-based assays for the identification of *A. pleuropneumoniae*, *H. parasuis* and *P. multocida* was achieved. These assays were all transferred as already validated assays operating in the referral services provided by the MRG to the Australian pig industry. Relevant PAHC staff were trained in all three assays (some in Australia and some in the Philippines). All assays were supported by QC materials (positive and negative controls, some reagents and other components) provided by the MRG. The assays were as follows:-

A) A multiplex PCR that identifies an isolate as *A. pleuropneumoniae* and then determines if the isolate is serovar 1, 5, 7 or 12. This assay is the assay developed within this project (see full details in Objective 2, Australian component). For practical reasons, the assay was transferred early in the project before the serovar 15 component had been developed by the UQ group. PAHC staff were able to establish the assay and show that the assay could identify *A. pleuropneumoniae* and successfully recognise serovars 1, 5, 7 and 12 (see Figure 7. 2B).

B) A PCR that specifically identifies *H. parasuis*. This assay was originally developed in the USA (Oliveira et al., 2001). A typical result achieved at PAHC is shown in Figure 7. 2 A

C) A PCR that specifically identifies *P. multocida* and then assigns the isolate one of eight lipopolysaccharide (LPS) genotypes. The assay was developed in a collaboration between the MRG and colleagues at Monash University (Harper et al., 2015). In the original format, the assay involves nine different reactions in the one tube (Harper et al., 2015) and is a very technically demanding assay to perform. Despite repeated efforts, the full version of the identification and typing assay could not be established at PAHC. However, a simplified version of the assay – a test that identifies an isolate as *P. multocida* but does not attempt any LPS typing was successfully established.

A – *H. parasuis* PCR



B – *A. pleuropneumoniae* identification and serotyping PCR

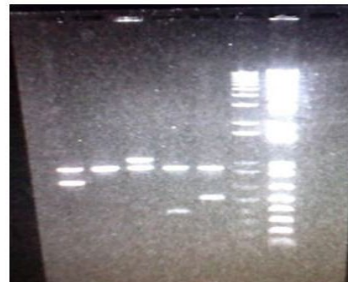


Figure 7.2. Results of PCR assays run at PAHC. Figure A shows the *H. parasuis* assay. Lanes 1 and 2 – Field isolates; Lane 3 – Reference strain of *H. parasuis*; Lane 4 - Molecular Weight Marker. The single band present in Lanes 1 and 2 (which matches the positive control in Lane 3) confirms the isolates as *H. parasuis*. Figure B shows the *A. pleuropneumoniae* PCR that confirms identity and recognises serovars 1, 5, 7 and 12. Lanes 1, 2, 3, 4, and 5 – Reference strains for *A. pleuropneumoniae* serovars 1, 3, 5, 7, and 12 respectively; Lanes 6 and 7 – Molecular Weight Marker. The common band present in Lanes 1 to 6 confirms the isolates are all *A. pleuropneumoniae*. In Lane 1, the lower band confirms isolate as serovar 1. In Lane 2, there is no other band, therefore this isolate is not serovar 1, 5, 7 or 12. In Lane 3, the upper band confirms isolate as serovar 5. In lane 4, the lower band confirms isolate as serovar 7. In lane 5, the lower band confirms isolate as serovar 12.

Considerable effort went into the establishment of a suitable assay for PCV2. A number of assays were established – a PCR, an immunohistochemistry (IHC) assay and a quantitative real-time PCR.

While a PCR was established, the assay was not highly useful in a diagnostic setting. The challenge for diagnostic laboratories is that PCV2 is a common infection. In circumstances where the level of PCV2 rises above a critical level, the infected animal can start to show the signs of one of PCV2 linked diseases – for example postweaning multisystemic wasting syndrome (Olvera et al., 2004). Hence, an assay that simply identifies the presence of PCV2 is not a highly useful assay in a diagnostic sense.

In contrast, the IHC assay is a simple assay that requires only a low level of technology and is a relevant assay in terms of providing information on the possible role of PCV2 in disease conditions (O'Dea et al., 2011). When used on an appropriate tissue such as lymphoid tissue, IHC allows the specific detection of PCV2 and an evaluation of whether lesions suggestive of a PCV2 linked disease are present in the tissue and associated with the presence of PCV2 (O'Dea, 2010). With appropriate training in Australia (at the laboratory of Dr O’Dea and the provision of positive controls and essential reagents, Dr Lola (project staff based at PAHC) was able to establish and validate (at an analytical level) the IHC assay for PCV2 at PAHC. The main disadvantage to all IHC assays is the need for experienced staff to produce the necessary tissue slides and then perform and read the results of the assay. Hence, an IHC assay is not easily adapted to a high throughput situation. In the case of the current project, the departure of Dr Lola from the project to alternative employment meant that use of the IHC in the field validation studies was not feasible. However, the reagents and protocols remain available within PAHC and could be used in future diagnostic work if suitable staff are available.

After considerable effort, a functioning real-time PCR for PCV2 has been established. The assay is a multiplex assay that detects a 100 base pair (bp) segment of the PCV2 and a section of the 18S rDNA of the pig. The pig target has to be positive to confirm that the sample has been appropriately prepared (termed an internal control). The amount of the 100 bp target that is detected can be quantified by comparison with a standard curve that is performed in each run. In the initial establishment of this assay, there were difficulties in producing an acceptable standard curve as defined by closeness of fit (R^2 value) and the

efficiency of the reaction (a calculation provided by the software that analyses the results). This version of the assay used a positive control which was a plasmid containing the PCV2 genome. A new version of the positive control was developed by having the 100 bp sequence constructed by a commercial supplier of short DNA sequences (in the same manner as primers are artificially constructed short segments of DNA). The advantage of the constructed positive control is that there is a precise knowledge of the amount of the control DNA, allowing the preparation of a dilution series that contains known amounts of the target DNA. With repeated testing and refinement of procedures, a functioning real-time PCR that gave a good closeness of fit and a good reaction efficiency was achieved (see Figure 7. 3). Unfortunately, this stage (a valid assay with all internal controls functioning appropriately) was only achieved late in the project. Hence, the assay has not been used on field samples collected in the validation study.

Overall, a suite of new relevant molecular assays have been established and fully validated at PAHC. These assays allow the confident recognition of key major bacterial pathogens (*A. pleuropneumoniae*, *H. parasuis* and *P. multocida*). As well, PAHC has a validated capacity to recognise *A. pleuropneumoniae* serovars 1, 5, 7 and 12 by PCR. This suite of assays represent a key new capacity for PAHC. The assays have been embedded at PAHC and are supported by relevant QC materials. These assays will prove of long term benefit to the pig industry of the Philippines.

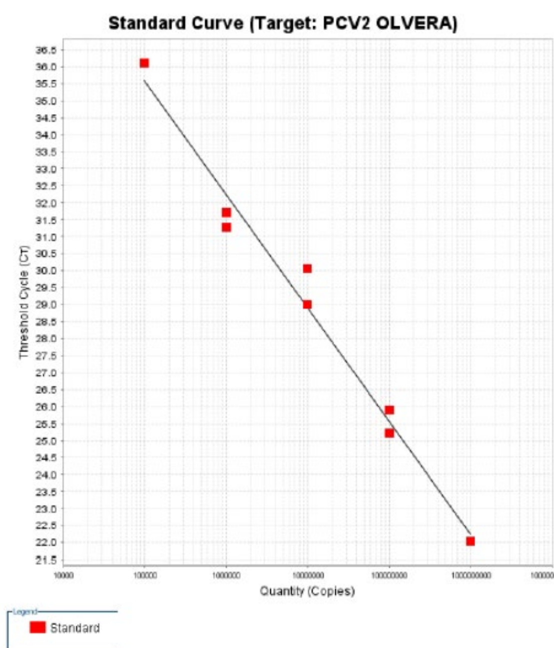


Figure 7.3. Results from a real-time PCV-2 PCR run showing the results of the standard curve. The standard curve had an acceptable R^2 value (0.977) and an acceptable efficiency of reaction (99.581%)

CLSU Component

The CLSU component of Objective 2 was focused on the development of LAMP assays for key bacterial pathogens – *A. pleuropneumoniae*, *H. parasuis* and *M. hyopneumoniae*. While existing assays were used for this work, each assay had to be implemented and validated within the operating environment of the CLSU research group.

For *A. pleuropneumoniae* and *H. parasuis*, the work at CLSU resulted in a specific assay that gave a positive result with the relevant target species and negative results with bacteria that were both close relatives and/or bacteria that are likely to be present in the respiratory tract of pigs. This specificity testing was done using bacteria and/or bacterial DNA supplied by the MRG Figure 7.4 illustrates the specificity of the *A. pleuropneumoniae* LAMP assay.

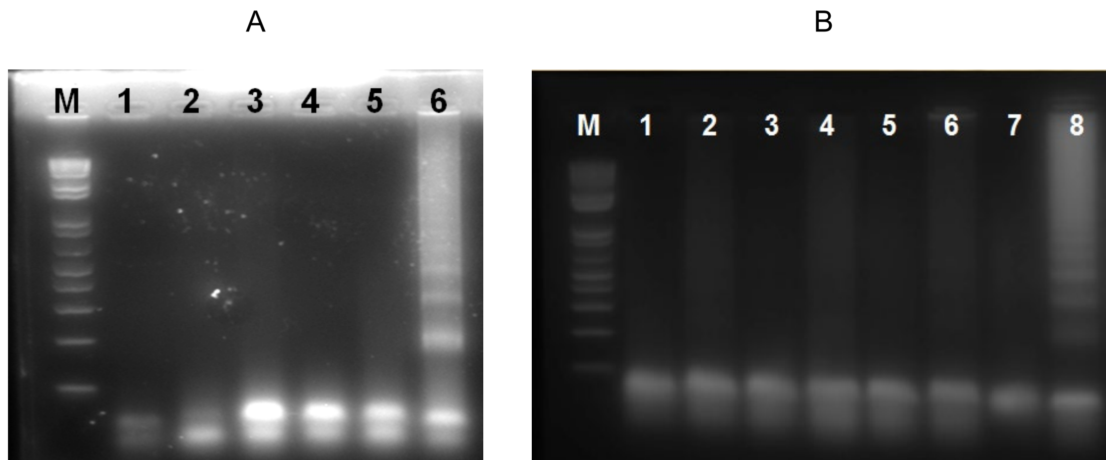


Figure 7.4. Results of specificity evaluation of LAMP for *A. pleuropneumoniae*.

A. Lane M - molecular weight marker; Lane 1- *Streptococcus suis*; Lane 2 – negative control (water); Lane 3 – *Haemophilus parasuis*; Lane 4 – *Mycoplasma hyopneumoniae*; Lane 5 – *Pasteurella multocida*; Lane 6 – *Actinobacillus pleuropneumoniae*. Only Lane 6 shows the typical multiple bands seen in a positive LAMP reaction.

B. Lane M - molecular weight marker; Lane 1 - *Actinobacillus equuli;* Lane 2 - *Actinobacillus indolicus ;* Lane 3 - *Actinobacillus minor;* Lane 4 - *Actinobacillus porcinus;* Lane 5 - *Actinobacillus rossi;* Lane 6 - *Actinobacillus suis;* Lane 7 - negative control (water)*;* Lane 8 = *Actinobacillus pleuropneumoniae*. Only Lane 8 shows the typical multiple bands seen in a positive LAMP reaction.

The specificity results for the *M. hyopneumoniae* LAMP were not as clear cut as for the *A. pleuropneumoniae* and *H. parasuis* assays. The assay gave no reaction with a collection of bacterial pig respiratory pathogens outside the genus *Mycoplasma.* Unfortunately, the *M. hyopneumoniae* LAMP assay gave false positive reactions with a number of field isolates of *M. hyorhinis* (see Figure 7.5).

Diagnostic laboratories have traditionally regarded *M. hyorhinis* as part of the normal respiratory flora of pigs (Clavijo et al., 2014). In recent times, there has been a recognition that, while the organism can indeed be a part of the normal flora, *M. hyorhinis* can be a cause of systemic disease in young piglets (Clavijo et al., 2014).

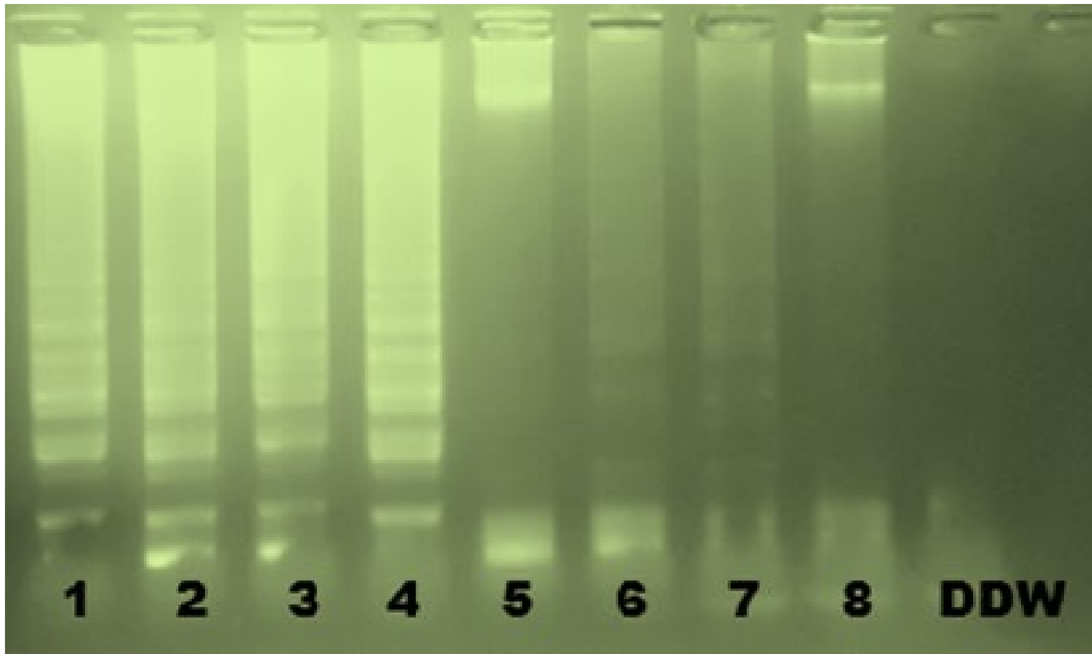


Figure 7.5. Results of a specificity evaluation of the LAMP assay for *M. hyopneumoniae.* Lanes 1 – 8 field strains of *Mycoplasma hyorhinis*. Lane DDW – negative control – double distilled water. The multiple bands seen in Lanes 1 to 4 are clear false positives. The low level of bands in Lanes 5 and 8 also should not be seen in a clear negative reaction.

The false positives associated with *M. hyorhinis* in the *M. hyopneumoniae* LAMP are a problem. It is not clear why the false positives have occurred. In the original publication describing the *M. hyopneumoniae* LAMP (Li et al., 2013), the assay was shown to not yield false positives when tested with a number of *M. hyorhinis* strains. Given this situation of false positives, the use of this assay in routine diagnostic laboratories does not appear to be a realistic option at this stage.

Australian Component

Multiplex PCR for identification and serotyping of A. pleuropneumoniae

The final version of the PCR developed in this work was evaluated with the reference strain for each of the 15 serovars and 411 field isolates (394 from Australia, 11 from Indonesia, five from Mexico and one from New Zealand) of *A. pleuropneumoniae*. These strains and field isolates were used to evaluate the serotyping performance of the assay. The ability of the assay to correctly identify an isolate as *A. pleuropneumoniae* was evaluated using 26 non-*A. pleuropneumoniae* species.

The assay was designed to provide a species level identification (is the isolate *A. pleuropneumoniae*?) and then to determine if the isolate was serovar 1, 5, 7, 12 or 15. If the tested isolate was *A. pleuropneumoniae*, the PCR was designed to produce a product that was 951 bp in size. If an isolate was not *A. pleuropneumoniae*, no product of 951 bp should be produced. The PCR was further designed to ensure that each of the five different possible confirmed serovar results would gave a unique size product – serovar 1 754 bp, serovar 5 1,114 bp, serovar 7 396 bp, serovar 12 557 bp and serovar 15 269 bp. Hence, an isolate that was *A. pleuropneumoniae* serovar 15 would give two bands – a 951 bp band (confirming the isolate as *A. pleuropneumoniae*) and another band of 269 bp. An isolate that was *A. pleuropneumoniae* but not serovar 1, 5, 7, 12 or 15 would give just one band (the species specific band of 951 bp). An isolate that was not *A. pleuropneumoniae* should give no band at all.

The multiplex PCR gave the expected results with all 15 serovar reference strains. The serovar 1, 5, 7, 12 and 15 reference strains gave the expected two bands (the species

specific band plus the seorvar specific band) while the other reference strains for the remaining 10 serovars gave only the species specific band. The results of the reference strains are shown in Figure 7. 6.

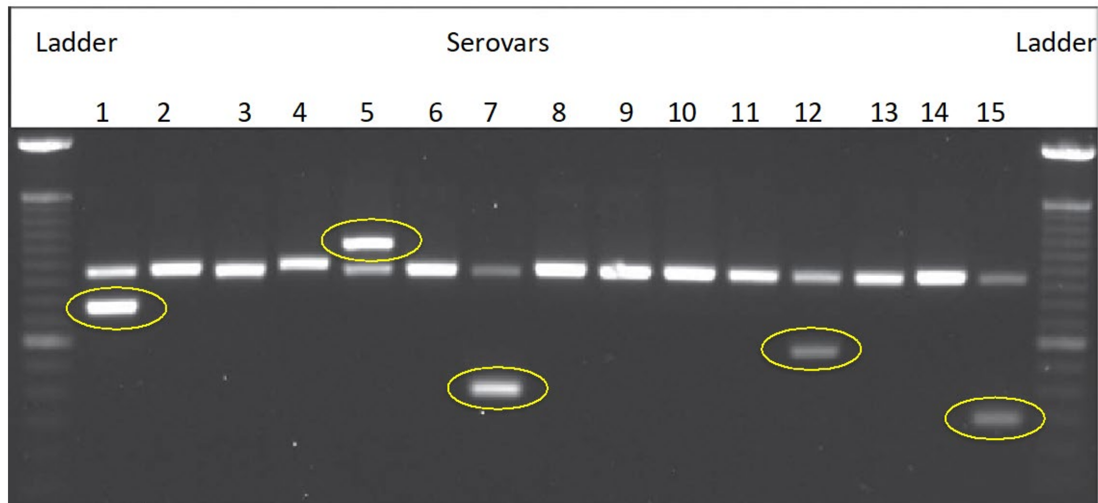


Figure 7.6. Results of use of multiplex identification/serotyping PCR on the 15 reference strains for the recognised serovars of *A. pleuropneumoniae*. Each serovar has given the species-specific band (as expected) of 951 bp. The reference strains for serovars 1, 5, 7, 12 and 15 show the specific additional serovar-specific band - serovar 1 754 bp, serovar 5 1,114 bp, serovar 7 396 bp, serovar 12 557 bp and serovar 15 269 bp (yellow circles).

For the majority of the field isolates, there was full agreement between the conventional serotyping and the new multiplex PCR for serovars 1 (n = 46), 5 (n = 81), 7 (n = 80), 12 (n = 16) and serovar 15 (n = 117). There were four isolates where the conventional serotyping (three being serovar 3 and one being serovar 6) where the multiplex PCR gave a different result – all four being identified as serovar 15. It is known that some serovar 15 isolates can be mis-identified by conventional serotyping as serovar 3, 6 or 8 (Gottschalk, 2007). A mis-identification by the conventional assay thus appears to be the most likely explanation for the only disagreement between conventional serotyping and the PCR assay developed in this study.

There were 25 isolates that could not be confidently serotyped due to cross-reactions in the conventional test – with 10 of those isolates being shown to be either serovar 7 (4 isolates) or 12 (six isolates). There were also another 25 isolates that gave no reaction in the conventional serotyping – with the multiplex assigning 23 of these to serovars 1 (one isolate), 5 (one isolate), 7 (four isolates) and 15 (17 isolates) with the remaining two isolates giving no serovar-specific band. Hence, the PCR markedly outperformed conventional serotyping – allowing the recognition of the serovar of 33 isolates of a total of 50 isolates that could not be confidently serotyped by conventional methods (due to either cross reactions or no reaction).

Importantly, the species-specific product (951 bp) was amplified in the multiplex PCR with all 411 *A. pleuropneumoniae* field isolates. None of the non-target species produced the species-specific band – the expected result.

The decision to develop a multiplex PCR to recognised serovars 1, 5, 7, 12 and 15 was made for a variety of reasons. Serovars 1, 5 and 7 are important serovars in terms of prevalence in a number of geographical regions including North America (Gottschalk and Taylor, 2006) and Australia (Blackall et al., 1999; Blackall and Pahoff, 1995). In addition, serovars 1 and 5 are widely recognised as being more virulent than other serovars (Gottschalk and Taylor, 2006). Primers targeting serovars 12 and 15 were included into the multiplex PCR as there has been considerable confusion about the capacity to separate serovars 12 and 15. In the formal description of serovar 15, Blackall *et al.* (2002) reported that isolates of serovar 15 were initially thought to be serovar 12. Serovar 15 is amongst the top one or two serovars of *A. pleuropneumoniae* found in Australian pigs

(Blackall et al., 2002), making correct recognition of this serovar an important issue in Australia. The fact that serovar 15 has now been reported in Japan (Koyama et al., 2007), Thailand (Tonpitak, 2010) as well as North, Central and South America (Broes et al., 2007; Gottschalk, 2007) increases the likelihood of laboratories in other countries incorrectly serotyping field isolates of this serovar. Importantly, it is known that mis-identification of serovar 15 as either serovar 12 (Blackall et al., 2002) or serovars 3, 6 and 8 (Gottschalk, 2007) is a common problem.

Overall, the PCR developed in this study is a key step forward in the ability of diagnostic laboratories around the world to correctly serotype *A. pleuropneumoniae*. As the common killed pleuropneumonia vaccines provide protection only against the serovar or serovars present in the vaccine (Gottschalk and Taylor, 2006), the ability to quickly and confidently serotype *A. pleuropneumoniae* isolates is a critical diagnostic capacity in the development and monitoring of effective, sustainable prevention and control programs for this major pig disease.

Typing of H. parasuis

This work involved two different approaches – typing with a focus on pathogenic and non-pathogenic isolates (virulence gene typing assays) and typing with a focus on being able to separate isolates of *H. parasuis* that had no epidemiological connection while grouping together *H. parasuis* isolates that were epidemiologically connected (MLST).

For the purpose of this study, the following definitions have been used – pathogenicity is the ability of an organism to cause disease (a yes / no state); virulence is the degree of pathogenicity of an isolate; virulence genes are genes that are thought to play a role in determining firstly if an isolate is pathogenic or not and then secondly (if pathogenic) the virulence of an isolate.

The virulence gene typing assays were first used on the 15 serovar reference strains of *H. parasuis*. These strains have been well characterised in terms of virulence (Kielstein and Rapp-Gabrielson, 1992) and this prior knowledge allowed an evaluation of the potential of the various virulence gene-associated PCRs to correctly predict the pathogenicity (and possibly the virulence) of a strain. Table 7.2 shows the results of all seven assays when applied to all 15 strains. The results of the *vtaA* PCR are shown in Figure 7. 7.

The results of the testing of the 76 field isolates is summarised in Table 7.3 The association of pathogenicity with these field isolates was based on the assumption that the experimentally confirmed pathogenicity levels of the reference serovar strains shown in Table 7.3 applied to all field isolates of the same serovar.

Table 7.2. Results of PCR assays for potential virulence markers in 15 reference strains of *H. parasuis*.

Serovar	Code	Virulence*	Result for indicate candidate virulence gene**					
			vtaA	*hhdA*	*hhdB*	*lsgB*	*fhuA*	*capD*
1	NR4	++	-	-	-	-	-	-
2	SW140	+	+	-	-	-	-	-
3	SW114	-	-	-	-	-	-	-
4	SW124	+	+	-	-	-	-	-
5	Nagasaki	++	+	+	+	+	+	+
6	131	-	-	-	-	-	-	-
7	174	-	+	-	-	-	-	-
8	C5	+	+	-	-	-	-	-
9	D74	-	-	-	-	-	-	-
10	H367	++	+	+	-	-	+	+
11	H465	-	+	-	-	-	-	-
12	H425	++	+	+	+	+	+	-
13	IA-84-17975	++	+	+	-	-	+	-
14	IA-84-22113	++	+	+	+	-	+	-
15	SD-84-15995	+	+	+	-	-	+	-

* The virulence results shown in this Table are those assigned by Kielstein *et al.* (1992). In that study, the virulence was scored as follows: + +, death of pigs within 96 h post-inoculation; +, clinical symptoms and systemic gross lesions of polyserositis and arthritis at necropsy; ±, mild clinical symptoms or gross lesions at necropsy; 0, no clinical symptoms or gross lesions at necropsy.
** In the *vtaA* assay, all strains gave the smaller (291 bp) band. A positive result in the *vtaA* assay indicates the presence of the larger (406 bp) band.

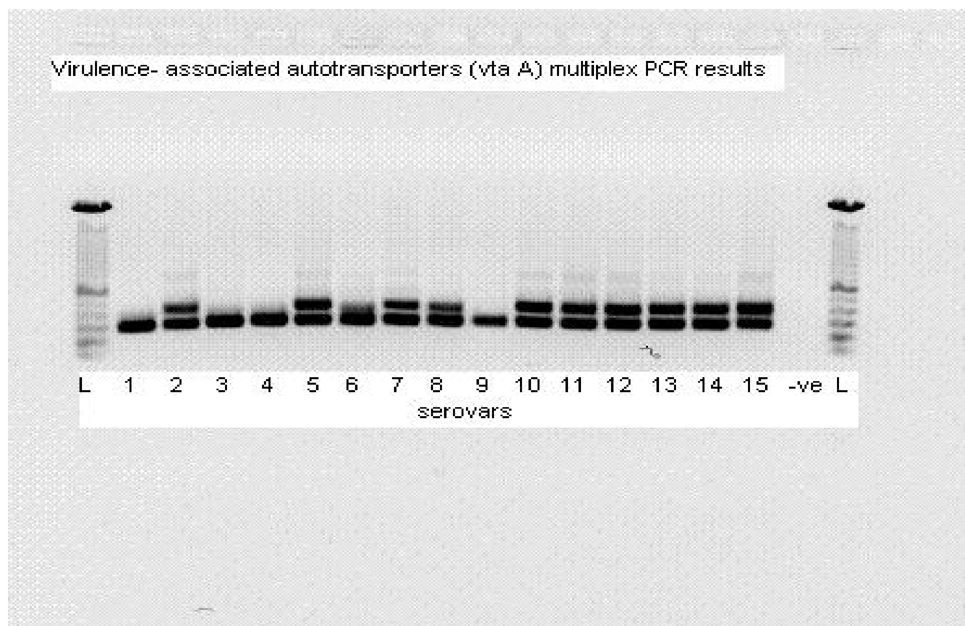


Figure 7.7. Results of the *vtaA* PCR for the 15 serovar reference strains of *H. parasuis*. Lanes marked L are molecular weight markers. All *H. parasuis* should give the smaller band (291 bp). Strains that are pathogenic are reported to be characterized by a larger second band of 406 bp. In this gel, the reference strains all give the expected smaller band and serovars 2, 5, 7, 8, 10, 11, 12, 13, 14 and 15 gave the second (larger – 406 bp) band that has been associated with pathogenicity.

The results of testing the reference strains show that there is no perfect correlation between the presence of any virulence gene and either pathogenicity or virulence. As an example, the serovar 1 reference strain was highly virulent but lacked any of the virulence genes. Conversely, the serovar 11 reference strain was non-pathogenic but was positive for *vtaA*.

The results of the testing of the 76 field isolates for the potential virulence genes is summarised in Table 7.2. The allocation of these field isolates as being pathogenic (and the associated virulence level) or not was based on the assumption that field isolates had the same virulence as the reference serovar strain shown in Table 7.2. This means that all serovar 5 isolates from the field were assumed to have a virulence score of ++ (the same as shown for the reference serovar 5 strain by Kielstein *et al.* (1992). The virulence levels were as follows ++ = highly virulent; + = virulent and 0 = non-pathogenic. The presence of *vtaA* or *hhdA* alone was significantly higher in the combined highly virulent and moderately virulent than in the non-pathogenic isolates (Fisher's exact test, $p < 0.05$). As well, the presence of either *vtaA* or *hhdA* or both was significantly higher in the combined highly pathogenic and moderately pathogenic than in the non-pathogenic isolates (Fisher's exact test, $p < 0.05$).

Both the work with the reference strains and the field isolate showed that no single virulence gene or indeed any combination of potential virulence genes could clearly, confidently and correctly predict the pathogenicity or virulence of any single strain of *H. parasuis*. As an example, strain NR4 (the reference strain for serovar 1) is highly pathogenic (Kielstein and Rapp-Gabrielson, 1992) but gave a negative reaction in all five virulence gene PCR assays. However, the use of the larger collection of field isolates did show a statistically significant association between pathogenicity and the presence of either *vtaA* or *hhdA.* Indeed, the presence of either or both of these genes resulted occurred in 80% of pathogenic field isolates while only 25% of non-pathogenic isolates had either or both of these genes. Hence, while the *vtaA* and *hhdA* PCR assays established in this work are helpful when looking at populations or collections of isolates, the assays are not suitable for the confident confirmation of an isolate as pathogenic.

Table 7.3 Results of PCR assays for potential virulence genes in 76 field isolates of *H. parasuis*.*

Presumed Virulence	Positive by PCR for the indicated virulence gene						
	vtaA	*hhdA*	*hhdB*	*lsgB*	*fhuA*	*capD*	*vtaA* and/or *hhdA*
Highly virulent (24 isolates)	16 (66.7%)	17 (70.8%)	4 (16.7%)	2 (8.3%)	8 (33.3%)	6 (25%)	19 (79.2%)
Moderately virulent (21 isolates)	14 (66.7%)	8 (38.1%)	5 (23.8%)	0	2 (9.5%)	0	17 (81%)
Combined highly and moderately virulent (45 isolates)	30 (66.7%)	25 (55.6%)	9 (20%)	2 (4.4%)	10 (22.2%)	6 (13.3%)	36 (80%)
Not pathogenic (20 isolates)	4 (20%)	2 (10%)	1 (5%)	0	0	0	5 (25%)
Unknown (11 isolates)	5 (33%)	0	0	0	0	0	0

* The presumed virulence is based on the assumption that the field isolates of a serovar have the same virulence as the reference strain – with the reference strain pathogenicity having been determined previously by Kielstein *et al.* (1992).

The virulence gene typing assays were meant to assist in handling a major challenge with the prevention and control of disease conditions associated with *H. parasuis*. As *H. parasuis* is a normal inhabitant of the nasal cavity of pigs (Aragon et al., 2012), the key question is whether the colonising strains are pathogenic or not. While the assays established in this work are helpful at the population level, the fact that individual strains cannot be confidently identified as pathogenic or not means that the challenge of understanding the pathogenicity of field isolates remains to be addressed.

In the MLST work on *H. parasuis*, a total of 36 field isolates were successfully typed. This required the sequencing of seven genes in each isolate. A total of 32 different STs were recognised in the 36 isolates examined. This means that almost all *H. parasuis* isolates were genetically distinct from each other, a remarkable level of genetic diversity. The limited degree of genetic diversity is perhaps best illustrated by comparing these results with the first results to flow from the initial creation of the MLST scheme for the related organism – *P. multocida.* The initial creation of the *P. multocida* MLST scheme was performed by the MRG and involved 66 Australian avian isolates (Subaaharan et al., 2010). This initial *P. multocida* MLST found 29 STs amongst 66 isolates – a lower number of STs in a far greater number of strains than found for *H. parasuis* in the current work.

Further, when the 36 Australian isolates were compared with the 127 isolates from other countries currently held in the public *H. parasuis* MLST website, all but one of the Australian isolates were unique genotypes not previously seen by overseas based researchers. These results mean that it would appear that MLST offers considerable potential as a means of tracking *H. parasuis*. The availability of MLST as a typing tool – with that typing apparently detecting a very wide genetic diversity – means that the use of MLST should provide considerable insights into disease outbreaks and would allow the confident tracking of particular genetic types of *H. parasuis*.

Objective 3. Implement systems that apply current technology and new tests developed in this project (Objective 2) to determine causes of respiratory disease outbreaks in the Philippines and Australia.

Philippines Component

Under this Objective, two large field studies were performed. As the two field studies were quite different in nature, the key results and implications of each study are presented separately.

Lung Scoring Study

This large study had two components. The components are interrelated but, for ease of presentation and flow, the key results and discussion on the two components are presented separately below.

The production systems, the biosecurity practices and the herd health providers

This component dealt with describing the pig production systems in the two provinces of Bulacan and Pampanga. The study also examined the biosecurity practices of the surveyed pig farms. Finally, this component sought to identify source of herd health advice (termed herd health providers) for pig farmers in Bulacan and Pampanga. The full, detailed results and a full discussion of this work are provided in the published paper that arose from this work (Alawneh et al., 2014). The following text provides an overview and discussion of the key results.

A summary of the descriptive statistics (farm profile, farmer profile and choice of herd health provider is provided in Appendix . Hierarchical cluster analysis showed two clusters within each farm type – with these clusters being distinguished by the health advisors used by the farmers (see Table 7.4). Small holder and commercial farmers had significantly different combinations of herd health advisors. The preferred health advisors for smallholder farmers were agriculture supply stores (63%), government livestock inspector/agricultural technicians (50%) and government veterinarians (28%). In general, commercial farms were more reliant on private service providers - private consultants (61%), on farm veterinarians (51%) and pharmaceutical company representatives (48%).

The two clusters within the small holder farmers were termed SA (96 farms) and SB (275 farms). The SA farms had on more productive sows, more pigs on the farm, were more likely to house pigs in separate pens as opposed to free range, used a private veterinarian as the main health provider, and were less dependent on pig farming as a main source of income (all $P<0.01$) than SB farms.

Table 7.4. Details of farm (*n* = 471) and farm manager profiles of the two production types (smallholder, *n* = 371; commercial, *n*= 100) and herd health provider types (smallholder A, SA, *n* = 96; smallholder B, SB, *n* = 275; commercial A, CA, *n* = 67; and commercial B, CB, *n* = 33) for participating pig farms from two provinces in Philippines.

	Smallholder farms			Commercial farms			S vs.C
Profile	SA	SB	P[a]	CA	CB	P[a]	P [a]
Located in Bulacan	52 (54)[b]	113 (40)	0.03	24 (36)	18 (55)	0.09	0.73
Located in Pampanga	44 (46)	162 (60)		43 (64)	15 (45)		
Number of productive sows			<0.01			<0.01	<0.01
Median; Min, Max	0; 0, 30	0; 0, 18		223; 0, 2580	1; 0, [illegible]		
Mean; 1st quartile, 3rd quartile	3; 0, 3	1; 0, 0		395; 121, 425	39; 0, [illegible]		
Total number of animals			<0.01			<0.01	<0.01
Median; Min, Max	14; 1, 92	6; 0, 77		1520; 1, [illegible]	22; 0, [illegible]		
Mean; 1st quartile, 3rd quartile	22; 4, 11	9; 4, 11		3102; 629, [illegible]	481; 6, [illegible]		
Housing type							
Separate pens	61 (64)	120 (44)	<0.01	61 (91)	27 (81)	0.21	<0.01
Pen adjacent to house	32 (33)	87 (32)	0.78	3 (4)	2 (6)	0.20	<0.01
Free range	1 (1)	61 (22)	<0.01	0 (0)	1 (3)	0.9	<0.01
Other	2 (2)	7 (3)	0.20	13 (19)	3 (9)	0.14	<0.01
Herd health provider							
None	2 (2)	1 (0)	0.86	0 (0)	0 (0)	1.0	0.61
Technical consultant contract grower	4 (4)	3 (1)	0.07	2 (3)	1 (3)	1.0	0.61
Private veterinarian (consultant)	72 (75)	0 (0)	<0.01	52 (78)	9 (27)	<0.01	<0.01
Livestock inspector	40 (42)	145 (53)	<0.01	3 (5)	15 (46)	<0.01	<0.01
On-farm veterinarian	4 (4)	4 (2)	0.21	50 (75)	1 (3)	<0.01	<0.01
Hog farmer association	1 (1)	0 (0)	1.0	6 (9)	4 (12)	0.11	<0.01
Government veterinarian	1 (1)	103 (38)	<0.01	7 (10)	11 (33)	0.01	0.06
Fellow pig farmer	1 (1)	61 (22)	<0.01	3 (5)	7 (21)	0.65	0.11
Farmer co-operative	1 (1)	3 (1)	0.67	0 (0)	1 (3)	0.65	1.00
Pharmaceutical company representative	6 (6)	8 (3)	0.21	38 (57)	10 (30)	0.02	<0.01
Agricultural supply store	14 (15)	220 (80)	<0.01	2 (3)	23 (70)	<0.01	<0.01
Farm manager profile							
Gender			0.89			0.61	0.06
Female	28 (29)	84 (31)		12 (18)	8 (24)		
Male	68 (71)	191 (69)		55 (82)	25 (76)		
Education			0.41			<0.01	<0.01
Elementary or can't read	11 (12)	25 (9)		3 (5)	4 (12)		
High school	45 (47)	154 (56)		3 (5)	13 (39)		
College	40 (42)	96 (35)		61 (91)	16 (49)		
Years of experience			0.19			<0.01	0.061
Median (Min, Max)	7 (1, 39)	7 (1, 42)		22 (1, 35)	8 (1, 36)		
Mean (1st quartile, 3rd quartile)	10 (5, [illegible]	9 (5, 9)		20 (12, 28)	11 (7, [illegible]		
Proportion of income derived from raising pigs			<0.01			<0.01	<0.01
<25%	39 (41)	79 (29)		1 (2)	9 (27)		
25-50%	19 (20)	95 (35)		40 (60)	9 (27)		
51-75%	8 (8)	2 (1)		4 (6)	0 (0)		
>75%	2 (2)	1 (0)		4 (6)	0 (0)		
Unknown	28 (29)	98 (36)		18 (27)	15 (46)		

Key: *n* is number of farms, Min minimum, Max maximum, S smallholder farms; C commercial farms.

Within the two clusters of commercial farms, termed CA (67 farms) and CB (33 farms), the CB had fewer pigs, and were more likely to use livestock inspectors, pharmaceutical company representatives (P = 0.02), government veterinarians (P = 0.01), and agricultural stores (all P<0.01 except as indicated) as their health provider.

Descriptive statistics of farm management and biosecurity practices are presented in Table 7.5. Overall, commercial farms showed a higher frequency of good biosecurity practices related to semen introduction and use of artificial insemination, vaccination of sows and grower pigs, contact with pig transport vehicles, entrance protocol and hygiene levels, and sick and dead pig management (P = 0.05) (all at P <0.01 except where indicated). Overall, within smallholder and commercial farm clusters, the biosecurity practices of the two A subclusters (CA and SA) were better than those of the B subclusters (SB and CB). The specific practices of relevance were: contact and mixing with other animals or rodents, contact and farm entrance protocol, transport vehicles entrance protocol and hygiene levels, semen introduction and use of AI, vaccination of sows and grower pigs and sick and dead pig management.

Farmer affiliation networks were established for all farms, just commercial farms, just smallholder farms as well as the two subclusters within each farm type. These networks are fully illustrated in the formal publication (Alawneh et al., 2014). For this report, as an illustration of the overall network is shown in Figure 7.8. This overall network was fragmented – with one large component that consisted of 468 farms, 10 herd health providors and 974 links. The same results were found with the two small holder farm sub-clusters – a fragmented network with one giant component. However, the two commercial farm subclusters both comprised of one giant component.

A focus of the current study was to identify those main herd health providers who were more centrally located in the overall network and within each of the identified farmer group network structures. Except for commercial farms of subcluster CA, agricultural stores were more centrally located within each network structure. Smallholder farmers affiliated with GV were also affiliated with LIAT (*Pearson's* correlation 0.40; p<0.05). In general commercial farms were more strongly linked with private herd health providers compared with other providers in the network.

Overall, four different clusters of pig farms were identified in Region 3. These subclusters were based on their production type (smallholder or commercial) and the choice of herd health providers. The study found subtle but significant management and biosecurity practice differences between the clusters. Although larger smallholder and commercial farms had relatively better on-farm biosecurity compared with smaller smallholder and commercial farms, on-farm biosecurity as whole was low. The study found associations between the different farm clusters and the herd health providers. These associations may have been influenced by local trade issues and farmer socioeconomic status. Two subgroups of herd health providers (government and private providers) and one solitary provider (agricultural stores) were identified. Within Region 3, government, private providers, and agricultural supply stores seem to operate independently. While agricultural supply stores provided herd health services to the majority of farmers, these stores had little or no interaction with other providers. It is possible that veterinary authorities could achieve marked improvements in pig production efficiency by encouraging collaboration between private and government herd health providers in Region 3.

Table 7.5. Descriptive statistics of farm management and biosecurity management practices classified by production type (smallholder, *n* = 371; commercial, *n* = 100) and herd health provider (smallholder A, SA, *n* = 96; smallholder B, SB, *n* = 275; commercial A, CA, *n* = 67; and commercial B, CB, *n* = 33) for participating pig farms from two provinces in Philippines.

	Smallholder farms			Commercial farms			S vs.C
	SA	SB		CA	CB		
Management and biosecurity practices	*n* (%)	*n* (%)	P[a]	*n* (%)	*n* (%)	P[a]	P [a]
Semen, reproduction and purchased pigs							
Artificial insemination used on farm	28 (29)	22 (8)	<0.01	59 (88)	12 (37)	<0.01	<0.01
AI under veterinary supervision	21 (22)	19 (7)	<0.01	49 (73)	12 (37)	<0.01	<0.01
Introduced or purchased pigs quarantined	32 (33)	26 (10)	<0.01	57 (85)	14 (42)	0.02	<0.01
Quarantine period - days			<0.01			<0.01	<0.01
Median; Min, Max	0; 0, 35	0; 0, 60		14; 0, 90	0; 0, 60		
Quarantine pen distance (m) from pens			<0.01			<0.01	<0.01
Median; Min, Max	0; 0,	0; 0, 150		20; 0,	0; 0, 200		
Vaccination –sows							
Actinobacillus pleuropneumoniae	8 (8)	0 (0)	<0.01	12 (18)	1 (3)	0.04	<0.01
Hog cholera virus	36 (38)	33 (12)	<0.01	50 (75)	16 (48)	0.05	<0.01
Haemophilus parasuis	0 (0)	0 (0)		2 (3)	1 (3)	1.00	<0.01
Mycoplasma hyopneumoniae	3 (3)	0 (0)	0.03	7 (10)	1 (3)	0.42	<0.01
Porcine circovirus type 2	1 (1)	0 (0)	0.25	8 (12)	1 (3)	0.28	<0.01
PRRS[b]	7 (7)	2 (1)	<0.01	41 (61)	9 (27)	<0.01	<0.01
Pseudorabies virus	7 (7)	2 (1)	<0.01	40 (60)	5 (15)	<0.01	<0.01
Swine influenza virus	1 (1)	0 (0)	0.26	8 (12)	3 (9)	1.00	<0.01
No Answer	23 (24)	234 (85)	<0.01	4 (6)	11 (33)	<0.01	<0.01
Vaccination-Grower pigs							
Actinobacillus pleuropneumoniae	12 (13)	4 (1)	<0.01	34 (51)	4 (12)	<0.01	<0.01
Hog cholera virus	73 (76)	156 (57)	<0.01	58 (87)	25 (76)	0.28	<0.01
Mycoplasma hyopneumoniae	5 (5)	4 (1)	0.07	26 (39)	6 (18)	0.07	<0.01
Porcine circovirus type 2	1 (1)	1 (0)	1.0	10 (15)	1 (3)	0.09	<0.01
PRRS[b]	12 (13)	16 (6)	0.04	46 (69)	11 (33)	<0.01	<0.01
Pseudorabies virus	2 (2)	3 (1)	0.58	31 (46)	8 (24)	0.04	<0.01
Swine influenza virus	0 (0)	0 (0)		9 (13)	1 (3)	0.15	<0.01
No Answer	19 (20)	108 (39)	<0.01	2 (3)	5 (15)	0.03	<0.01
Feeding and water (grower-finishers)							
Commercial feed			<0.01			<0.01	<0.01
Made on farm feed						<0.01	<0.01
Swill feed							
Swill fed to pigs	37 (39)	80 (29)	0.16	0 (0)	4 (12)	0.01	<0.01
Swill cooked before you feed it	14 (15)	43 (16)	0.90	0 (0)	4 (12)	0.01	<0.01
Swill type							
Commercial product	5 (5)	24 (9)	0.28	0 (0)	1 (3)	0.34	0.02
Table scraps from home	31 (32)	58 (21)	0.04	0 (0)	3 (9)	0.04	<0.01
Food from external sources such as restaurants	2 (2)	21 (8)	0.07	0 (0)	2 (6)	0.12	0.11
Rice by products mixed with water	17 (18)	53 (19)	0.77	0 (0)	2 (6)	0.12	<0.01
Water supply							
Town supply	21 (22)	77 (28)	0.29	4 (6)	3 (9)	0.43	<0.01
Well in property	72 (75)	192 (70)	0.39	63 (94)	30 (91)	0.43	<0.01

Table 7.5. Descriptive statistics of farm management and biosecurity management practices classified by production type (smallholder, *n* = 371; commercial, *n* = 100) (Continued)

Management of sick and dead pigs							
Farm workers can recognise sick and healthy pigs	94 (98)	263 (96)	0.79	67 (100)	33 (100)	1.0	<0.01
Sick pigs separated from healthy pigs	31 (32)	21 (8)	<0.01	53 (79)	18 (55)	<0.01	<0.01
Sick and dead pigs recorded	11 (11)	6 (2)	<0.02	51 (76)	11 (33)	<0.01	0.22
Disposal method of dead pigs			<0.01			0.57	0.05
Burn	12 (12)	6 (2)		2 (3)	2 (6)		
Bury	80 (83)	264 (96)		58 (87)	29 (87)		
Feed to fish	4 (4)	5 (2)		8 (12)	2 (6)		

Key: *n* is total number of farms, Min minimum, Max maximum,S smallholder farms; C commercial farms.

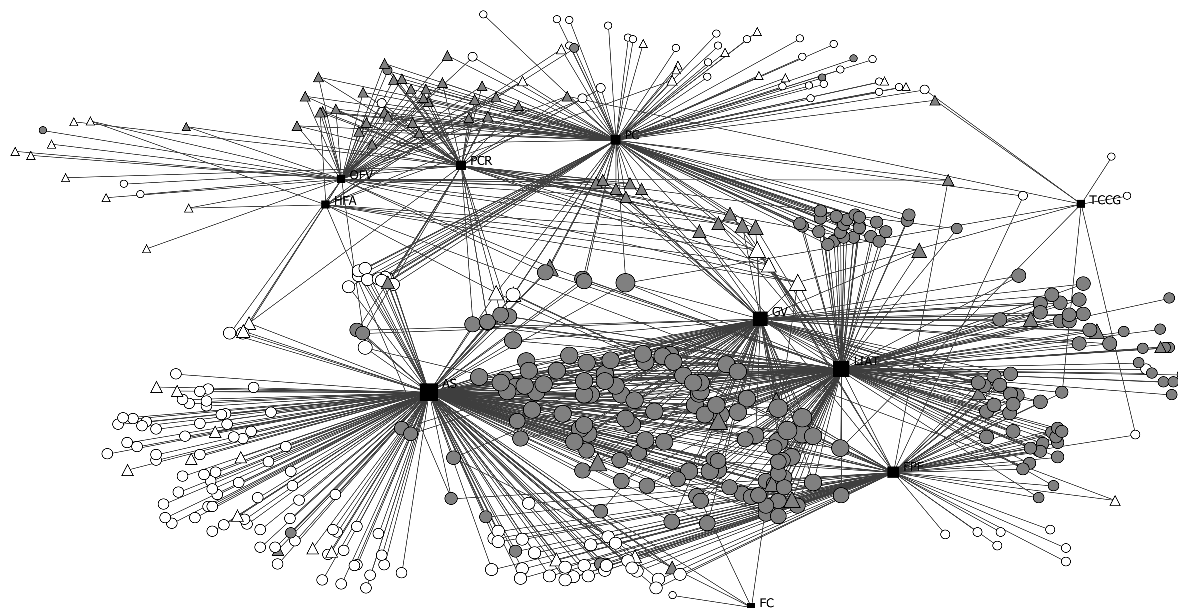


Figure. 7.8. Social network analysis of pig farm affiliation with herd health providers for the study participants in two provinces in Philippines. Network constructed using spring-embedded layout algorithm in Ucinet 6.421. The graph represents the overall network with empty circles representing Bulacan smallholder farms, solid circles representing Pampanga smallholder farms, empty triangles representing Bulacan commercial farms, solid triangles representing Pampanga commercial farms, the squares representing herd health providers, the size of the shapes are proportional to farm and HHP eigenvector value, Key: FC Farmer Co-operative; HFA Hog Farmer Association; LIAT Livestock Inspector – Agricultural Technician; PCR Pharmaceutical company representative; GV Government Veterinarian; OFV On Farm Veterinarian; PC Private Consultant - veterinarian; TCCG Technical Consultant Contract Grower; FPF Fellow Pig Farmer; AS Agricultural Supply store.

Prevalence and Risk Factors

This aspect of the overall lung score study was focused on the prevalence of the difference lung scores and an analysis of the risk factors.

A total of 2,489 lungs (representing 547 farms) were examined. The results of 602 lungs were excluded from the analysis (mainly because the source farm could not be identified or because the source farm was outside the two relevant provinces). Hence the final number of lungs included in the study was 1,887 from a total of 471 farms.

A summary of the lung and pleurisy scores are shown in Table 7.6. For the purpose of this study, the following definitions were applied:-

A) a pig with a lung score greater than the median of all pigs was classified as high lung score (HLS) pig.

B) a pig with a pleurisy score of >1 was classified as high pleurisy score (HPL) pig.

A total of 48% (909/1887) of the lungs examined gave a high lung score, 22% (408/1887) gave a high pleurisy score, 51% (970/1887) showed acute cranio-ventral pneumonia lesions, and 28% (520/1887) had chronic cranio-ventral lesions.

Although the apparent prevalence of high lung scores did not differ between provinces (P=0.19), the prevalence of high pleurisy scores was lower in Pampanga (P=0.04) compared with Bulacan. The prevalence of high lung scores (P<0.01) and high pleurisy scores (P< 0.01) was greater in commercial farms than in small holder farms (Table 3).

Table 7.6. Details of lung and pleuritis scores.*

Province	Production system	Number of Farms	Number of Lungs (Range)	Mean lung score (SD)	Mean pleuritis score (SD)
Bulacan	Smallholder	162	491(1-70)	9 (12)	0.5 (1.0)
	Commercial	44	309 (1-52)	14 (13)	1.0 (1.2)
	Total	206	800 (1-70)	11 (13)	0.8 (1.1)
Pampanga	Smallholder	209	441 (1-37)	7 (11)	0.3 (0.8)
	Commercial	56	646 (1-138)	14 (14)	0.7 (1.1)
	Total	265	1087 (1-138)	11 (13)	0.6 (1.0)
Total		471	1887 (1-138)	11 (13)	0.6 (1.0)

*SD = standard deviation.

The significant risk factors for being a high lung score farm, after adjusting for the effect of other covariates, are shown in Table 7.7. Commercial pig farms with lung lesions were 3.46 times more likely to be a high lung score farm compared with small holder farms that had no lung lesions. Farms that did not vaccinate against hog cholera, did not have a market pen, or surrounded by piggeries located within 500 m were also at higher risk of being a high lung score farm.

Table 7.7. Estimated risk ratios from a multivariable logistic regression model assessing the association of farm-level exposure with high lung scores in 1,887 finisher pigs originating from 416 farms in the Philippines.*

Variable	No. of pigs with low lung score (%)	No. of pigs with a high lung score (%)	Risk Ration (95 % CI)	P [b]
Production system and lung lesion				**<0.01**
Smallholder farms, no lung lesion	262 (79)	70 (21)	Reference	
Smallholder farms, with lung lesion	16 (41)	23 (59)	2.80 (1.91 - 3.75)**	<0.01
Commercial farms, no lung lesion	31 (44)	39 (56)	2.19 (1.42 – 3.17)	<0.01
Commercial farms, with lung lesion	7 (23)	23 (77)	3.46 (2.35 – 4.34)	<0.01
Grower-finishers vaccinated against Hog Cholera				**<0.01**
Yes	223 (69)	102 (31)	Reference	
No	93 (64)	53 (36)	1.74 (1.28 – 2.35)	0.01
Market pen used on farm				**<0.01**
No	273 (74)	97 (26)	Reference	
Yes	43 (43)	58 (57)	1.79 (1.18 – 2.57)	<0.01
Surrounded by piggeries within 500 meters				**0.01**
No	193 (71)	78 (29)	Reference	
Yes	123 (62)	77 (38)	1.52 (1.14 – 2.03)	<0.01

* CI Confidence Interval

**Interpretation: after adjusting for the effect of other variables in the model, small holder farms with lung lesions records reported for that farm were 2.8 (95% CI 1.91–3.75) times more likely to be classified as HLS farms compared with smallholder farms and no lung lesions.

The significant risk factors for being a high pleurisy score farm, after adjusting for the effect of other covariates, are shown in Table 7.8. Commercial pig farms were at a higher risk than smallholder farms. Farms that practice feedback or allow commercial livestock vehicles on farm were at higher risk of being HPS farms.

Table 7.8. Estimated risk ratios from a multivariable logistic regression model assessing the association of farm-level exposure with pleuritis in 1,887 finisher pigs from 471 pig farms in the Philippines.*

Variable	No. of pigs with low pleurisy score (%)	No. of pigs with high pleurisy score (%)	RR (95% CI)	P [b]
Production system				**<0.01**
Smallholder	334 (90)	37 (10)	Reference	
Commercial farms *	63 (63)	27 (27)	2.69 (1.61 – 4.42)	<0.01
‘Feedback’ carried out on this farm				**0.02**
No	398 (87)	57 (13)	Reference	
Yes	9 (56)	7 (44)	3.27 (1.49 – 5.63)	0.01
Commercial livestock vehicles allowed on farm				**0.03**
Yes	188 (91)	18 (9)	Reference	
No	219 (83)	46 (17)	1.83 (1.07 – 3.20)	0.03

*CI = confidence interval.

* Interpretation: after adjusting for the effect of other variables in the model commercial farms were 2.69 (95% CI 1.61 – 4.42) times more likely to be classified as HPL farms compared with smallholder farms.

Figure 7.9 shows the spatial clustering of high risk pig farms (lung scores and pleurisy) imposed on a density map showing the study population of commercial and smallholder farms. Two primary spatial clusters of high risk farms were identified. One primary spatial cluster A 9.5 km-radius cluster (P = 0.01) was observed in the Southeast of Bulacan. and comprised of 39 farms (17 commercial and 22 smallholder farms). Another secondary cluster (9.7 km-radius) (P = 0.33) was identified in North of Pampanga and included 31 farms (22 commercial and 9 smallholder farms).

Overall, this study has shown that pigs from commercial farm groups were at higher risk of being classified as having a high lung score or a high pleurisy score. As well, the presence of a market pen on farm, vaccinating grower finisher pigs against hog cholera and the presence of another piggery within 500 m all increased the risk of high lung score pigs. The risk for high lung pleurisy pigs was associated the use of feedback, and access by commercial livestock vehicles onto the farm. If pig producers were informed about these risk factors, a marked reduction of prevalence in the scores might be achieved by targeted intervention programs. Farmer should be encouraged and supported to review and improve on-farm disease control programmes to reduce risk of high lung and pleurisy scores in their herds.

While this study informs pig producers in the Philippines about potential factors influencing the health and productivity of their herds, it also provides valuable insights for veterinary authorities to evaluate the feasibility of implementing routine inspection of pig lungs at slaughter to monitor herd health in the Philippines.

Validation Study

Eight farms participated in the study, four in Bulacan and four in Pampanga. One farm used an all-in-all-out system while the remaining seven farms used a continuous flow system. Farm visits were conducted weekly from 17th September to 10th December 2013. Visits alternated between Pampanga and Bulacan; farms in Pampanga were visited seven times, and those in Bulacan six times. On each occasion an apparently healthy pig, a pig showing signs suggestive of *M. hyopneumoniae* (suspect MHYO) and a pig showing signs

suggestive of *A. pleuropneumoniae* (suspect APP) were selected from each farm (See Table 7.9).

Table 7.9 Frequency distribution of healthy, suspect *Mycoplasma hyopneumoniae* and suspect *Actinobacillus pleuropneumoniae* pigs by farm and province.

Province/Farm	Healthy	Suspect *M. hyopneumoniae*	Suspect *A. pleuropneumoniae*	Total
Bulacan				
BF	6	6	6	18
PF	6	6	6	18
RF	6	6	6	18
SF	6	6	6	18
Subtotal	24	24	24	72
Pampanga				
AA	7	7	7	21
PG	7	7	7	21
RD	7	7	7	21
SC	7	7	7	21
Subtotal	28	28	28	84
Total	52	52	52	156

All pigs were between 13 and 17 weeks of age and had not been treated with any antibiotic in the preceding month. Clinical signs reported for pigs in each category (apparently healthy, suspect MHYO, suspect APP) are shown in Table 7.10. A summary of the lesions seen in the lungs of the pigs at slaughter is presented in Table 7.11.

Detailed histopathological examination has been performed on the range of tissues collected. At the time of preparation of this report, a detailed analysis of the results of the histopathology has not been completed. A summary of histological lesions of bronchopneumonia and peribronchial cuffing for dorsocaudal and cranioventral lobes for each category of pig (healthy, suspect *M. hyopneumoniae* and suspect *A. pleuropneumoniae*) is shown in Appendix 11.11.

The results of the culture of the lungs (both dorsocaudal and cranioventral lobes) are presented in Table 7.12. Despite a deliberate selection of pigs that, on the basis of clinical signs, were likely to be positive for *A. pleuropneumoniae*, no lung sample yielded *A. pleuropneumoniae*. All *P. multocida* and *H. parasuis* isolates obtained by culture were confirmed by the relevant species-specific PCR assay at PAHC.

Table 7.10. Frequency distribution of clinical signs for each category of pig (healthy, suspect *Mycoplasma hyopneumoniae* and suspect *Actinobacillus pleuropneumoniae*).

Category	Clinical Signs	Yes - No. (%)	No - No. (%)
Healthy			
	Other pigs coughing in pen	16 (30.8)	36 (69.2)
	Antibiotics within one month	0 (0.0)	52 (100.0)
Suspect *M. hyopneumoniae*			
	Coughing	52 (100.0)	0 (0.0)
	Increased respiratory rate	32 (61.5)	20 (38.5)
	Fever	1 (1.9)	51 (98.1)
	Huddling	1 (1.9)	51 (98.1)
	Loss of appetite	19 (36.5)	33 (63.5)
	Retarded growth/ill thrift	26 (50.0)	26 (50.0)
Suspect *A. pleuropneumoniae*			
	Cough when forced to move	48 (92.3)	4 (7.7)
	Sudden onset prostration	21 (40.4)	31 (59.6)
	Mouth breathing	37 (71.2)	15 (28.8)
	Foamy discharge from mouth	0 (0.0)	52 (100.0)
	Fever and loss of appetite	10 (19.2)	42 (80.8)
	Stiffness	4 (7.7)	48 (92.3)
	Vomiting and diarrhoea	4 (7.7)	48 (92.3)
	General cyanosis	0 (0.0)	52 (100.0)
	Chronic cough	31 (59.6)	21 (40.4)
	Dog sitting	24 (46.2)	28 (53.8)
	Thumping	49 (94.2)	3 (5.8)

Table 7.11. Frequency distribution of post mortem findings for each category of pig (healthy, suspect *Mycoplasma hyopneumoniae* and suspect *Actinobacillus pleuropneumoniae*).

Post mortem finding	Healthy	Suspect *M. hyopneumoniae*	Suspect *A. pleuropneumoniae*	Total
A. pleuropneumoniae - like lesions				
No	48 (92.3)	35 (67.3)	33 (63.5)	116 (74.4)
Active	2 (3.8)	15 (28.8)	18 (34.6)	35 (22.4)
Resolving	2 (3.8)	2 (3.8)	1 (1.9)	5 (3.2)
Pleurisy score				
0	32 (61.5)	25 (48.1)	30 (57.7)	87 (55.8)
1	7 (13.5)	9 (17.3)	7 (13.5)	23 (14.7)
2	11 (21.2)	13 (25.0)	10 (19.2)	34 (21.8)
3	2 (3.8)	5 (9.6)	5 (9.6)	12 (7.7)
Pericarditis				
Absent	47 (90.4)	40 (76.9)	44 (84.6)	131 (84.0)
Present	5 (9.6)	12 (23.1)	8 (15.4)	25 (16.0)
Cranioventral pneumonia status				
No lesion	4 (7.7)	0 (0.0)	0 (0.0)	4 (2.6)
Acute	38 (73.1)	43 (82.7)	38 (73.1)	119 (76.3)
Chronic	10 (19.2)	9 (17.3)	14 (26.9)	33 (21.2)

Table 7.12. Frequency distribution of positive cultures for dorsocaudal, cranioventral or either lobe for each category of pig (healthy, (healthy, suspect *Mycoplasma hyopneumoniae* and suspect *Actinobacillus pleuropneumoniae)*.*

Culture Result/Lobe	Healthy	Suspect M hyo	Suspect APP	Total
Pasteurella multocida				
Dorsocaudal lobe	12 (23.1)	13 (25.0)	17 (32.7)	42 (26.9)
Cranioventral lobe	14 (26.9)	16 (30.8)	17 (32.7)	47 (30.1)
Either lobe	15 (28.8)	17 (32.7)	20 (38.5)	52 (33.3)
Suspect *Streptococcus suis*				
Dorsocaudal lobe	0 (0.0)	5 (9.6)	3 (5.8)	8 (5.1)
Cranioventral lobe	6 (11.5)	5 (9.6)	8 (15.4)	19 (12.2)
Either lobe	6 (11.5)	6 (11.5)	8 (15.4)	20 (12.8)
Haemophilus parasuis				
Dorsocaudal lobe	0 (0.0)	1 (1.9)	2 (3.8)	3 (1.9)
Cranioventral lobe	0 (0.0)	2 (3.8)	2 (3.8)	4 (2.6)
Either lobe	0 (0.0)	2 (3.8)	3 (5.8)	5 (3.2)
Bordetella bronchiseptica				
Dorsocaudal lobe	2 (3.8)	8 (15.4)	6 (11.5)	16 (10.3)
Cranioventral lobe	3 (5.8)	7 (13.5)	12 (23.1)	22 (14.1)
Either lobe	4 (7.7)	11 (21.2)	12 (23.1)	27 (17.3)

* No pig in any category yielded *A. pleuropneumoniae*.

Each of the lung samples was examined at CLSU by the following tests:- PCR for *A. pleuropneumoniae*, *H. parasuis* and *M. hyopneumoniae;* LAMP for *A. pleuropneumoniae*, *H. parasuis* and *M. hyopneumoniae.* The results for these tests for samples from both the dorsocaudal and cranioventral lobes are presented in Table 7.13.

The ApxIV ELISA was used on all 156 serum samples and only 17 positive reactions were obtained – with those positive reactions occurring in all three groups of pigs (six healthy pigs, six suspect *M. hyopneumoniae* pigs and five suspect *A. pleuropneumoniae* pigs).

Table 7.13. Frequency distribution of positive results from polymerase chain reaction (PCR) and isothermal loop amplification (LAMP) assays for dorsocaudal and cranioventral lobes for each category of pig (healthy, suspect *Mycoplasma hyopneumoniae* and suspect *Actinobacillus pleuropneumoniae*).

	Dorsocaudal lobe				Cranioventral lobe			
Test positive	Healthy	Suspect MHYO	Suspect APP	Total	Healthy	Suspect MHYO	Suspect APP	Total
A. pleuronpneumoniae PCR	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
A. pleuronpneumoniae LAMP*	36 (69.2)	36 (69.2)	39 (75.0)	111 (71.2)	34 (66.7)	30 (60.0)	29 (59.2)	93 (62.0)
M. hyopneumoniae PCR	2 (3.8)	1 (1.9)	3 (5.8)	6 (3.8)	5 (9.6)	4 (7.7)	5 (9.6)	14 (9.0)
M. hyopneumoniae LAMP	20 (38.5)	16 (30.8)	18 (34.6)	54 (34.6)	16 (30.8)	13 (25.0)	14 (26.9)	43 (27.6)

In looking at the formal analysis of the comparison of test pairs, it is important to understand that there is no single statistic that provides a complete picture of the agreement between two dichotomous tests. However, the kappa statistic is the most widely used. It indicates the proportion of agreement beyond that expected by chance. It is affected by both bias (the extent to which two tests agree on the proportion of positive cases) and prevalence of the attribute the tests aim to detect so both of these factors should be considered when interpreting kappa statistics (Sim and Wright, 2005). When there is a large bias kappa is higher than when bias is low or absent. When the prevalence of the attribute is either very high or very low (rather than ~50%), chance agreement is also high, so kappa is reduced. McNemar's chi-squared test was used to test for bias by testing the null hypothesis that the proportion of positive responses was the same in both tests. This test is commonly used for this purpose but it ignores results in the agreement diagonal. The bias index was calculated. This index adjusts the discrepancy between the numbers in the two discordant cells by the total number of samples. A bias index of 0 indicates no bias, whereas a large value e.g. 50% indicates that the proportion of samples testing positive differs markedly between the two tests. The prevalence index was also calculated. The value of this index increases as the prevalence moves away from 50% (in either direction).

Adjusted kappas can be calculated; these statistics are known as BAK (bias adjusted kappa) and PABAK (prevalence and bias adjusted kappa). These should not be used instead of kappa but can be considered along with kappa to indicate the extent to which kappa has been influenced by prevalence and bias. For any given set of marginal totals, there is a maximum value that kappa can take. This value is known as the maximum attainable kappa, and its value should also be considered when interpreting kappa. Consideration of the percent positive and percent negative agreement can be used to indicate where and how much improvement is required when kappa is suboptimal.

The results for the pair-wise comparison of the three assays for *A. pleuropneumoniae* (culture performed at RADDL Region 3, PCR and LAMP performed at CLSU) and the two assays for *M. hyopneumoniae* (PCR and LAMP performed at CLSU) for samples from the dorsocaudal lobe are shown in Tables 7.14 and 7.15 and for samples from the cranioventral lobe in Tables 7.16 and 7.17.

The tests evaluated in this study for *A. pleuropneumoniae* were culture, PCR, LAMP and serology. Both culture and PCR were negative for all lung samples examined. In contrast, LAMP gave many more positives (111 dorsocaudal and 93 cranio-ventral lobes). The formal statistical analysis provided strong supportive evidence, for both lobes ($p < 0.001$ and high bias indices of 71 and 62%), that the number of positive tests by LAMP was markedly different than the other two tests. As the *A. pleuropneumoniae* serology test (an independent assay looking for antibody to a toxin produced by *A. pleuropneumoniae*) gave only a few positive results, it would appear that the LAMP results are at odds with all three other diagnostic assays.

Table 7.14. Statistics from McNemar's chi-squared test comparing results from pairs of diagnostic tests from the dorsocaudal lobes.*

Organism Test 1	Test 2	No. samples	No. (%) T1 +	No. (%) T2 +	No. (%) T1+/T2+	No. (%) T1+/T2-	No. (%) T1-/T2+	No. (%) T1-/T2-	McNemar's χ^2	Bias index	Prevalence index
Actinobacillus pleuropneumoniae											
Culture	PCR	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
LAMP	Culture	156	111 (71.1)	0 (0.0)	0 (0.0)	111 (71.2)	0 (0.0)	45 (28.8)	<0.001	71.2	28.8
LAMP	PCR	156	111 (71.1)	0 (0.0)	0 (0.0)	111 (71.2)	0 (0.0)	45 (28.8)	<0.001	71.2	28.8
Mycoplasma hyopneumoniae											
PCR	LAMP	156	6 (3.8)	54 (34.6)	1 (0.6)	5 (3.2)	53 (34.0)	97 (62.2)	<0.001	30.8	61.5

*T1 = Test 1, T2 = Test 2, McNemar's χ^2 = *p* value from McNemars chi-squared test.

Table 7.15. Statistics (kappa) comparing results from pairs of diagnostic tests for samples from the dorsocaudal lobes.*

Organism Test 1	Test 2	% Observed agreement	% Expected agreement	% Positive agreement	% Negative agreement	Kappa	Max kappa	PABAK	BAK
Actinobacillus pleuropneumoniae									
Culture	PCR	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
LAMP	Culture	28.8	28.8	0	28.8	0	0	-0.42	-0.55
LAMP	PCR	28.8	28.8	0	28.8	0	0	-0.42	-0.55
Mycoplasma hyopneumoniae									
PCR	LAMP	62.82	64.2	3.3	77	-0.04	0.14	0.26	-0.2

* Max kappa - maximum attainable kappa given observed marginal totals, PABAK – prevalence and bias adjusted kappa, BAK – bias adjusted kappa.

Table 7.16. Statistics from McNemar’s chi-squared test comparing results from pairs of diagnostic tests from the cranioventral lobes.*

Organism Test 1	Test 2	No. samples	No. (%) T1 +	No. (%) T2 +	No. (%) T1+/T2+	No. (%) T1+/T2-	No. (%) T1-/T2+	No. (%) T1-/T2-	McNemar's χ^2	Bias index %	Prevalence index %
Actinobacillus pleuropneumoniae											
Culture	PCR	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
LAMP	Culture	150	93 (62.0)	0 (0.0)	0 (0.0)	93 (62.0)	0 (0.0)	57 (38.0)	<0.001	62	38
LAMP	PCR	150	93 (62.0)	0 (0.0)	0 (0.0)	93 (62.0)	0 (0.0)	57 (38.0)	<0.001	62	38
Mycoplasma hyopneumoniae											
PCR	LAMP	156	14 (9.0)	43 (27.6)	9 (5.8)	5 (3.2)	34 (21.8)	108 (69.2)	<0.001	18.6	63.5

*T1 = Test 1, T2 = Test 2, McNemar's χ^2 = *p* value from McNemar’s chi-squared test.

Table 7.17. Statistics (kappa) comparing results from pairs of diagnostic tests for samples from the cranioventral lobes.*

Organism Test 1	Test 2	% Observed agreement	% Expected agreement	% Positive agreement	% Negative agreement	Kappa	Max kappa	PABAK	BAK
Actinobacillus pleuropneumoniae									
Culture	PCR	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
LAMP	Culture	38	38	0	38	0	0	-0.24	-0.45
LAMP	PCR	38	38	0	38	0	0	-0.24	-0.45
Mycoplasma hyopneumoniae									
PCR	LAMP	75	68.41	31.6	84.7	0.21	0.41	0.5	0.16

* Max kappa - maximum attainable kappa given observed marginal totals, PABAK – prevalence and bias adjusted kappa, BAK – bias adjusted kappa

A similar marked and statistically supported difference in the number of positive samples for *M. hyopneumoniae* by the two applied diagnostic tests (PCR and LAMP) also occurred. For the dorso-caudal samples, only 3.8% dorso-caudal samples tested positive for *M. hyopneumoniae* using the PCR, but 34.6% samples were positive using the LAMP. There was strong evidence based on McNemar's chi-squared test (p <0.001) and the bias index (30.8%) that the proportion of positive responses differed between the two tests.

The results of the cluster analysis for lung score, pleurisy score and the various diagnostic tests are shown in Table 7.18

Table 7.18. Intra-class correlation co-efficients (ICC) and associated p-values indicating degree of clustering of conditions by farm.*

Condition	ICC	p value
Lung Score ≥ 25	0.06	0.07
Pleurisy Score ≥ 1	0.18	0.001
Pericarditis	<0.01	0.50
Presence of *A. pleuropneumoniae* -like lesions	<0.01	0.50
Positive reaction in ApxIV ELISA	0.36	0.002
Presence of *P. multocida*	0.11	0.02
Presence of suspect *S. suis*	0.00	0.50
Presence of *B. bronchiseptica*	0.14	0.01
Presence of *H. parasuis*	0.21	0.19
Positive LAMP reaction in LAMP for *A. pleuropneumoniae* in dorsocaudal lobe	<0.01	0.50
Positive LAMP reaction for *A. pleuropneumoniae* in cranio-ventral lobe	0.03	0.22
Positive PCR reaction for *M. hyopneumoniae* in dorsocaudal lobe	<0.01	0.50
Positive PCR reaction for *M. hyopneumoniae* in cranio-ventral lobe	<0.01	0.50
Positive LAMP reaction for *M. hyopneumoniae* in cranio-ventral lobe	0.05	0.11
Positive LAMP reaction for *M. hyopneumoniae* in dorsocaudal lobe	0.06	0.11
Positive Bacterial culture	0.07	0.04

*Positive bacterial culture = presence of any of the recognised potential respiratory pathogens – *B. bronchiseptica, H. parasuis, P. multocida* and *S. suis.*

In this form of analysis, significant clustering means that positive pigs are not found randomly across the eight farms but are more likely to come from certain farms and not be found on others. An intra-class correlation coefficient (ICC) of more than 0.25 is unusually high. The results show a high degree of clustering of ApxIV antibody positive pigs by farm. Moderate clustering was shown for the presence of *P. multocida,* and for a pleurisy score of ≥1. As well, there was some clustering of *B. bronchiseptica* and the presence of at least one bacterial species.

Crude associations between results from the ApxIV ELISA, and culture results and lung score, pleurisy score, presence of pericarditis and active/resolving *A. pleuropneumoniae* like lesions (outcome variables) were assessed using random effects logistic regression, with the farm fitted as the random effect. Associations with *H. parasuis* were not assessed due to the small number of culture positive pigs.

These crude analyses suggested only strong associations between *P. multocida* and cranio-ventral pneumonia (ie a lung score of >25), *P. multocida* and *A. pleuropneumoniae*-like lesions and *B. bronchiseptica* and cranio-ventral pneumonia (ie a lung score of >25) (see Tables 7.19 and 7.20).

Table 7.19. Odds ratios of associations between culture and ApxIV ELISA results and a pig having a lung score of at least 25, after accounting for clustering by farm. P values in bold are overall Wald p values for that variable

Bacteria	OR (95% CI)	p value
P. multocida	4.33 (2.03 - 9.24)	<0.001
B. bronchiseptica	2.96 (1.14 - 7.70)	0.026
Suspect *S. suis*	1.13 (0.43 - 2.98)	0.81
Significant bacteria present*		
0	Ref	
1 species	3.62 (1.76 - 7.46)	<0.001
2 species	15.38 (3.19 - 74.21)	0.001
3 species	1.99 (0.11 - 34.61)	0.637
Significant bacteria present*	4.44 (2.22 - 8.85)	<0.001
ApxIV ELISA Positive	1.68 (0.56 - 5.10)	0.356

*Significant bacteria = presence of any of the recognised potential respiratory pathogens – *B. bronchiseptica, H. parasuis, P. multocida* and *S. suis.*

Table 7.20. Odds ratios of associations between culture and ApxIV ELISA results and a pig having a either active or resolving *A. pleuropneumoniae*-like lesions, after accounting for clustering by farm. P values in bold are overall Wald p values for that variable

Bacteria	OR (95% CI)	p value
P. multocida	1.97 (0.94 - 4.14)	0.072
B. bronchiseptica	0.31 (0.09 - 1.10)	0.069
Suspect *S. suis*	0.69 (0.22 - 2.22)	0.538
Significant bacteria present*		**0.913**
0	Ref	
1 species	1.11 (0.51 - 2.38)	0.799
2 species	1.27 (0.39 - 4.11)	0.686
3 species	n/a	
Significant bacteria present	1.10 (0.54 - 2.27)	0.792
ApxIV ELISA Positive	1.24 (0.41 - 3.76)	0.706

*Significant bacteria = presence of any of the recognised potential respiratory pathogens – *B. bronchiseptica, H. parasuis, P. multocida* and *S. suis.*

A one mode circular network diagram (see Figure 7.9) was used to show the co-occurrence of post mortem lesions, bacteria on lobe-specific culture and presence of antibodies to ApxIV toxin (both a positive reaction or a suspect reaction). These conditions are represented as nodes (circles) where the size of the node is proportional to the number of pigs with the condition (number also written in/near node). Edges (lines) join nodes where the two connected conditions co-occur in one or more pigs (number of pigs on edge). Edges joining same category of condition (bacteria/lesion seen at slaughter/ApxIV result) have the same colour as the node, those across categories e.g. lung score of >25 and the presence of *P. multocida* in the cranio-ventral lung lobe in the same pig are a blended colour. For example Figure 7.9 shows that, of the 156 pigs in the study, 81 pigs had a lung score of at least 25, 42 pigs were positive on culture for *P. multocida* in the dorso-caudal lobe. Furthermore, of the 42 pigs that yielded *P. multocida* in the dorso-caudal lobe, 34 pigs also had a lung score of >25.

Overall, the validation study proved a complex and challenging study. The specific planned objectives of the study were to evaluate the performance of a number of the project established laboratory based diagnostics (specifically PCR and culture assay for *A. pleuropneumoniae* and PCR and LAMP for *M. hyopneumoniae*). The nature of the planned study – selecting apparently clinically affected pigs as well as healthy pigs, sampling those pigs at slaughter, performing lung soring, collecting and processing lung and other tissue samples – meant that the study was also an evaluation of the overall diagnostic systems put into place by the project. The study also featured some future proofing – duplicate and back up samples were collected and stored – providing a key biological resource for future studies.

In looking at the specific planned objectives, one major objective was to compare culture and PCR for the detection of *A. pleuropneumoniae*. As the capacity for detection by LAMP was available at CLSU, this assay was also included. Despite specifically seeking to identify pigs suffering from clinical illness associated with infection by *A. pleuropneumoniae*, not one *A. pleuropneumoniae* positive was obtained by culture or PCR. In contrast, a relatively large number of samples were positive for the *A. pleuropneumoniae* LAMP. As an additional test, an ELISA for antibodies to ApxIV was used. The advantage of the ApxIV antibody test is that ApxIV is an in vivo toxin that is not produced when *A. pleuropneumoniae* grows in artificial media (Schaller et al., 1999). Hence, antibodies to ApxIV are only produced by infection and not vaccination (Dreyfus et al., 2004). The ApxIV antibody ELISA used in this study is a commercial version of a validated assay (Dreyfus et al., 2004). The results from the ELISA assay (only 17 positives in the 156 serum samples and a correlation with farms) suggests that most of the pigs in this study had not been exposed to *A. pleuropneumoniae*. It is possible that the LAMP results (a large number of positive samples) can be explained the assay being far more sensitive than either culture or PCR and by the fact that the LAMP positives reflect early infection that has not yet caused a rise in antibodies. However, the literature indicates that the ApxIV ELISA detects antibodies within two to three weeks and that herds are detected before clinical signs or pathological lesions are detected (Dreyfus et al., 2004). It is not possible – on the basis of the results from this study – to definitively identify if the LAMP assay *A. pleuropneumoniae* is a far more sensitive test that serology, PCR and culture or if the assay is giving false positives. Given this uncertainty over the assay, it would not be prudent to adopt the use of this assay in the Philippines veterinary diagnostic systems at this stage. Further evaluation and validation studies are required.

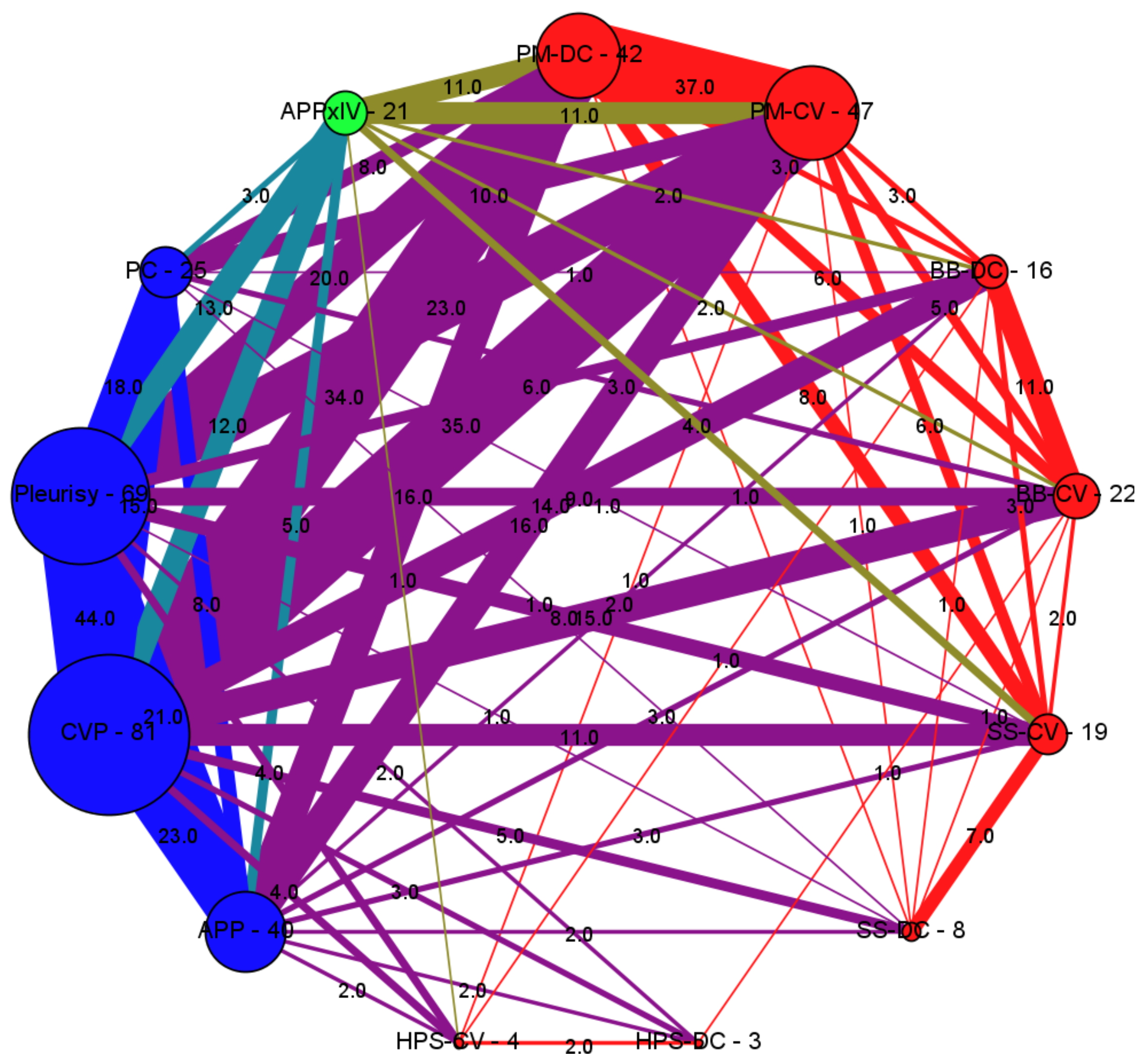


Figure 7.9 Circular network showing co-occurrence of post-mortem findings, bacteria on lobe-specific culture and antibodies to ApxIV toxin for all pigs (n = 156). Nodes are proportional to the number of pigs affected with that condition and number is stated within/beside the node. Edges are proportional to the number of pigs in which the two connected conditions were found and the number is stated mid-way along the edge. APP = *A. pleuropneumoniae* like lesions at slaughter, either active or resolving; APPxIV = antibodies to *A. pleuropneumoniae* ApxIV detected; BB-CV = *B. bronchiseptica* isolated from cranio-ventral lobe; BB-DC = *B. bronchiseptica* isolated from dorso-caudal lobe; CVP = Lung score ≥ 25; HP-CV = *H. parasuis* isolated from cranio-ventral lobe; HP-DC = *H. parasuis* isolated from dorso-caudal lobe PC = pericarditis seen at slaughter, Pleurisy = pleurisy seen at slaughter; PM-CV = *P. multocida* isolated from cranio-ventral lobe; PM-DC = *P. multocida* isolated from dorso-caudal lobe; SS-CV = suspect *S. suis* isolated from cranio-ventral lobe; SS-DC = suspect *S. suis* isolated from dorso-caudal lobe.

The two assays used to detect *M. hyopneumoniae* – a PCR based on existing published and apparently valid assay (Caron et al., 2000) and a published LAMP (Li et al., 2013) – showed a marked, significant difference in the number of positive results – the PCR assay gave a total of 20 positive results (14 being cranio-ventral lobe samples and 6 being dorso-caudal samples) while the LAMP gave a total of 97 positive results (43 being cranio-ventral lobe samples and 54 being dorso-caudal samples). Culture for

M. hyopneumoniae is a difficult task that is beyond the capacity of a front-line regional diagnostic laboratory such as the RADDL Region 3 laboratory. Furthermore, the available commercial serological tests cannot distinguish between infected and vaccinated animals. Hence, there is no realistic option of another assay to help determine which of the assays used in this study (PCR or LAMP) is giving results that reflect the true infection status of the examined pigs. It is well accepted that the cranio-ventral lobes are the most common site of *M. hyopneumoniae* lesions and thus *M. hyopneumoniae* colonisation (Thacker et al., 2001). Hence, while not definitive, the fact that the LAMP assay gave numerically more positive results with dorso-caudal lobes (54) as opposed to cranio-ventral lobes (43) is counter to the expected situation. Overall, as with the *A. pleuropneumoniae* assays, it is not possible to reach any conclusions on which of the two *M. hyopneumoniae* assays (PCR or LAMP) is “correct” – the far higher number of positives given by LAMP may be a reflection of a more sensitive assay or may be a result of false positives. The original description and validation of the *M. hyopneumoniae* LAMP was an extensive study that indicated the assay was species specific and would not give a positive with any other *Mycoplasma* spp found commonly in pigs (Li et al., 2013). As well, the LAMP assay was used on clinical samples (nasal swabs) and gave essentially the same results as a real-time PCR (Li et al., 2013). Given this background, the findings of the current study (far more positives than a PCR and far more positives in the dorso-caudal lobe than the cranio-ventral lobe) are difficult to explain. As with the *A. pleuropneumoniae* LAMP assay, the overall conclusion is that the uncertainty of the validity of the results given by this assay mean that the LAMP assay for *M. hyopneumoniae* developed within this project is not yet suitable for routine applications.

The validation study did demonstrate the appropriateness of the basic protocols (tissue sampling, lung scoring, bacterial isolation and identification) established in this project. A particularly encouraging result was the total agreement between the phenotypic and genotypic results for *P. multocida* and *H. parasuis*. All isolates of these two species that were identified by the abbreviated, cost effective phenotypic system developed within the project (and used at RADDL Region 3) were shipped as pure cultures and/or DNA extracts to PAHC and all were confirmed by the relevant species specific PCR. This is strong evidence of the effective performance of a number of the project developed protocols and most importantly confirmation of the technical expertise of the Philippines project staff. Importantly, the isolation and identification of *H. parasuis* is clear evidence of the ability of RADDL Region 3 (field and laboratory staff) to work together to isolate and identify an organism that is very difficult to obtain. Indeed, as *H. parasuis* is a more demanding organism (in terms of growth requirements) than *A. pleuropneumoniae* (Blackall and Norskov-Lauritsen, 2008), the results of this study have shown that *A. pleuropneumoniae* – if present – would have been isolated and identified.

The validation study has also provided new information on porcine respiratory disease in the Philippines. The study has shown a strong association between the presence of *P. multocida* and the occurrence of a lung score of >25 and between the presence of *P. multocida* and the presence of *A. pleuropneumoniae*-like lesions. The widely accepted view of *P. multocida* is that the organism is a secondary pathogen or an opportunistic pathogen (Brockmeier et al., 2002). However, some studies that have used genetic typing methods to delve below species level (to recognise clones or genetic variants) have provided some evidence that some forms of porcine *P. multocida* may be primary pathogens. In Australia, several outbreaks (one pneumonic pasteurellosis and the other a septicaemic pasteurellosis) have been shown to be associated with a single strain (within each outbreak) isolated in pure culture in the absence of any other bacteria (Blackall et al., 2000). This Australian study showed that despite examining multiple colonies on primary plates from multiple tissues sourced from multiple pigs that only a single genotype of *P. multocida* was present (Blackall et al., 2000). Given that *P. multocida* can be commonly found in the upper respiratory tract of pigs (Brockmeier et al., 2002), the presence of a single genetic type across so many pigs, tissues and colonies argues against a simple secondary role in these Australian cases (Blackall et al., 2000). Rather,

the presence of a single genetic type (although the types were different between the pneumonic and septicaemic cases) argues for a primary role of a particular form of *P. multocida* in these cases. Using different genetic methods, a similar conclusion – that *P. multocida* can be a primary pathogen – has been reached in a large UK-based study that looked at over 150 porcine *P. multocida* isolates (Davies et al., 2003). The UK study showed a very limited range of genetic types of *P. multocida* were associated with pneumonic disease and concluded that this was evidence of a primary role for *P. multocida* in causing pneumonic disease in pigs (Davies et al., 2003).

The results from the current study – an association between *P. multocida* and lung lesion scores of >25 and an association with the presence of *A. pleuropneumoniae*-like lesions is clearly not conclusive evidence of a primary pathogen role for *P. multocida*. However, the study does add to the body of evidence suggesting that *P. multocida* may indeed be a primary agent of porcine respiratory disease. Importantly, the cultures of *P. multocida* have been stored and are available for future genetic typing studies.

Overall, the validation study providing convincing evidence of the suitability of the overall diagnostic systems and protocols developed in this project. While the original planned objectives - a clear direct comparison of several molecular diagnostic assays that would allow a confident adoption of these molecular assays – was not achieved, the study should be regarded as a success.

Objective 4: To establish novel mechanisms for communication among industry, government and research institutions in the Philippines on pig health.

A key component of this Objective was the establishment and active engagement of a stakeholder focussed Project Overview Group that had a broad representation. The Group met annually over the period of the project to overview progress on the project. As well, additional meetings to address significant issues were held. The Committee membership was as follows:-

Dr Teodulo M. Topacio (Chair) (Nat'l Ad. Com. Animal Disease Control & Emergencies)

Dr Wlfredo P. Resoso (Philippine College of Swine Practitioners)

Dr Minda S. Manantan (National Meat Inspection Service)

Mr Felix G. Valenzuela (Livestock Development Council)

Dr Zosimo L. de Leon (National Federation of Hog Farmers, Inc.)

Mr Edwin G. Chen (Pork Producers Federation of the Philippines, Inc.)

Dr Arturo T. Calud (Private Swine Practitioner; Leader of Swine Production Performance Project).

A range of communication activities occurred across the life of the project. These activities include the following:-

- Presentations on the project were made to the National Advisory committee on Animal Disease Control and Emergencies;
- Presentations on the project were made to the Swine Foundation;
- Updates on the project were provided to Monitor (PCAARRD newsletter), the Animal Husbandry Journal and the SWINE newsletter of the industry group PROPORK;
- Project fact and information sheets were prepared and disseminated;
- A project homepage was developed and maintained within the PCAARRD website

The stakeholder analysis survey was the major activity of this Objective. The survey involved 356 respondents from 29 cities and municipalities in Bulacan (13 cities/ municipalities of the total of 24) and Pampanga (16 cities/municipalities of the total of 22) provinces. Respondents included: smallholders (n=96), commercial raisers (n=54), LI/AT (n=53), livestock trader (n=113), veterinarians (n=26), slaughterhouse operators (n=13).

Table 7.21 presents the results of the aspects of the survey that dealt with the preferred communication channels and trusted sources of information on respiratory diseases. These results emphasise the key role that government staff (livestock inspectors/ agricultural technologists and veterinarians) play as key trusted sources of information to all the industry stakeholders. As the project has worked closely with these government staff, this is conformation that the outcomes of the project will be delivered via a source – government staff – that is a widely trusted information resource. The key role of government staff needs to be maintained in all future communication and extension efforts (regardless of whether those efforts are focused at smallholder or commercial pig raisers).

The survey results also highlighted the following:-

- 93% of respondents claimed a high level of knowledge of respiratory diseases in pigs
- 89% of respondents considered respiratory diseases to be a serious problem

- 73% of respondents would react to the first signs of respiratory disease by beginning medication
- 89% of respondents would report an outbreak of respiratory disease to a government animal health person.

The above results contain some very positive aspects (the self-perceived level of knowledge, the recognition of the importance of respiratory disease and the high level of willingness to contact relevant government authorities). However, the fact that a high percentage of respondents would react to an apparent respiratory disease problem by medication is not a very positive aspect. There is clearly a need to get information and support to pig farmers so that the response to a disease outbreak will focus more on sustainable prevention and controls programs (accurate diagnosis, appropriate vaccines, improved biosecurity) rather than an immediate adoption of antimicrobial medication.

As part of the communication activities and advocacy, special meetings with the Project Overview Group were conducted purposely to report and promote the significant accomplishments of the project particularly on the results of respiratory disease surveillance in Bulacan and Pampanga, bacterial isolation activity at RADDL Region 3, lung scoring study and on development of LAMP assays.

The members of the National Advisory Committee for Animal Disease Control & Emergencies, the highest policy making body within the BAI, found great value in the results of lung scoring study specifically the critical role of the agrivet supply store as a key player in providing information on disease management and prevention for backyard pig raisers. The study reported and documented that backyard farmers directly rely on the animal health related services/advice of the agrivet supply store. Thus, it was impressed to the BAI and the other extension providers especially from the local government units that activities pn training and dissemination of information on animal health need to include the owners of agrivet supply stores.

The National Meat Inspection Service (NMIS) found the lung scoring as useful method to enhance and strengthen the regulatory function of regional meat inspectors and even the veterinarians assigned by the local government units. The NMIS expressed willingness to adopt the lung scoring technique promoted by the project to be part of their regulatory services.

The project through PCAARRD made representations to the private hog industry groups to promote and advocate the outputs of the project and entice their participation in future R&D activity. The PROPORK and NFHFI are the two major hog federations which are also members of the Project Overview Group.

Figure 7.10. Interactions between Philippine project team members and key industry stakeholders.

Also under this Objective, three manuals on 1) Lung scoring: A tool for improving swine respiratory disease investigation, diagnosis and control, 2) Field sample collection: A quick reference for swine respiratory disease investigation and diagnosis, and 3) Disease outbreak investigation: A guide for animal health workers were developed and published through PCAARRD (see Figure 7.11). These manuals were developed based on the need to enhance the regional and national field workers to strengthen their skills and capacities to conduct proper disease surveillance, investigation and diagnosis. The manuals were disseminated to RADDLs, BAI, the veterinary schools of various State Colleges and Universities and the Provincial Veterinary Offices of Bulacan, Pampanga and Laguna.

Figure 7.11. The three Manuals produced by the Project.

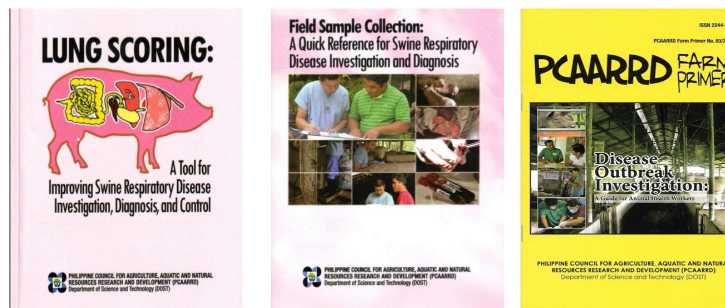


Table 7.21. Data from stakeholder analysis survey dealing with preferred communication channels and trusted sources of information on respiratory diseases.

Respondent	Preferred Communication Channel	Mean (SD)	Trusted source of information	Mean (SD)
Smallholder Pig Raiser	Brochures & flyers, pamphlets	3.49 (1.04)	Livestock Inspector – Agricultural Technologist	2.16 (1.02)
Commercial Pig Raiser	Seminars & fora	2.12 (1.16)	Seminars & fora	2.62 (1.39)
Veterinarian	Seminars & fora	2.27 (1.42)	Government veterinarians	2.63 (1.43)
Livestock Trader	Television	2.30 (1.43)	Government veterinarians	2.53 (1.45)
Livestock Inspector – Agricultural Technologist – Meat Inspector	Seminars & fora	2.03 (1.29)	Government veterinarians	2.47 (1.42)
Slaughterhouse Operator	Seminars & fora	3.00 (1.41)	Government veterinarians	2.22 (1.39)
Total		2.53 (1.29)		2.43 (1.35)

8 Impacts

Scientific impacts – now and in 5 years

Australian Based Work

There has been a rapid industry adoption of the multiplex assay for the identification and serotyping of *A. pleuropneumoniae*. This rapid adoption has been driven by the fact that the MRG is the national reference centre for this pathogen. The MRG provides the identification (at species level) and seorvar confirmation that underpins the entire porcine pleuropneumonia vaccine programs in Australia. The need for a serotyping service is driven by the fact that the widely used inactivated pleuropneumonia vaccines provide protection only against the serovars in the vaccine. Hence, when an outbreak occurs, there is a need to isolate and serotype the causative agent to determine if the off-the-shelf registered product (typically containing serovars 1, 7 and 15) is appropriate. Furthermore, there is a need to confirm if multiple serovars are present (and if those serovars are covered by the off-the-shelf product). If the decision is to use an autogenous vaccine (ie a farm specific vaccine) there is still a need for serotyping of the farm isolate as part of the formal registration process for such autogenous products. Hence, regardless of the final decision (off-the-shelf or autogenous), the typing services provided by the MRG underpin and guide the entire national program for pleuropneumonia vaccines.

The traditional serotyping process is slow, costly and involves the use of animals to produce the required sera. Hence, the MRG switched across to the molecular approach as soon as possible. The development of the multiplex PCR progressed in a staged manner – firstly the serovar 1, 5, 7 and 12 assay was developed and validated. This assay entered the user pays reference service provided by the MRG in October 2011. A total of 72 *A. pleuropneumoniae* isolates were serotyped by the serovar 1, 5, 7 and 12 multiplex. A number of isolates (over 20 in number) did not react to the serovar specific components of the assay and had to be serotyped by the traditional approach (and were all identified as serovar 15). In November 2013, the full multiplex (serovars 1, 5, 7, 12 and 15) was adopted for use in the reference service. Since that time, 95 isolates of *A. pleuropneumoniae* have been fully serotyped. To date, only one isolate has not given a reaction in the multiplex (with that isolate also being non-typable in the conventional assay.

The adoption of the multiplex serotyping assay will continue in Australia for the foreseeable future. The adoption of the serovar 15 component of the assay is likely to greatly increase in other countries over the next five years. Initially, serovar 15 was recognised by industry funded research undertaken by the MRG (Blackall et al., 2002). As overseas based diagnostic laboratories have adopted the traditional serotyping technologies used by the MRG, there has been a growing recognition that serovar 15 is widespread around the world (unless laboratories have access to the specific antisera, isolates of serovar 15 are typically wrongly serotyped). Using the Australian reference strain, isolates of serovar 15 are now recognised in a number of major pig producing countries – Canada (Gottschalk and Lacouture, 2014; MacInnes et al., 2008), Japan (Koyama et al., 2007) and Thailand (Tonpitak, 2010). In Canada, the laboratories still are unable to confidently identify serovar 15 – simply reporting that isolates that cross-react with serovars 3, 6, 8 and 15 were present in 15% of Ontario swine herds (MacInnes et al., 2008). The increasing profile of serovar 15 in major pig producing countries and the inability of confident recognition of this serovar by conventional serotyping means that the multiplex PCR developed in this project (or modified versions of the assay) is likely to be a widely adopted technology around the world in five years time.

Philippines Based Work

There is evidence already that the Philippines based component of this project is having scientific impact. The formal publication describing the pig production systems, the biosecurity practices and the herd health providers was published in 2014 (Alawneh et al., 2014). Despite this very recent appearance in the scientific literature, the publication has already been cited in the publications, with one of those publications being about pig production in a northern European setting (Backhans et al., 2015) and two involving studies on pig production in developing countries (Leslie et al., 2015; Mokoele et al., 2015). These formal citations, appearing so quickly after the formal publication of the ACIAR work, are clear evidence of the scientific impact of this work.

Further evidence of the scientific impact of the project has been the recent decision by an Australian based autogenous vaccine producer to actively explore the possibility of establishing a vaccine production facility in Pampanga. This Australian company is a heavy user of the serotyping and genotyping services provided in Australia by the MRG. Because of their prolonged use of the specialised diagnostic services provided by the MRG, the company is aware of the value in the guidance provided by these technologies in refining and targeting autogenous vaccines for pig respiratory diseases. This company has monitored the various conference papers arising from the project and sees the opportunity to provide targeted killed/autogenous bacterial vaccines to the Philippines pig industry with those vaccines being supported by the diagnostic systems developed in this project. This is a very positive sign – it is clear evidence that industry stakeholders (in this case a vaccine manufacturer) are aware of the value of the diagnostic systems developed in this project and see an opportunity to use those systems to support an vaccine option (autogenous, farm-linked vaccines) that is not currently easily available to Philippine pig producers.

As time goes by, there is likely to be a far greater interest in accurate, effective diagnostic systems as a means of ensuring that vaccination programs are targeted and sustainable. The key point is that vaccines provide a far more sustainable option than a reliance on antimicrobial agents but need to be used in a setting where an accurate diagnosis has been made – a porcine pleuropneumonia vaccine cannot control a problem that is actually enzootic pneumonia. Hence, there will be more industry stakeholders using the diagnostic systems established by this project and more sustainable and effective respiratory disease control programs in place.

Capacity impacts – now and in 5 years

The project has had a marked impact on the capacity of Philippine scientists involved in the project. Four of the project scientists had the opportunity to undergo intensive two week long hands-on training with partner researchers at the University of Queensland (Drs David, Lola, Manangiit and Retes). In addition, specialized training was organized for Dr Lola at the Animal Health Laboratories in Perth on the detection of PCV2 by immunohistochemistry. The professional development and professional network of a number of the Philippines scientists was enhanced by the staff attending major international pig conferences – Drs Balayut and Basinang attended the International Pig Veterinary Conference in South Korea while Dr Azul and Lola attended the Asian Pig Veterinary Congress in Vietnam.

The professional staff involved with the project in management and communication roles also had the opportunity to engage in professional development and extending their professional network. Ms Corales and Mr de Castro attended the Asian Pig Veterinary Congress in Vietnam with Ms Corales presenting two posters covering her communications research within the project.

Project leaders had the opportunity to visit both the University of Queensland and the then Department of Education, Employment, Development and Innovation (now the Department

of Agriculture) in Queensland to gain insight into research management. A particular focus of these visits was to examine the then newly developing research relationship between the University of Queensland and the Department – the organisations had recently negotiated the creation of the Queensland Alliance for Agriculture and Food Innovation – a research where the Department funds University staff to undertake work in key, identified research areas. These exchanges involved Drs Villar as well as Drs Dazo and Lapuz and meetings with senior management of both the University and the Department.

The professional and scientific staff involved with the attendance at international meetings gained new capacities in the skills and expertise needed to develop scientific presentations. As the ability to communicate effectively with colleagues is a key capacity, these new skills are an important part of overall capacity enhancement of the broad Philippines research team.

A major enhancement in capacity was shown with the performance of the two large field studies under Objective 3. These studies – while designed in full collaboration between the Australian and Philippines research teams – were performed by the Philippines team. Both studies required considerable logistical skills, large teams of people in the field and in the associated laboratories and significant co-ordination across numerous stakeholders (farmers, slaughterhouses, government regulators and so on). The two studies, as shown in the main body of this report, produced considerable new knowledge and understanding of the pig industry, the biosecurity/health practices of the pig industry in Region 3, the risk factors for respiratory disease and the role and interaction of various pathogens in causing respiratory disease. Importantly, the studies showed the capacity of the Philippines team to undertake and successfully complete highly technical, highly interactive studies. The generic skills gained by the Philippines team in large scale project management and co-ordination will serve the animal industries (pig and other food animals) of the Philippines into the future.

The skills developed by the Philippines team are broad skills and with a focus on integrated approaches that link both field and laboratory aspects to diagnose diseases. These new skills approaches will include appropriate field issues (survey design, clinical examination, sample collection, necropsies, field data collection) and laboratory issues (increased skills in conventional and molecular diagnostic methodologies). Hence, the fact that these skills are now embedded in core government staff ensures that the impact will continue to be relevant well into the future.

There was also a significant capacity increase within the Australian research team, with all team members gaining from the experiences and interactions with the Philippines team and the Philippines pig industry. Members of the team were able to gain experiences in diseases which are exotic to Australia – porcine epidemic diarrhea and porcine respiratory and reproductive syndrome. In the laboratory context, the development of new serotyping tool required the research staff at the MRG to gain a new range of skills in the application of molecular biology methods to identify gene sequences in previously uncharacterized genes.

As the core of the Australian research team has a long term commitment to pig research, the improved capacity gained in the current project will stimulate new research ideas and new areas of opportunity for industry relevant and industry funded research well into the future.

Community impacts – now and in 5 years

The project has involved direct interaction with producers via on visits, sample collection, disease investigation and surveys. These direct interactions reinforced the existing interactions between farmers and government technical officers, veterinarians and scientists. However, the main community impacts were indirect, through increasing capacity for disease diagnosis, thereby increasing production or increased assurance of disease-free status, enabling access to new markets.

The improvements in pig respiratory disease diagnosis delivered in this project will support more effective and more sustainable prevention and control programs within the Philippines pig industry. This will improve the production efficiency of all sectors of the pig industry and the welfare of both pigs and people ensuring greater economic returns to producers and the community.

The communication research undertaken has clearly shown the central role of advice and information provided by government officers for both smallholder and commercial producers. Future activities to improve pig production efficiency will achieve maximum community impact by recognizing the central trusted role of local government officers.

In the Australian context, the improved diagnostic tools for serotyping *A. pleuropneumoniae* isolates are in routine use and are helping guide and improve sustainable control of porcine pleuropneumonia via vaccination (as opposed to the non-sustainable approach of antimicrobial treatment). The improved diagnostic tools developed for Australia will enhance the profitability of the pig industry by reducing the negative impact of disease and will help ensure that the pig industry remains an important part of the Australian rural community.

Economic impacts

The project has been able to achieve immediate, albeit small, economic impacts within the life of the project. These impacts are linked to the front-line diagnostic activities of RADDL Region 3 and PAHC. Both laboratories now use the isolation and identification techniques established by the project for the isolation and identification of the key bacterial pathogens associated with porcine respiratory disease. These techniques were shown to be effective in the field studies under Objective 3 and have replaced the use of high cost, less effective commercial identification kits that are imported from outside the Philippines. The savings gained from ceasing the use of these expensive kits have been re-invested back into the general diagnostic activities of the relevant laboratories.

In the longer term, there is a strong likelihood of on-going economic benefits flowing to the Philippines. At around the time this project commenced, the Philippine swine industry was the eighth largest swine industry in the world and was worth AUS$2.5 billion (estimated in 2006). A key feature of the Philippine pig industry is that the 'backyard' industry is a major component of the overall industry, representing somewhere around 66% of the total industry. This ranks the backyard sector, in itself, as the 13th largest pig industry in the world. The total pig mortality rate in 2008 was estimated to be 3.75% in Luzon, with at least half caused by respiratory diseases. As a 1% mortality equates to 150,000 pigs (10,000 tonnes of pig carcass), a reasonable estimate is that respiratory disease was causing the loss of over 18,000 tonnes of pig carcass, a loss of considerable significance.

The key initial step in reducing the economic losses associated with pig diseases is the establishment of an effective diagnostic system (with both field and laboratory components optimised). The current project has made considerable progress in this optimisation of diagnostic systems. Hence, in the next five to ten years, effective prevention and control programs can be implemented and monitored. These programs, made possible by the optimised diagnostic systems based around the current project will reduce mortality, enhance average daily gain (growth rate) and feed conversion efficiency and reduce the need for costly antibiotics: an overall enhancement of industry profitability. The potential entry of a new supplier of autogenous/killed vaccines for bacterial respiratory pathogens highlights how improved diagnostic systems can open up entirely new opportunities for sustainable, effective prevention and control programs.

In Australia, a similar laboratory-focused economic impact has already occurred. The MRG is the national reference centre for the typing of *A. pleuropneumoniae*. The new diagnostic molecular tool developed in this project has reduced the time required for serotyping by between 2 to 4 days. The new test requires lees technical time input and does not require the use of unique typing sera produced from rabbits. Hence, the typing services that

underpins the entire Australian vaccination program for porcine pleuropneumonia is now delivered with a shorter turn around time and with less costs.

The economic impact of respiratory diseases on the Australian pig industry is widely recognized. It has been shown that, in 2004, a 1,000 sow piggery that is disease free will have a typical profit of around $951/sow/year. The same piggery with a moderate disease problem will make a profit of $825/sow/year, while with a severe disease status the profit falls to only $65/sow/year. The improved diagnostic service for porcine pleuropneumonia achieved in this project will be a key support to help move piggeries from the severe disease category to the moderate disease category.

Social impacts

It has been estimated that pig meat is the main protein source for over 90 million Filipinos. Indeed, the pig industry is the second most valuable agricultural product (rice is the most valuable) in the Philippines. The large dominance of smallholder farms in supplying this key food means that the improved government provided diagnostic and animal health services developed in this project will have significant social impacts. While these social impacts are not evident at the current time, the coming years will see Increased pig production efficiency will have clear social benefits within the Philippines: increased profitability for pig producers, increased reliability (reduced risk) for keepers of small numbers of animals and more stable prices for producers and consumers. Improved health and welfare of pigs also means a better and safer product for human consumption.

A key longer term social impact revolves around the issue of animal welfare. Effective disease reduction programs improve animal welfare, which is an increasing community concern and an increasing constraint on international trade.

The social impact of this project within the Australian context will flow in the coming years and will be based on the increased efficiency and hence increased profitability of the pig industry. The presence of a vibrant and profitable pig industry adds to the strengths and capacities of rural Australian communities.

Environmental impacts

In terms of the current, just finalized project, no clearly identified positive environmental impacts have occurred.

However, in the next five years, the project will help reduce the negative environmental impacts of both the Philippines and Australian pig industries. These improvements will flow because the project has delivered the diagnostic systems to support improved prevention and control programs for respiratory disease. The increased production efficiency that flows from better prevention and controls means that less feed is required and less effluent/waste is produced per unit of production.

Another key positive environmental impact will be the reduced usage of antibiotics by the pig industries of both countries. The environmental impact of the presence of antimicrobial agents in piggery waste is an emerging issue. The issue is both the active agents themselves as well as the possibility of antibiotic resistance genes moving from pig faecal bacteria to environmental organisms. These environmental organisms can then survive for much longer periods than the pig faecal bacteria and ultimately move the resistance gene back into another organism capable of infecting either humans and/or animals.

The project will reduce the need to use of antibiotics in two ways. Firstly, rapid and effective diagnostic systems allow quick and effective treatment regimes to be used. Secondly, accurate diagnosis allows the use of effective vaccination programs that do not rely on antibiotics.

Communication and dissemination activities

A range of communication and dissemination activities were undertaken in this project. The activities occurred in three main areas – industry stakeholder communication, peer communication activities and communication research. The formal report on the communication research activities has been presented earlier in this report under Objective 4. The following sections deal with the two other activity areas - industry stakeholder communication and peer communication activities

Industry Stakeholder Focused Activities

A range of activities were undertaken in this area. Unless otherwise noted, all of these activities were organised and delivered by the Philippines research team.

Formal publications providing information on the project have been published as follows:-

Newsletter and Website Activities

- "PHL-AUS Joint R&D to abate swine respiratory diseases" (2010) *The PCARRD Monitor* Vol 38
- Joint RP-Aussie R&D to abate swine respiratory diseases (2010) *ProPork Swine Newsletter* Vol 11
- PH-Aussie governments join R&D efforts to abate swine respiratory diseases (http://www.pcarrd.dost.gov.ph/CIN/swine/index.php?option=com_content&view=article&id=537:ph-aussie-governments-join-rad-efforts-to-abate-swine-respiratory-diseases)
- "Collaboration Visit", *Vet Connect* Summer Edition, 2010 (a newsletter produced by the School of Veterinary Science, The University of Queensland)

Seminars and Presentations

- Presentation to National Advisory Committee on Animal Disease Control and Emergencies, February 2011, PCARRD, Los Banos
- Presentation to Annual Consultation Meeting of the Swine Production Performance in the Philippines project, March 2011, PCARRD, Los Banos
- Presentation to Seven (7) Swine Association Presidents in Bulacan, April 2011, Provincial Veterinarian's Office in Bulacan
- Presentation to backyard raisers in 21 municipalities and 3 component cities of Bulacan Province alongside the PVO initiative to educate the merits of biosecurity in farm management, started 26th April 2011 and ongoing
- Presentation to National Federation of Hog Farmers – Manilla, August 2011.
- Presentation and hands on training in lung scoring and field sample collection to staff at PVO Bulacan and PVO Pampanga in November 2011. Participants totalled 47 and included agricultural technologists/livestock inspectors, local government veterinarians and veterinary students undertaking clinical training at RADDL Region 3.
- Presented the lung scoring study at the Annual Swine Production Performance meeting of the Swine Foundation in March, 2012. The activity was attended by about 80 participants (i.e. commercial farm owners, farm veterinarians, pharmaceutical company representatives and consultants).
- Provided hands-on training in lung scoring to local government staff within the Bulacan and Pampanga provinces in December 2012.

- Provided hands-on training in lung scoring for Elanco animal health technical staff in July 2012.
- Provided hands-on training in lung scoring for Catmon Multi-purpose Livestock Co-operative in July 2012.
- Presentation of outputs of lung scoring and its value as a tool in respiratory disease monitoring to the following meetings:-
    - National Advisory Committee on Animal Disease Control and Emergencies, March 2013
    - Swine Production Performance Project, April 2013
    - Hog Congress, April 2013
    - Annual Meeting of PROPORK members, April 2013

Brochures and Flyers

- Fact and Information sheets on project developed and disseminated to relevant industry stakeholders.

Formal Publications

The project produced three formal publications that targeted professionals directly working with the Philippines pig industry. These formal publications involved input from the full Australian and Philippines research teams and are as follows:-

- Lung Scoring - A tool for improved swine respiratory disease investigation, diagnosis and control.
- Field Sample Collection - A quick reference for livestock inspectors for swine respiratory disease investigation and diagnosis
- Swine Respiratory Disease Investigation – A guide for livestock inspectors and agricultural technologists.

The Lung Scoring Manual has been specifically promoted to the National Meat Inspection Service (NMIS) for improved post-mortem inspection of slaughtered pigs in slaughterhouses in the Philippines.

The formal peer review literature publications and the formal conference presentations are listed under Section 10.

Peer Communication Activities

A number of formal presentations at regional, national and international meetings were made by Australian and Philippines research team members. These formal professional communication activities are listed in detail under Section 10 “Publications produced by Project”. The following informal peer-level communication activities were also undertaken:-

- Lectures by Australian researchers (Drs Blackall and Parke) to faculty and students at University of the Philippines (Los Banos), Central Luzon State University and De La Salle Araneta University
- Lecture by Dr Blackall to staff at Bureau of Animal Industry, Quezon City

9 Conclusions and recommendations

Conclusions

The structure of this project involved parallel but interconnected activities that were focussed on achieving improved investigation, diagnosis and technical support for the control of porcine respiratory diseases in both Australia and the Philippines. As the sustainable technology options available in the two countries and the current levels of diagnostic support differed in the two countries, the following conclusions are presented in a country orientated sequence.

The Philippines

The initial activities undertaken in this project highlighted that the basis of an effective investigative and diagnostic system within the government provided services existed but that technical gaps and limitations meant that the services were not optimal. The key gaps and limitations were identified and then, within the resources available to the project, activities were undertaken to address those limitations and gaps.

In both the field based activities (regional level) and the laboratory services (at the regional and national level), the research team produced a series of protocols, forms and documents that addressed the gaps and limitations in the existing systems. The field based activities focussed on both on-farm investigations as well as establishing a formal approach (lung scoring) that allows the general respiratory health of slaughter pigs to be assessed and monitored. In the laboratories, novel but sustainable isolation and identification technologies for key bacterial and viral respiratory pathogens were established and validated. Novel molecular assays that involve little technology were also established in this activity.

The new systems were evaluated in two large field trials. These trials provided the following key information/knowledge:-

- The integrated field and laboratory diagnostic systems developed in this project are robust and capable of delivering relevant information to key stakeholders;
- A detailed knowledge of farm management practices in the area of biosecurity and general herd health;
- Lung scoring is a valuable tool that provide insight and knowledge on the respiratory health of pigs
- An understanding of the risk factors associated with both pneumonia (as shown by a high lung score at slaughter) and pleurisy (again as shown by a high pleurisy score at slaughter);
- An understanding that a high lung score is more commonly associated with commercial farms as opposed to smallholder farms;
- Further validation and evaluation work is required before the alternative molecular assays established in this project (LAMP assays for *A. pleuropneumoniae*, *H. parasuis* and *M. hyopneumoniae*) can be used in routine applications;
- An association between *P. multocida* and high lung lesion scores and an association between *P. multocida* and the presence of *A. pleuropneumoniae*-like lesions. These are associations only and not necessarily cause and effect. However, the associations add to the body of evidence suggesting that *P. multocida* may indeed be a primary agent of porcine respiratory disease.

Australia

The key result for the Australian based studies was the development, validation and routine use of a molecular alternative for the combined identification and traditional serotyping of *A. pleuropneumoniae* of serovars 1, 5, 7, 12 and 15. This new diagnostic tool has markedly improved the sustainability (in terms of economics and animal welfare issues) of the typing services that underpin the Australian vaccination programs to prevent and control porcine pleuropneumonia.

The typing assays evaluated for *H. parasuis* have been shown to be of value at a population level to understand potential virulence differences across the species and to follow disease outbreaks. However, while suitable tools at the population level studies looking at virulence levels, the tools are not useful at an individual strain level to confidently predict the pathogenicity of an individual strain.

Recommendations

On the basis of the achievements of the current project, the following recommendations are made:-

1. A follow-up project that seeks to “Scale Out” and to “Scale Up” should be considered.
2. The “Scale Out” component of a follow-up project should focus on establishing the type of integrated field and laboratory services developed in Region 3 within the current project to new Regions.
3. The “Scale Up” component of a follow-up project should seek to build on the expertise developed in the current project to undertake a holistic approach to achieving better health and disease control in the Philippines pig industry.
4. The “Scale Up” component of a follow-up project should be based in Region 3, with support from PAHC) to take advantage of the skills and expertise developed within the research team of the current project.
5. If a follow-up project does occur, that project should seek to address why the LAMP assays established in the current project functioned acceptably in the laboratory but appeared to be have challenges when applied in the field situation.
6. If a follow-up project does occur, further studies looking at the potential role of *P. multocida* as a primary respiratory pathogen of pigs should be included.
7. Due to the positive outcomes of the replacement of the conventional serotyping of *A. pleuropneumoniae* in the current project, any follow-up project should have a focus (within the Australian component) of seeking alternative non-serological methods to replace conventional serotyping of *H. parasuis* and *P. multocida*.

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List of publications produced by project

The following text provides the formal peer review publications and the conference presentations linked to this project. For the case of completeness, the publications and conference presentations associated with the linked John Allwright Fellow (Ms Denise Dayo from RADDL Region 3) are shown as well.

Formal Peer Review Publications

Alawneh, J.I.B., T., Parke, C., Lapuz, E., David, E., Basinang, V., Baluyut, A., Villar, E., Lopez, E.L., Blackall, P.J. 2014. Description of the pig production systems, biosecurity practices and herd health providers in two provinces with high swine density in the Philippines. Preventative Veterinary Medicine 114, 73-87.

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Formal Peer Review Publications linked to John Allwright Fellowship

Dayao, D.A.E., Gibson, J.S., Blackall, P.J., Turni, C. 2014. Antimicrobial resistance in bacteria associated with porcine respiratory disease in Australia. Veterinary Microbiology 171, 232-235.

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Conference Presentations linked to John Allwright Fellowship

Dayo, D., Turni, C., Gibson, J.S., Blackall, P.J. 2012. Towards a method for antibiotic sensitivity testing of *Haemophilus parasuis*. In: 6th International Veterinary Vaccines and Diagnostic Conference (Cairns, Australia).

Dayao, D.A.E., Gibson, J.S., Blackall, P.J., Turni, C. 2014. Antimicrobial resistance in respiratory bacterial pathogens in Australia. In: 81st Philippines Veterinary Medical Association Conference (Palawan, Philippines).

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11 Appendixes

11.1 Appendix 1: Detailed laboratory protocol for the quantification of Porcine Circo-Virus Type 2.

Introduction

This detailed laboratory protocol is based on a published assay – Olvera *et al.* (2004) – see full reference details at the end of this protocol.

Reagents and materials

- TaqMan® Universal PCR Master Mix (cat. 4304437, 200 reactions)
- Eukaryotic 18S rRNA Endogenous Control (5' VIC®/3' MGB probe, primer limited) (cat. 4319413E, 2500 reactions)
- PCV2 plasmid DNA (PCV2pGEMt)
- PCV2F primer (see Table at end of protocol)
- PCV2R primer (see Table at end of protocol)
- PCV2S (5' FAM - 3' TAMRA) probe (see Table at end of protocol)

Reaction components (25 µl reaction) – set up each sample in duplicate

	Volume per sample
PCV2F primer (22.5 µM)	1 µl
PCV2R primer (22.5 µM)	1 µl
PCV2S (5' FAM-3' TAMRA) probe (4 µM)	1 µl
TaqMan® Universal PCR Master Mix	12.5 µl
IC (18s) control	0.4 µl
PCR-grade water	6.60 µl
Template	2.5 µl

To be included in run

- 10^9 to 10^4 copies of PCV2 plasmid DNA (PCV2pGEMt) in duplicate
- Negative control reaction of PCR-grade water in duplicate

Cycling conditions

Rotor-Gene 6000 (Qiagen) or equivalent

Description	Cycles	Temperatures (°C)	Time
UNG incubation	1	50 °C	2 minutes
Amplitaq gold activation	1	95 °C	10 minutes
Denaturation	40	95 °C	15 seconds
Annealing/ Extension	40	60 °C	1 minute
Hold			

Acquire green and yellow channels at end of the extension step. The run time is approximately 100 minutes

Run analysis

After the completion of the run:

1. In the Analysis window, select Quantification
2. Select the channel Green press ‘show’ and then Yellow channel press ‘show’
3. Normalise the background fluorescence using the following parameters:

Parameter	Value/Function
Threshold	0.01
Begin normalising	Cycle 1
Normalisation method	Dynamic tube
Reaction efficiency	Acceptable range 90-110%
M (Std curve slope)	-3.6 to -3.1
R^2 Value	0.99
No Template Control Threshold	1%

Preparation of PCV2 standard

1. Measure concentration of PCV2 pGEMt plasmid DNA (Plasmid containing PCV2 virus genome) twice and take average value.

Note: use spectrophotometer parameters for double stranded DNA (dsDNA)

2. Calculation of plasmid concentration:

$$\text{Number of copies} = \frac{(\text{Amount (ng)} \times 6.022\times10^{23})}{(\text{*Length in bp*} \times 1\times10^{9} \times 660\text{**})}$$

*: Plasmid: (pGEMt) from Promega = 3000 bp in size

Insert: PCV2 genome = 1770 bp in size

Total (Plasmid & insert) = 4770 bp

**As dsDNA has a molecular size of 660 Daltons/base pair

For additional information see (http://scienceprimer.com/copy-number-calculator-for-realtime-pcr)

Example:

Measure concentration:

159.5 ng +160.5 ng = 160 ng

$$\text{Number of copies} = \frac{(160 \text{ (ng)} \times 6.022\times10^{23})}{(4770 \text{ bp} \times 1\times10^{9} \times \text{*}660)} = 3.06\times10^{10}$$

*: dsDNA: 660 Daltons/base pair

Dilution	**Source of plasmid DNA for dilution**	**Initial conc. (copies)**	**Volume of plasmid DNA (µl)**	**Volume of diluent (µl) (nuclease-free H_2O)**	**Final Volume (µl)**	**Final conc. in (copies)**
1. (1:3)	Stock	3.06x $x10^{10}$	2 µl	4 µ	6 µl	$1x10^{10}$
2. (1:10)	Dilution 1	$1x10^{10}$	5 µl	45 µl	50 µl	$1x10^{9}$
3. (1:10)	Dilution 2	$1x10^{9}$	5 µl	45 µl	50 µl	$1x10^{8}$
4. (1:10)	Dilution 3	$1x10^{8}$	5 µl	45 µl	50 µl	$1x10^{7}$
5. (1:10)	Dilution 4	$1x10^{7}$	5 µl	45 µl	50 µl	$1x10^{6}$
6. (1:10)	Dilution 5	$1x10^{6}$	5 µl	45 µl	50 µl	$1x10^{5}$
7. (1:10)	Dilution 5	$1x10^{5}$	5 µl	45 µl	50 µl	$1x10^{4}$

Preparation of PCV2 primer and probe working concentration

Formula:

(Total volume of working dilution (µL) x conc. of dilution (µM))/conc. of stock solution (µM) = volume of stock required (µL)

PCV2F

Stock conc. = 100 µM

Working conc. = 22.5 µM

To prepare 100 µL of a 22.5 µM primer working concentration from a 100 µM primer stock;

(100 µL x 22.5 µM)/100 µM = 22.5 µL

Use 22.5 µL of PCV2F primer stock (100 µM), aliquot 77.5 µL of sterile nuclease-free H_2O into a new 1.5 ml tube and add 22.5 µL of your PCV2F primer stock.

PCV2R

Stock conc. = 100 μM

Working conc. = 22.5 μM

Use 22.5 μL of PCV2R primer stock (100 μM), aliquot 77.5 μL of sterile nuclease-free H_2O into a new 1.5 ml tube and add 22.5 μL of your PCV2R primer stock.

PCV2S (probe)

Stock conc. = 100 μM

Working conc. = 4 μM

Use 4 μL of PCV2S probe stock (100 μM), aliquot 96 μL of sterile nuclease-free H_2O into a new 1.5 ml tube and add 4 μL of your PCV2S probe stock.

Table 1
Sequence, localisation and properties of primers and probe designed for the PCV2 real time PCR by primer express

Oligo	T_m (°C)	%GC	bp	Sequences (5′ → 3′)	Location in PCV2 genome
PCV2F	60	63	19	CCAGGAGGGCGTTGTGACT	1535 → 1553
PCV2R	59	55	20	CGCTACCGTTGGAGAAGGAA	1633 → 1614
PCV2S	68	52	25	AATGGCATCTTCAACACCCGCCTCT	1612 → 1592

T_m is calculated by the neighbour joining method (Primer Express v.1.5 software).

Reference:

1. Olvera, A., Sibila, M., Calsamiglia, M., Segalés, J. & Domingo, M. 2004, Comparison of porcine circovirus type 2 load in serum quantified by a real time PCR in postweaning multisystemic wasting syndrome and porcine dermatitis and nephropathy syndrome naturally affected pigs. *Journal of Virological Methods,* **117**:75-80.

11.2 Appendix 2: Multiplex polymerase chain reaction assay to identify and serotype isolates of *Actinobacillus pleuropneumoniae* of serovars 1, 5, 7, 12 and 15.

Preparation of samples for the polymerase chain reaction (PCR)

From an overnight culture grown on an appropriate medium, a 1 μl loopful of growth was suspended in 200 μl of water. The suspension was vortexed and cooled on ice for 5 min. This was followed by heating at 98°C for 5 min, cooling on ice for 5 min and a second round of heating for 5 min at 98°C. After centrifugation for 2 min at 17,380 x *g*, the supernatant was collected and stored at -20°C. A 2 μl aliquot of the supernatant was used for PCR analysis.

Multiplex PCR

The 50 μl reaction mix consisted of 10 mM Tris-HCl pH 8.3, 1.5 mM Mg Cl_2, 50 mM KCl, 0.005% Tween 20, 0.005% Nonidet P-40, 0.125 mM of each deoxynucleoside triphosphate (dNTP) (Roche, Mannheim, Germany) and 1 U of *Taq* polymerase (Roche, Mannheim, Germany). The sequences and concentrations of the primers used in this PCR reaction are provided in Table 11.2.1.

The primers used in this PCR confirm the isolate as *A. pleuropneumoniae* and then determine if the isolate is serovar 1, 5, 7, 12 or 15. To the master mix described above, 2 μl of DNA template was added.

The PCR reaction was run on an Eppendorf thermal cycler with the following cycling conditions: first cycle at 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 66°C for 20 sec and 72°C for 2 min with a final cycle at 72°C for 10 min. Amplification products were visualised by electrophoresis using 10 μl of the amplified product in 1% agarose gel (Progen, Australia) containing 50 ng/ml ethidium bromide in Tris-Acetate-EDTA buffer (0.04 M Tris-acetate, 0.01M EDTA) at 70 V for 2 hour and photographed under UV illumination.

Table 11.2.1 Primers used in the multiplex assay to identify and serotype *A. pleuropneumoniae*.

Primer name	Sequence	Conc. used (µM)	Amplicon size (bp)	Reference
LPF	5' AAG GTT GAT ATG TCC GCA CC 3'	0.2	951	Gram and Ahrens (1998)
LPR	5' CAC CGA TTA CGC CTT GCC A 3'	0.2		
Ap1F	5' GGG CAA GCC TCT GCT CGT AA 3’	0.2	754	Angen *et al*. (2008)
Ap1R	5' GAA AGA ACC AAG CTC CTG CAA T 3’	0.2		
Ap5F (A)#	5' TTT ATC ACT ATC ACC GTC CAC ACC T 3’	0.2	1114	Lo *et al*. (1998)
Ap5R (B) #	5' CAT TCG GGT CTT GTG GCT ACT AAA 3’	0.2		
Ap7F	5' GGT GAC TGG CGT ACG CCA AA 3’	0.2	396	Angen *et al*. (2008)
Ap7R	5' GGG CTG CAG ACT GAC GTA A 3’	0.2		
Ap12F alt	5' **GAT** GGT TCT CCA GAT GAC TCT GAA A 3’*	0.3	557	Angen *et al*. (2008)*
Ap12R alt	5' **TG**G CTA TTG GAT GAA GAT GAC TCA T 3’*	0.3		
Ap15 cF	5’ GGG GAT CGA AAG GCT ATG G 3’	0.2	269	This study
Ap15 eR	5’ CTG CGG TAA TCG CTA CCA TTA TCC 3’	0.2		

Original primer name according to Lo *et al*. (1998) in brackets

* Additional bases in the serovar 12 primers that were added are shown in bold

Appendix 11.3: Details of the five virulence-associated polymerase chain reaction assays for *Haemophilus parasuis*

Preparation of DNA from reference strains and field isolates

From an overnight culture grown on BA/SN (Turni and Blackall, 2007), a 1 µl loopful of growth was suspended in 100 µl of water. The suspension was heated at 98°C for 5 min, followed by cooling on ice for 5 min. After centrifugation for 5 min at 17,380 x *g,* the supernatant was collected and stored at -20°C.

Polymerase chain reaction assays

The details of the reaction mixes and the cycle conditions of the five assays are set out below. All assays were run on an Eppendorf thermal cycler. Amplification products were visualised by electrophoresis using 10 µl of the amplified product and 2 µl EZ Vision dye (Amresco) in 1% agarose gel (Progen) containing 1% TAE buffer at 80V for 1 hour.

The *vtaA* PCR used a 25 µl reaction mix that consisted of 5.0 µl of 5 x GoTaq Reaction buffer (Omega), 2mM $MgCl_2$, 0.4 mM dNTPs, 0.8 µM of primer YADAF1 5' – TTTAGGTAAAGATAAGCAAGGAAATCC - 3', 0.8 µM of primer PADHR1 5' - CCACACAAAACCTACCCCTCCTCC - 3', 400 nM of primer YADAF3 5' – AATGGTAGCCAGTTGTATAATGTTGC - 3', 400 nM of primer PADHR3 5' – CCACTGTAATGCAATACCTGCACC - 3, 1 U GoTaq (Promega) and 10 ng DNA template as previously described (Olvera et al., 2011). The cycling conditions were as follows: first cycle at 94˚C for 5 min, followed by 25 cycles of 94˚C for 45 sec, 64˚C for 45 sec and 72˚C for 60 sec with a final cycle at 72˚C for 7 min. This assay can result in two amplicons – one of 406 base pairs (bp) and one of 291 bp. Those isoates/strains giving both bands are termed *vta* group 1 and are reported to be virulent (Olvera et al., 2011) while those strains/isolates giving only the 291 bp band are termed *vta* group 3 and are regarded as aviruent (Olvera et al., 2011).

Mix (2X) (ThermoScientific), 2 mM $MgCl_2$, 0.4 mM dNTPs, 0.3 µM µM of primer MP_A1 5' - GGTTCTAGTTCACAAACAGCCAATAC - 3', 0.3 µM µM of primer MP_A2 5' - GATATTTACCCCTGCCTTCATTGTATC - 3', 0.3 µM of primer MP_B1 5 '- ATCTTGCCCTGATTAGAGAGTAGGAGT - 3', 0.3 µM of primer MP_B2 5' - GTGAATATAGCCCTTATCCAAATAGGC - 3 and 10 ng DNA template as previously described (Assavacheep et al., 2012). The expected amplicons were of 964 bp for *hhdA* and 557 bp for *hhdB* (Assavacheep et al., 2012).

The *lsgB* PCR used a 20 µl reaction mix that consisted of 10 µl of Fermentas PCR Master Mix (2X) (ThermoScientific), 2 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 µM µM of primer *lsgB-F1* 5' - ATGAATTTGATTATTTGTATGACTCCATTT - 3', 0.4 µM of primer *lsgB-R1* 5'- CTATTGGCATGTGTAGTCAATTACTTC -3', and 100 ng DNA template (Martínez-Moliner et al., 2012). The cycling conditions were as follows: first cycle at 94˚C for 3 min, followed by 35 cycles of 94˚C for 30 sec, 55˚C for 45 sec and 72˚C for 90 sec with a final cycle at 72˚C for 7 min.

The *fhuA* PCR used a 20 µl reaction mix that consisted of 10 µl of Fermentas PCR Master Mix (2X) (ThermoScientific), 2 mM $MgCl_2$, 0.4 mM dNTPs, 0.8 µM µM of primer *SSH1U* 5' - ATGGTTTGGTTGTAATGGAGTATC - 3', 0.8 µM µM of primer *SSH1L* 5' - AACAACGCCAGCTAGGCTTGTACT -3', and 100 ng DNA template as previously described (Zhou et al., 2010). The cycling conditions were as follows: first cycle at 94˚C for 5 min, followed by 30 cycles of 94˚C for 30 sec, 51˚C for 30 sec and 72˚C for 45 sec with a final cycle at 72˚C for 10 min. The expected amplicon was 563 bp (Zhou et al., 2010).

The *capD* PCR used a 20 μl reaction mix that consisted of 10 μl of Fermentas PCR Master Mix (2X) (ThermoScientific), 2 mM $MgCl_2$, 0.4 mM dNTPs, 0.8 μM μM of primer capD-F 5' - CGAAGGGAGTGTTTCTATCA - 3', 0.8 μM μM of primer capD-R 5' - GAGTTTCTCACCAGGTCTAA -3', and 100 ng DNA template as previously described (Wang et al., 2013). The cycling conditions were as follows: first cycle at 95˚C for 5 min, followed by 30 cycles of 94˚C for 30 sec, 51˚C for 30 sec and 72˚C for 45 sec with a final cycle at 72˚C for 10 min. The expected amplicon was 958 bp (Wang et al., 2013).

Appendix 11.4: Details of the multi-locus sequence typing technique for *H. parasuis*

Preparation of DNA from reference strains and field isolates

From an overnight culture grown on BA/SN (Turni and Blackall, 2007), a 1 µl loopful of growth was suspended in 200 µl of PrepMan Ultra reagent (Applied Biosystems, Foster City CA) and heated at 100°C for 10 min on a heating block. After cooling, the suspension for 3 min it was spun at 17,380 x *g* for 3 min, the supernatant collected and stored at -20°C until used.

MLST

The primers and PCR conditions were as previously described (Olvera et al., 2006). Partial sequences of seven housekeeping genes - β chain of ATP synthase (*atpD*), malate dehydrogenase (*mdh*) translation initiation factor IF-2 (*infB*), ribosomal protein b subunit (*rpoB*), 6-phosphogluconate dehydrogenase (*6pgd*), glyceraldehyde-3-phosphate dehydrogenase (*g3pd*) and fumarate reductase B (*frdB*) (as per Table 11.4.1 below). A reaction volume of 25 µl was prepared containing 2.5mM $MgCl_2$, 200 µM dNTPs, 0.4 µM of each forward and reverse primer, 1.5 U *Taq* polymerase and 10 ng of DNA template. The PCR conditions were 5 min at 95°C, 35 cycles of 1 min at 95 °C, 30 s at 48° C and 30 s at 72°C, with the final step for 10 min at 72°C.

To confirm the acceptability of the amplification, a 10 µl aliquot of the PCR product was mixed with 2 µl EZ-Vision dye (Amresco) and run on a 1% agarose gel (80V for 30 mins) with a 100 bp ladder to confirm the specific fragment size for each gene. Purification of the PCR product was done in a reaction volume of 10 µl consisting of 1 µl Exosap-IT, 8 µl DNA water and 1 µl of PCR product. This was held at 37°C for 30 min and 10 sec followed by holding for 15 mins and 10 sec at 80°C.

The 12 µl sequencing reaction was prepared adding of 4 µl of the purified PCR product to 4 µl of DNA water and finally 4 µl of the equivalent primer. All Sequencing was performed by the Australian Genome Research Facility (AGRF) at the University of Queensland. Sequence Alignment software (SeqED, v 1.5.2.2) was then used to import and align the forward and reverse complimentary sequences. The database available for *H. parasuis* from the PubMLST web site (http://pubmlst.org/hparasuis) was used to assign alleles for each typed isolate. The final seven allele profile then defined a sequence type (ST). All novel alleles were submitted to the designated curator (Dr Karen Register).

Table 11.4.1 Primers for the MLST PCR

Gene	Primer sequences	Fragment size (bp)
atpD	atpDF 5'-CAAGATGCAGTACCAAAAGTTTA-3' atpDR 5'-ACGACCTTCATCACGGAAT-3'	470
infB	infBF 5'-CCTGACTAYATTCGTAAAGC-3' infBR 5'-ACGACCTTTATCGAGGTAAG-3'	599
mdh	mdh-up 5'-TCATTGTATGATATTGCCCC-3' mdh-dn 5'-ACTTCTGTACCTGCATTTTG-3'	537
rpoB	rpoBF 5'-TCACAACTTTCICAATTTATG-3' rpoBR 5'-ACAGAAACCACTTGTTGCG-3'	501
6pgd	6pgdF 5'-TTATTACCGCACTTAGAAG-3' 6pgdR 5'-CGTTGATCTTTGAATGAAGA-3'	553
g3pd	3gpdF 5'-GGTCAAGACATCGTTTCTAAC-3' 3gpdR 5'-TCTAATACTTTGTTTGAGTAACC-3'	564
frdB	frdBF 5'-CATATCGTTGGTCTTGCCGT-3' frdBR 5'-TTGGCACTTTCGATCTTACCTT-3'	582

Appendix 11.5: Details of design, questionnaire, data management and analysis for the cross-sectional study

Study design

This was a cross-sectional study conducted between October 2011 and March 2012. The target population was finisher pigs in Bulacan and Pampanga provinces while the source population was finisher pigs that presented for slaughter during the study duration at 29 cooperating slaughterhouses in the Bulacan and Pampanga provinces (the total number of abattoirs in the region was 32). The study population was finisher pigs presented for slaughter where farm of origin was located in Bulacan or Pampanga provinces and selected by the specified sampling strategy. To identify a risk factor with an odds ratio of three or greater, assuming a statistical power of 80%, a level of significance of 95%, and 80% of unexposed farm have pigs with lung lesions (based on the findings of slaughterhouse lung score training conducted in the period between March and July 2011), and a prevalence of the risk factor in the population of 10%, 750 farms were required (Abramson, 2011). To account for logistical difficulties and potential loss of farm traceability, 175 herds (19% of the desired sample size) were further added to the desired sample size.

Farm questionnaire

The responses to the farm questionnaire were gathered by face-to-face interviews conducted by four experienced interviewers (two teams each comprised of two interviewers) between October 2011 and March 2012. The questionnaire was written in English and translated to the appropriate dialect, as required, at the interview. To reduce information bias, the questionnaire was pretested on regional and provincial veterinary officers and animal health advisors with expertise in the Philippines pig production systems. The questions were asked exactly as stated in the questionnaire and only non-directive guidance provided. The validity of the collected questionnaire data was confirmed during follow-up visits to six farms (three in each province). To reduce misclassification bias that could arise from coding errors, the interviewers and the first author checked and corrected impossible coding of categorical variables (n = 80) and unreliable outlier values for continuous variables (n = 3).

Statistical analysis – farm questionnaire

Farm profile, farmer demographics, and farm management practice data were described, and the associations with production type were assessed using Fishers exact χ^2test for categorical variables or Wilcoxon rank sum test to test the equality of the medians for continuous variables.

Agglomerative hierarchical cluster analysis (Everitt et al., 2001) was used to identify similar subgroups of farms (both smallholder and commercial) that clustered based on the source of their herd health advice – termed herd health providers (HHPs).

Social network metrics, analyses and figures were derived using R (R Development Core Team, 2012), Ucinet 6.421 (Borgatti et al., 2002) and NetDraw 2.123 (Borgatti et al., 2002). The current study sought to explore the ‘ego-centric’ network of the farmers and then use that network to identify those main HHP (‘more centrally located’) in the overall network and within each of the identified farmer group network structures (i.e. ‘socio-centric’ network). This was done by using centrality measures which provide a metric that quantify farmer interaction or ‘dependence’ on one or more HHP. Because each farmer could have more than one HHP, the analysis also examined whether pairs of farmers who engage with one HHP are more likely to also engage with another.

Lung scoring

Lungs from finisher pigs were scored over a 25-week period between October 2011 and March 2012 in Bulacan and Pampanga provinces with 110 intensive lung scoring sessions being performed. Lung scoring days and slaughterhouses were randomly allocated (one slaughterhouse per day) using multistage stratified random sampling process and a systematic random sampling process was used to select the sampling units (i.e. individual finisher pigs for lung scoring).

Due to the nature of processing slaughtered pigs the following method was employed to identify pigs during slaughter: the sample start-up pig and each of the subsequent pigs to be sampled were tagged with a green flag (flags consisted of a bamboo stick, 30cm long and 3 mm in diameter, with a 5 cm × 5 cm. Pigs that were not included in the study sample were tagged with a black flag. The tagging order was determined by the order in which lungs was ready for scoring. Once scoring of a green tagged pig was completed the lung scorer replaced the green tag with a black one.

The lung scoring system used in this study was based on the methodology of Straw and Leman (Straw et al., 1986). The degree of consolidation in each lung lobe was assessed by estimating the percentage of the volume of each lobe of the lung with visual and palpable signs of consolidation. The percentages were adjusted to a scale of 1 to 10 for the cranial and cardiac lobes, and a scale of 1 to 5 for the caudal and intermediate lobes. The total lung score for a pig was the sum of the score for all lobes. The maximum cranioventral lung score possible was 55. In order to identify tissue damage that could potentially be associated with *A. pleuropneumoniae*, chronic lesions observed primarily on the diaphragmatic (caudal) lobes involving a central abscess with or without pleurisy were scored yes if observed. Pleurisy was recorded as a score of zero – no pleurisy, one - fibrous adhesions between the lung lobes, two – pleurisy lesions over the caudal and or cranioventral lobes and also pleuritic lesions on the rib cage, or three – the lungs were severely adhered to the rib cage and difficult to remove without tearing the lung tissue. Pericarditis was recorded as yes if present. To reduce inter-assessor variability in lung score, throughout the study duration all lung scoring was conducted by one experienced veterinarian.

Data management and statistical analyses Lung Scoring

Farm records were excluded from this is study if there was inaccuracy in details supplied by livestock trader, or farms were located in other provinces, untraceable, or refused to participate. Moreover, a farm was also excluded if the date of slaughter was six weeks after pigs were sold to a trader. A six-week interval was selected based on the fact that respiratory lesions (particularly *M. hyopneumoniae* infection) of pig lungs can resolve 8 - 12 weeks after lesions develop (Kobisch et al., 1993).

The percentile distribution of lung scores of all pigs slaughtered on all slaughterhouse visits and originating from the same farm was calculated. Arbitrarily, a pig with a lung score greater than the median of all pigs was classified as high lung score (HLS) pig. For pleurisy scores, a pig with a score > 1 was classified as high pleurisy score (HPS) pig.

Descriptive statistics were determined for the lung and pleuritis scores for the study population (mean, median, first and third quartiles, minimum and maximum values, where applicable). These were compared to the descriptive statistics for the pig lung and pleuritis scores from the farms excluded from the analysis using χ^2 or Wilcoxon rank sum tests, respectively. Statistical significance was declared at alpha level of 0.05 or less. The prevalence (apparent prevalence) of HLS and HPS scores for the pooled data and for each production system or province was calculated as the proportion of HLS and HPS pigs, respectively. The 95% confidence interval was estimated using exact binomial method. The production system was classified as either commercial or smallholder in accordance with definition used by the Philippine Bureau of Animal Statistics (Philippines Statistics Authority, 2011). By this definition, a commercial system (C) piggery comprised a pig farm which

satisfies at least one of the following conditions: 1) at least 21 head of adult pigs; 2) at least 41 head of grower pigs; or 3) at least 10 head of adults and 22 head of grower pigs, while a smallholder system (S) piggery comprised a farm raising one to 41 pigs (mixed age) in their household.

Data were aggregated at the farm level. A farm was classified as being at high risk of HLS if at least 50% of lungs examined at slaughter had a HLS than the population median. A farm was classified as being at high risk of HPS if 50% of lungs had pleuritis score ≥ 1. In two separate modelling processes, the association between candidate explanatory variables and HLS or HPS scores were assessed using univariable mixed-effects logistic regression models with slaughterhouse fitted as a random effect. Correlation between potential predictor variables was investigated using Pearson's χ^2 tests. The overall fit of the final model was evaluated using the Hosmer and Lemeshow goodness of fit test. Because odds ratios (OR) may lead to an overestimation of the size of the main effect, crude and adjusted ORs were converted to crude or adjusted risk ratios (RR) using the technique of Beaudeau and Fourichon (Beaudeau and Fourichon, 1998).

To estimate the proportion of HLS (or HPS) risk in the total population associated with explanatory variables in the final model, the population attributable fraction (PAF) was calculated (Williamson, 2010). The PAF estimates the proportional reduction in average disease risk that would be achieved by eliminating the exposure of interest (Szklo and Nieto, 2007). Although interpretation of PAF requires an assumption of a causal relationship between exposure and the outcome variable, it also has application when causation is uncertain (Benichou et al., 1998). For exposure variables where a causal link has not been established, PAFs can be used to speculate on the importance of a given set of exposure variables as a disease determinant until causality is established. Average achievable reduction of prevalence (AARP) was calculated by multiplying adjusted PAF by the prevalences of HLS or HPS, respectively (Benichou, 2001).

For spatial analyses, a farm was classified as being at high risk of ‘respiratory disease’ if at least 50% of lungs examined at slaughter had a higher lung score than the population median, or 50% of lungs had pleuritis score ≥ 1. The number of high risk farms per square kilometre was geographically interpolated using kernel smoothing techniques (Bowman and Azzalini, 1997). The analyses were conducted using the spastat package (Baddeley and Turner, 2005) in R. The location of the study population (smallholder and commercial farms) was plotted as points over this density map to show the spatial distribution of the study population.

Spatial clusters of respiratory problem farms were assessed using the Spatial Scan Test performed in SaTScan™ version 9.1.1 software (Kulldorff and Information Management Services Inc., 2009), based on a purely spatial Bernoulli distributional assumption model and scanning for circular clusters with a maximal population threshold of 50% of sites. In this process time remained dormant and the observed number of high risk farms in a cluster was compared to the distribution of expected number of high risk farms if spatial location of all such farms were independent. The statistical significance of clusters was determined through 9999 permutations and significant clusters were declared at an alpha ≤ 0.05.

Appendix 11.6: Details of study protocol for the validation study

Case definition of a respiratory disease affected pig and apparently healthy pig

Respiratory disease affected pig

On the day of selecting pigs for tissue and blood sampling, a pig showing signs consistent with respiratory disease and eligible for sampling was defined as follows:

Suspected *M. hyopneumoniae* pig:

1- Growing pig between 13 to 17 weeks of age

2- Chronic (weeks to months), persistent, non-productive cough

3- Excessive dust, irritating gases, or concurrent infections result in more severe coughing

4- Signs of pneumonia (e.g. increased respiratory rate, fever, huddling, loss of appetite)

5- Retarded growth and ill thrift in the face of near-normal appetites

6- The pig had not had been treated with any additional antibiotics (parental or oral) for his pressing respiratory problem or for any other health issue(s) at least one month ***before the sampling day***. Antibiotics that are used routinely in feed or water as supplements do not qualify as an additional antibiotic treatment, therefore, the pig is still eligible for sampling.

Suspected *A. pleuropneumoniae* pig:

1- Growing pig between 13 to 17 weeks of age

2- Shallow, non-productive cough

3- Sudden onset of prostration

4- High temperatures, apathy, anorexia, stiffness, and perhaps vomiting and diarrhoea

5- Marked dyspneoa with mouth breathing and, perhaps, a foamy, bloody discharge from the mouth and nose

6- Generalized cyanosis

7- Chronic cough and Retarded growth and ill thrift

8- The pig had not had been treated with any additional antibiotics (parental or oral) for his pressing respiratory problem or for any other health issue(s) at least one month ***before the sampling day***. Antibiotics that are routinely used in feed or water as supplements do not qualify as an additional antibiotic treatment; therefore, the pig is still eligible for sampling.

Apparently healthy pig:

On the day of selecting pigs for tissue and blood sampling, a healthy pig was a growing pig between 13 to 17 weeks of age showing no clinical signs consistent with respiratory disease as stated above, showing no signs of any other disease or ill health, and had not received any additional antibiotic treatments at least one month before the day of sampling.

Sample size

Based on available resources, the total number of pigs to be tested for the study was 150. The ratio of sick to healthy pigs was to be maintained at 2:1.

Sampling frame

Potential collaborating farms were identified by the PVOs. Lung scoring in a number of provincial slaughterhouses was conducted in pigs from these farms until a picture of the lung score status of the farms was determined. Two low lung score and two high lung score farms were selected from each province. On-farm disease investigations and selection of pigs

On-going on-farm disease investigations using project disease investigations documents were conducted on a weekly basis. Four farms visits were conducted on the Tuesday or Wednesday of each week, (alternating between Bulacan and Pampanga) by three veterinarians and one technician. Throughout this study, each on-farm disease investigator (ODI) was assigned to one farm in each province. They were responsible for conducting a detailed on-farm disease investigation and identifying pigs meeting the three case definitions at each visit to the assigned farm. Twelve pigs were sampled each week.

Overview of study protocol

Two weeks before the study start date (termed pre-study period), a detailed disease investigation using the protocols developed in this project was performed for all participating farms. This period was required to allow for review and retraining of the ODIs to ensure consistent case definitions between them, to establish baseline information about the health status (or disease status) of pigs at the participating farms at that time, and to inform farm management (person who is in direct contact with the animals) about the study outline, objectives and targeted pigs which fitted the case definitions.

On-farm selection of pig samples

The ODIs identified two diseased pigs (following the case definition provided for *A. pleuropneumoniae* and *M. hyopneumoniae*) and one apparently healthy pig during disease investigation). Farms were assigned a colour code to distinguish the origin of the pigs after they left the farm. Each pig was marked on the back using a non-toxic spray-paint of the colour assigned to their farm using the letter “A” if it had *A. pleuropneumoniae* signs, the letter “M” if it had *M. hyopneumoniae* signs and “H” if it was apparently healthy.

Slaughter protocol

On farm visit days, pigs that were identified by the ODIs on their on-farm disease investigation and then selected based on the case definitions were necropsied and samples harvested at the Philippine Abattoir Development Corporation (PADC) in San Fernando, Pampanga on the same day of pigs selection. The pigs were euthanased humanely and the necropsy performed on sick (and healthy) pigs documented using study-specific data recording forms. For each necropsy, digital photographs of the pig before and after it was euthanased were taken. Photographs taken before euthanasia were of the pig’s head (face and upper airways) and body (cranial, lateral and caudal views). After euthanasia, the following photographs were captured: of the subcutaneous tissue, thoracic cavity, lungs, heart and pericardium, abdominal cavity, intestine, liver, kidneys and spleen. To minimize confusion with the containers and samples collected, RADDL and PAHC labelled all the containers using a province, farm, pig and sample specific code designed for the study.

Pig selection and blood and tissue sampling days

A total of 12 pigs were selected weekly and laboratory samples were harvested (blood and tissue; Table 11.6.1), dispatched and processed at RADDL Region 3 within working days of the week (Mon to Friday). The rationale behind this is that fresh samples (mainly tissue samples) are required for culture and that cultured samples will have to be examined 24 – 72 hours after processing. If Tuesday was a public holiday then on-farm disease investigations and tissue and blood sampling were conducted on the Wednesday.

Sample type, collection, handling and transport:

Blood and tissue samples (cranio-ventral (CV) and dorso-caudal (DC) lobes of the lungs, tonsil, mediastinal lymph node, spleen and liver) were collected from pig. Serum was extracted from the blood and stored at -80C for future PCV2 testing. One set of CV and DC lobes of the lungs was used for *A. pleuropneumoniae* and *M. hyopneumoniae* testing, another set for histopathology and the third set was stored at -80C as back-up samples. The tonsil, mediastinal lymph node, spleen and liver were stored at -80C or preserved in 10% buffered formalin for future PCV2 testing (see Table 11.6.2 for details). When a pig had no visible lesions on the relevant part of the lung then the tissue sample was comprised of normal sections only.

Serum and tissue samples were labelled as per project document guidelines (unique farm ID, unique pig ID, sample type, date). PAHC and RADD laboratory personnel prepared detailed SOPs for this study (see appendices) that were followed when collecting and dispatching the required samples.

Sample destinations

All harvested samples were sent to RADDL. RADDL personnel checked that all samples were correctly labelled then stored the backup sample and used the working sample for culture. They then prepared the working and histopathology samples as per PAHC SOPs (see appendices) and dispatched them to PAHC on Thursdays on a fortnightly basis. The *A. pleuropneumoniae* PCR was performed at PAHC and the DNA extract sent to CLSU for *M. hyopneumoniae* LAMP/PCR testing.

Culture and processing of samples at RADDL

Culture for *A. pleuropneumoniae* as well as other possible pathogens such as *P. multocida* and *H. parasuis* was conducted when the working sample arrived at RADDL Region 3 and processed by laboratory staff. Each sample was accompanied with a completed sample processing form. The form included farm and pig IDs, sampling date, tissue type, tissue size, schematic diagram indicating the site(s) on the lung lobe where the sample was taken from, complete history, and necropsy notes. RADDL harvested tissue healthy part and the diseased part of the working sample. Both samples were cultured on the same plate (the plate was divided to two halves, one half for the healthy tissue and the other for the diseased tissue). The plate will be clearly labelled as per RADDL lab SOPs.

Table 11.6.1. Description of required sample type, quantity (Qt.), sample collection method, sample destination, collection instructions, purpose and sampling fixation method (if applicable) from health and respiratory disease affected pigs from participating farms in Region 3.

Sample type	Qt.	Approx. size (minimum)	Collection method	Destination	Collection instructions	Purpose	Storage
Blood serum	1		10 ml tube	RADDL then to PAHC	Kept on ice at collection	To held frozen awaiting use in the qPCR for PCV2 and possible biochemistry tests.	Freeze -80°C
Lung section from CV lobes	1	2 cm X 2 cm	200 ml screw top container – suitable for holding formalin	RADDL then to PAHC	10% buffered formalin	Histopathology -Mhyo	10% buffered formalin
Lung section from CD lobes	1	2 cm X 2 cm	200 ml screw top container – suitable for holding formalin	RADDL then to PAHC	10% buffered formalin	Histopathology - APP	10% buffered formalin
Lung section from CV lobes	1	2 cm X 4 cm	Sterile 100 ml screw top container	RADDL	Kept on ice at collection	Backup sample (stored at RADDL not for processing)	Freeze -80°C

Table 11.6.1 (cont.). Description of required sample type, quantity (Qt.), sample collection method, sample destination, collection instructions, purpose and sampling fixation method (if applicable) from health and respiratory disease affected pigs from participating farms in Region 3.

Sample type	Qt.	Approx. size (minimum)	Collection method	Destination	Collection instructions	Purpose	Storage
Lung section from CV lobes	1	2 cm X 4 cm	Sterile 100 ml screw top container	RADDL then to PAHC	1- Sample contain healthy and diseased sections (if any) 2- Kept on ice at collection	Working sample. RADDL will use this sample for culture and then dispatch the same sample to PAHC to run the PCRs and DNA extraction	Freeze -80°C
Lung section from CD lobes	1	2 cm X 4 cm	Sterile 100 ml screw top container	RADDL	Kept on ice at collection	Backup sample (stored at RADDL not for processing)	Freeze -80°C
Lung section from CD lobes	1	2 cm X 4 cm	Sterile 100 ml screw top container	RADDL then to PAHC	1- Sample contain health and diseased section (if any) 2- Kept on ice at collection	Working sample. RADDL will use this sample for culture and then dispatch the same sample to PAHC to run the PCRs and DNA extraction	Freeze -80°C
Tonsil, Mediastinal LN, Spleen, Liver	1	1.5 cm X 1.5 cm of each organ	200 ml screw top container – suitable for holding formalin	RADDL then to PAHC	10% buffered formalin	Potential samples for PCV2 IHC assay	10% buffered formalin

Appendix 1 Standard Operating Procedures in the Collection of Samples for Animal Disease Diagnosis for the ODIs

A. What to prepare or bring before necropsy

1- Necropsy knife
2- Bone cutter
3- Scissors
4- Rat-toothed forceps
5- Scalpel and blade
6- Permanent pen
7- Lung score form
8- Cooler with coolant
9- Masks
10- Detergent soap
11- Boots
12- Screw cap sterile sample containers – 2 pcs
13- 10% formalin in 100ml container
14- Ethyl Alcohol in 100 container
15- 5 ml sterile screw cap sample container for each tonsil, mediastinal lymph node, liver, and spleen
16- 10 ml vacutainer (red cap)
17- Necropsy form
18- Camera for documentation
19- Gloves
20- Scrub suits & plastic apron
21- Alcohol lamp
22- 75% Alcohol
23- Metal trays – 4 pcs

B- Wear protective clothing – scrub suit, mask, gloves, boots before collection of samples and necropsy

C- Prepare and label collection containers with farm and pig IDs, date, your initials, tissue or fluid to be placed in the container before the necropsy

D- Perform physical examination of the pig

E- Observe structures of the head for any abnormalities

F- Perform the necropsy

I. Collect 3 to 5 ml blood at the jugular vein before euthanizing the pig

II. External examination of the pig

III. Examine the joints for any inflammation

IV. Observe structures of the head for any abnormalities

V. Examine the external genitalia for any abnormalities

VI. Cut and open skin to expose viscera and body cavities

G- Open the thoracic region by breaking the ribs and spreading apart the thoracic wall

I. Note for the presence of thoracic fluid

II. Examine serous membrane for displacement, adhesions, perforations and exudates

III. Examine the heart for presence of pericarditis and fluid

IV. Change gloves

V. Examine the lungs and do lung score

H- Collect samples as indicated in Table 3

I. Place the scissors, forceps and scalpel in the container with ethyl alcohol

II. Light the alcohol lamp and heat the scissors, forceps and scalper before cutting the tissues

III. Place the tissue in the designated and labelled container

IV. Examine the tonsil for any haemorrhage and sample

V. Examine the mediastinal lymph nodes and sample

VI. Collect samples as follows

1. 2.0 cm x 4.0 cm lung tissue from cranioventral lobe of the lungs for BI and PCR
2. 2.0 cm x 2.0 lung tissue from cranioventral lobe of the lung for backup
3. 2.0 cm x 4.0 cm lung tissue from dorsocaudal lobe of the lungs for BI and PCR
4. 2.0 cm x 2.0 cm lung tissue from dorsocaudal lobe of the lungs for backups
5. 2.0 cm x 2.0 cm lung tissue from cranioventral lobe of the lungs for histopathology
6. 2.0 cm x 2.0 cm lung tissue from dorsoventral lobe of the lungs for histopathology
7. 1.5 cm x 1.5 cm portions of spleen, mediastinal lymph node, and tonsil placed in individual 10 ml screw cap tubes

I- Countercheck if the samples collected are complete. See Table 3

J- Complete the label of the samples at the top and side of the container. Use permanent black fine tip marker pen. It should contain the following information:

1. Code of the farm and province
2. Pig status and identifier
3. Date of collection
4. Type of tissue in the container

E.g. AAFBH51 (Farm AA, Bulacan, Healthy pig, number 51)

7/30/13

LCV –PAHC (lung cranio ventral lobe for PAHC)

K- Place in big zip lock all containers with tissues that will go to PAHC then put in coolant. except for the container with formalin

L- Place in another big zip lock that will go to RADDL then put in coolant

M- Blood samples in vacutainers should be in slant position. If clotted, place in upright position and just keep it at room temperature until it reach the laboratory. RADDL will process the serum

N- Samples submitted in the laboratory should be accompanied with filled up necropsy form, lung scored form and laboratory examination request form

O- RADDL will take charge in storing and assorting the samples to be submitted at PAHC a day after the collection.

Appendix 2 Sample Submission to Philippine Animal Health Center - Instructions for RADDL

Checklist of tissues:

1- 1.5cm x 4.0 cm lung tissue from cranioventral lobe of the lungs placed in screw cap container, properly labelled with permanent marker. The lung tissue should be the sample used in the collection of inoculum for bacterial culture

2- 1. 5cm x 4.0 cm lung tissue from dorsocaudal lobe of the lungs placed in screw cap container, properly labelled with permanent marker. The lung tissue should be the sample used in the collection of inoculum for bacterial culture

3- 1.0cm x 1.0 cm lung tissue from cranioventral lobe of the lungs placed in 10% neutral buffered formalin for histopathology. Properly labelled with permanent marker.

4- 1.0cm x 1.0cm lung tissue from dorso-caudal lobe of the lungs placed in 10% neutral buffered formalin for histopathology. Properly labelled with permanent marker.

5- Serum sample placed in microcentrifuge tube. Properly labelled with permanent marker.

6- 1.5cm x 1.5cm portions of spleen, mediastinal lymph node and tonsil placed in individual screw cap containers and properly labelled with permanent marker.

Packaging and dispatch:

All sample containers should be placed in a cooler with gel coolant or its equivalent during transport. In cases where the samples are not yet for dispatch, the tissue samples and serum should be stored in the freezer or -20 biological refrigerator. The samples should be accompanied with a properly completed submission form.

Submission at PAHC Specimen Reception:

1. When submitting the samples at the specimen reception, give the submission form (and samples) and emphasize that the samples are for ACIAR project
2. PAHC form will be attached to the submission form and will be given PAHC case number as case identity of the samples
3. The samples will be endorsed to Dr Lilia Retes or Dr Rachel Azul for processing

Processing of samples:

1. The histopathology samples will be endorsed to Dr Lola of UP CVM for processing and reading. The tissue samples will be encoded in the ACIAR submission log book for documentation. The submission forms will be filed in a folder
2. The samples will be temporarily stored at the -20 biological refrigerator. The reagents and materials for DNA extraction will be prepared
3. Bacterial DNA extraction based on Qiagen 's DNeasy Blood and Tissue Kit protocol - 2 sets will be prepared. One set of DNAs will be dispatched to CLSU and the other set will run for App multiplex PCR.
4. The remaining tissue samples will be stored at the -80 freezer at the cold storage room of PAHC for future *Pasteurella* serotyping studies. The specific location of the samples in the -80 freezer will be written at the ACIAR submission log book for documentation.

5. The results of the assays- App multiplex will be reported in ACIAR PCR result forms. 2 soft copies will be printed out- one copy will filed on a folder, and the other copy will be dispatched to RADDL. An electronic version of the result can be requested if needed
6. The histopathology result will be reported in ACIAR pathology result forms. Two soft copies will be printed out- one copy will filed on a folder, and the other copy will be dispatched to RADDL. An electronic version of the result can be requested if needed
7. The results will be available/released 5-7 days after receipt of samples at PAHC

Appendix 11.7: Details of two typical standard operating procedures – Spot indole test and the complete growth medium (BA/SN)

Spot indole test

This spot test is used in place of a conventional indole test (in which the organism is grown overnight in a tryptone water type medium). This spot test is rapid and easily applied at a diagnostic level.

1. Add a drop of Kovac's Indole reagent (current batch is Merck Cat # 1.09293.0100) to a piece of filter paper placed in an empty petri dish. Wear gloves when handling this chemical.
2. From a fresh overnight agar culture, use a loop to rub a visible amount of growth onto the area with the indole reagent.
3. An immediate (5 secs) pink colour indicates a positive reaction.
4. A lack of pink colour after 5 secs is a negative reaction.
5. Reactions should not be read after 10 secs.

Control Strains

The indole positive control strain is the *Pasteurella multocida* PM 55.

The indole negative control strain is *Bordetella bronchiseptica* BR 976

Complete growth medium - BA/SN Agar

BA/SN is a good all round medium for the growth of pure cultures of growth-factor dependent (i.e. nicotinamide adenine dinucleotide requiring) bacteria such as *A. pleuropneumoniae* and *H. parasuis*. While not normally used as a primary isolation medium, it can function in this role. When used in this way, it will support luxurious growth of many types of bacteria. A variation of BA/SN that is selective and reduces the growth of competing organisms is available as a primary isolation medium (see Selective BA/SN protocol).

BA/SN can be used to look for the presence of *H. parasuis* in nasal swabs. In this application, small iridescent colonies are selected as possible *H. parasuis* and are checked for satellitic growth on blood agar.

Preparation Details

1. Weigh 20.0 g (+/- 0.05 g) **BBL Blood Agar Base** (**Becton Dickinson 4311037**) into a 500 ml bottle.
2. Add 475 ml deionised water (measured with a measuring cylinder).
3. Mix well: make sure the powder is completely lifted from the bottom of the bottle. Also make the sure cap is not leaking.
4. Autoclave at 121°C for 20 minutes.
5. Remove agar from autoclave, tighten lid and cool in the 50°C waterbath for at least 30 minutes.
6. In a laminar flow cabinet, aseptically combine the supplements for the required volume of agar (see Table 11.7.1), in a sterile container.
7. Remove molten agar from 50°C waterbath and within the laminar flow cabinet add supplements, mix and pour the plates.

8. Allow plates to dry in the cabinet for 30 minutes and store in sealed plastic bags at 4°C.
9. Incubate one plate at 37°C and one plate at room temperature for 48 hours for a sterility check.
10. Discard unused plates after 4 weeks.

Table 11.7.1: Supplements for BA/SN agar

SUPPLEMENTS	500 ml	100 ml
1% reduced nicotinamide adenine di nucleotide	1.25 ml	0.25 ml
0.05% Thiamine HCl	5 ml	1 ml
Heat Inactivated Chicken Serum or Horse Serum (56^{0}C/30 minutes)*	5 ml	1 ml
Oleic acid - Albumin Complex	25 ml	5 ml

*Horse serum currently used comes from JRH BioSciences, catalogue number 12449-500M.

Oleic acid – Albumin (O-A) Complex

1. Make a sodium oleate solution by combining 0.3 ml of oleic acid (any general purpose grade reagent quality will do) with 25 ml of 0.05N NaOH.
2. Make an albumin solution by dissolving 23.75 g of bovine albumin fraction V (current source is JRH Biosciences Cat # 85040) in 475 ml of normal saline (add gradually and avoid heating).
3. Add 25 ml of sodium oleate solution to albumin solution.
4. Adjust pH to 6.8.
5. Filter (depth filtration best) and dispense aseptically in 100 ml amounts.
6. Incubate in 37°C water bath overnight.
7. Incubate in 56°C water bath for 30 minutes.
8. Store at 4°C.
9. Dispense aseptically in laminar flow cabinet as required.

Thiamine solution (0.05% aqueous)

1. Accurately weigh 0.05 g of thiamine (Sigma – Cat # T 4625) (also called Aneurine Hydrochloride) into a 200 ml beaker.
2. Add 100 ml of distilled water and dissolve thiamine.
3. In a laminar flow cabinet sterilise the solution using a 0.22 µm filter and a 50 ml syringe.
4. Store at 4°C.
5. Always dispense aseptically in a laminar flow cabinet.

1% NADH

1. Dissolve 1 g of NADH, disodium salt grade II from Roche into 100 ml of distilled water
2. In a laminar flow cabinet, filter sterilise (using a 0.22 μm filter and a 50 ml syringe) and aliquot into 1 ml lots.
3. The aliquots are stored frozen.
4. Thawed aliquots are kept refrigerated and are used within a month.

Appendix 11.8: Typical identification table for certain pathogenic bacteria associated with pig respiratory diseases.

Key characters for the differentiation of the growth factor dependent species of *Actinobacillus* and *Haemophilus* found in pigs. Data based on Gottschalk et al. (2003), Kilian and Frederiksen (1981), Møller and Kilian (1990) Møller et al. (1996) and Tonpitaka et al (2007). + = $\geq$ 90% positive; (+) = > 90% positive or weak positive - = $\leq$ 10% positive; V = 11 – 89% positive

Characteristic	*Actinobacillus pleuropneumoniae*	*Actinobacillus porcitonsillarum*	*Actinobacillus minor*	*Actinobacillus porcinus*	*Actinobacillus indolicus*	*Haemophilus parasuis*
Haemolysis	V	+	-	-	-	-
CO_2 improves growth	-	-	-	-	-	-
Symbiotic growth	V	+	+	+	+	+
Catalase	V	-	-	-	+	+
Urease	+	+	+	-	-	-
Indole	-	-	-	-	+	-
Acid from						
Lactose	-	ND	+	V	V	-
Maltose	+	ND	+	V	+	+
(-)-D-mannitol	+	-	-	V	-	-
B-galactosidase	+	+	+	+	+	+
Host	Pig	Pig	Pig	Pig	Pig	Pig

Appendix 11.9: List for Reference Quality Control Strains

A set of live QC bacteria were sent to the Philippines to assist in the validation of various diagnostic assays.

Some of the strains are actually formal international reference strains while others are filed isolates that have been identified by definitive molecular tests (either a species specific PCR or 16S rDNA sequencing).

The full decode of all the isolates/strains hipped to the Philippines is shown below:-

Species	**Strain**
Actinobacillus pleuropneumoniae serovar 3	1421
Haemophilus parasuis serovar 3	SW 114
Staphylococcus hyicus	BR 540
Bordetella bronchiseptica	BR 976
Streptococcus suis	BR 844
Escherichia coli	ATCC 25922
Staphylococcus aureus	ATCC 29213
Staphylococcus aureus	ATCC 245923
Pasteurella multocida subspecies *multocida*	NCTC 10322

Some of the international strains have recognised roles as QC strains for antibiotic sensitivity testing or as serovar reference strains or as formal taxonomic type strains. These roles are shown below: -

Species	Strain	Role
Actinobacillus pleuropneumonia serovar 3	1421	Reference strain for serovar 3
Haemophilus parasuis serovar 3	SW 114	Reference strain for serovar 3
Escherichia coli	ATCC 25922	CLSI Antibiotic QC strain for Disc Diffusion and MIC technology
Staphylococcus aureus	ATCC 29213	CLSI Antibiotic QC strain for MIC technology
Staphylococcus aureus	ATCC 25923	CLSI Antibiotic QC strain for disk diffusion technology
Pasteurella multocida subspecies *multocida*	NCTC 10322	Formal Taxonomic Reference strain for species and sub species

The references for the standardised antimicrobial sensitivity testing are as follows:-

1. Clinical and Laboratory Standards Institute (CLSI). 2013. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; Second Informational Supplement. CLSI Document VET01-S2 (Wayne, Pennsylvannia, Clinical and Laboratory Standards Institute).

2. Clinical Laboratory Standards Institute (CLSI). 2013. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals: Approved Standard - Fourth Edition. CLSI Document VET01-A4 (Wayne, Pennsylvannia, Clinical and Laboratory Standards Institute).

Appendix 11.10: Examples of New Protocols and Procedures for Disease Investigations

A series of documents that formalise the on-farm disease investigation processes as well as slaughterhouse-based disease checks (lung scoring) have been developed by the project team and are in active use. Examples of the documents are listed below and provided on the following pages:-

A) Respiratory disease investigation checklist

B) Necropsy worksheet

C) Lung scoring form

Respiratory Disease Investigation Checklist

ACIAR Project AH/2009/022

Improved investigation, diagnosis and technical support for the control of respiratory diseases of pigs in the Philippines and Australia

RESPIRATORY DISEASE INVESTIGATION CHECKLIST

Date: ______________

Farm Code: _____________

Farm visit number: ____

Name of investigator (s): ____

Province: Municipality/City: Barangay:

Name of farm: ____

Owner: ____

Farm address: ____

Phone No.: Fax No.:

Main office address: ____

Phone No.: Fax No.:

Consulting Veterinarian: Phone No.

Respiratory disease investigation checklist Version 1.3 (25 May 2011) 1

ACIAR Project AH/2009/022

Improved investigation, diagnosis and technical support for the control of respiratory diseases of pigs in the Philippines and Australia

RESPIRATORY DISEASE INVESTIGATION CHECKLIST

Farm Code:

Farm visit number:

Longitude: Latitude:

Type of farm: Commercial Semi-commercial ☐

Backyard ☐ Contract Grower ☐

Introduced pigs and semen:

Pig type	Introduced?	Source and location of pigs	Date of last introduction	Number of pigs or Doses of semen
Gilts	Yes/No			
Boars	Yes/No			
Semen	Yes/No			
Grower pigs	Yes/No			

ACIAR Project AH/2009/022

Improved investigation, diagnosis and technical support for the control of respiratory diseases of pigs in the Philippines and Australia

RESPIRATORY DISEASE INVESTIGATION CHECKLIST

Farm Code: **Farm visit number:**

Biosecurity measures:

	Yes	No	Details
Have you seen feral pigs in contact with domestic pigs?			
Can visitors contact your pigs without your knowledge?			
Are visitors supplied with clean boots and overalls?			
Are ACIAR project staff supplied with clean boots and overalls?			
Do you do artificial insemination on your farm?			
If you do artificial insemination on your farm, have you consulted a veterinarian/adviser on disease prevention for introduced semen?			
Are introduced pigs kept separate for a period of time (quarantined) and observed for signs of disease?			
How long are introduced pigs kept in quarantine?			
How far is the quarantine area away from the main piggery?			
Do all of the people who work with the pigs on your farm know what swill is?			
Is swill fed to pigs?			
Is 'Feedback' carried out on this farm, if so for what disease(s)?			
Do you see rats and or mice in contact with your pigs?			
Do you regularly clean pens and/or remove manure where your pigs are housed?			
Are the Livestock vehicles used to transport your pigs to the slaughterhouse allowed to enter the farm?			
Do the Livestock vehicles ever have pigs on them when they arrive at your farm?			
Can you recognise 'sick' and 'healthy' pigs?			
Are sick and dead pigs recorded for each age-group of pigs as part of a health monitoring program?			
Is there another piggery close to this piggery, if so how close is it?			

ACIAR Project AH/2009/022

Improved investigation, diagnosis and technical support for the control of respiratory diseases of pigs in the Philippines and Australia

RESPIRATORY DISEASE INVESTIGATION CHECKLIST

Farm Code: **Farm visit number:**

Reference	**Boar**	**Dry or gestating sow**	**Gilt**	**Lactating sow**	**Sucker (piglet)**	**Weaner (Weaning to 10 weeks of age)**	**Grower Finisher (10+ weeks of age to slaughter)**
Number of live pigs (healthy & sick)							
Number of dead pigs at the day of inspection							
Pig health/disease status, enter number of pigs affected in each group							
Respiratory disease							
Sneezing							
Coughing							
Mouth breathing							
Thumping							
Nasal discharge							
Huddling - because pigs are sick (not healthy suckers)							
Twisted snout / epistaxis							
Sudden death (pig has good body condition)							
Locomotor disease (lameness, paralysis, etc)							
Neurological disease (paddling, ataxia, etc)							
Weight loss (emaciated, decreased growth rate, etc)							
Pig feeds used -Home mix (H) -Commercial (C) -Swill (S)							
Antibiotics in feed, please specify e.g. Tetracycline							
Antibiotics in water, please specify							
Injectable antibiotics given routinely, please specify							

4

Respiratory disease investigation checklist Version 1.3 (25 May 2011)

ACIAR Project AH/2009/022
Improved investigation, diagnosis and technical support for the control of respiratory diseases of pigs in the Philippines and Australia

RESPIRATORY DISEASE INVESTIGATION CHECKLIST

Farm Code: **Farm visit number:**

Vaccination Schedule for respiratory disease:

Birth to Slaughter (sucker, weaner, grower and finisher)

Disease	Brand name	Age at 1st dose	Brand name	Age at 2nd dose	Brand name	Age at 3rd dose

Selected gilts and selected boars

Disease	Brand name	Age 1st dose	Brand name	Age 2nd dose	Brand name	Age 3rd dose

ACIAR Project AH/2009/022

Improved investigation, diagnosis and technical support for the control of respiratory diseases of pigs in the Philippines and Australia

RESPIRATORY DISEASE INVESTIGATION CHECKLIST

Farm Code: **Farm visit number:**

Vaccination Schedule for respiratory disease (continued):

Sows

Disease	Brand name	Frequency of vaccination or timing in relation to breeding cycle

Boars

Disease	Brand name	Frequency of vaccination and timing

Have Respiratory diseases been previously diagnosed (and test method) for this farm: Yes/No

If yes provide details of disease and time.

____

____

Necropsies performed on farm and samples submitted to veterinary laboratory:

Pig class	No. of necropsies	Sample type & details	Number of samples	Laboratory	Date of submission	Date report received from lab	Date report to farmer
Boars							
Dry sows							
Gilts							
Lactating sows							
Suckers							
Weaners							
Growers							

NECROPSY WORKSHEET

ACIAR PROJECT AH/2009/022: RESPIRATORY DISEASE

Necropsy Worksheet

Date: ____________________

Name of investigator: ____________________

Owner / Farm: ____________________

Address: ____________________

Species: ____________________
Identification: ____________________
Date of death: ____________________
Sex: __________ Age: __________ Weight: __________ kg
Number affected: __________ Number dead: __________
Euthanased: Yes / No Method: __________
Freshly dead ☐ Mild decomposition ☐

Circumstances of Death: ____________________

Necropsy Commenced: am/pm Date ___ / ___ / ___

External Findings: ____________________

Body Condition:
Hydration: __________ Fat Deposits: __________ Muscle Mass: __________

Internal Findings:
Body Cavities (abnormal fluids, mesentery, thorax, abdomen): ____________________

Respiratory System (Nasal cavity, larynx, trachea, pleura, lungs): ____________________

1

Necropsy worksheet Version 1 (23 February 2011)

ACIAR PROJECT AH/2009/022: RESPIRATORY DISEASE

Necropsy Worksheet

Cardiovascular System (Heart, pericardium, heart valves):__________

Nervous System (Brain, meninges):__________

Haemolymphatics (tonsils, lymph nodes, spleen, thymus):__________

Sensory Organs (Eyes, ears):__________

Musculoskeletal (joints):__________

Digestive System (peritoneum, mouth, tongue, oesophagus, stomach, intestines, liver):__________

Samples Saved/ Tests Requested:

Microbiology:__________

Histopathology:__________

Photos:__________

Tissues for Researchers:__________

Carcase Disposition:__________

Tentative Diagnoses:

1. __________
2. __________
3. __________
4. __________

Comments & Clinical Problems Answered at Necropsy:__________

2

Necropsy worksheet Version 1 (23 February 2011)

LUNG SCORING FORM

ACIAR RESPIRATORY DISEASE INVESTIGATION – LUNG SCORING FORM

Slaughterhouse: ________________ Slaughterhouse Visit Number: _____ Start Date: _______

Lung Number: ____________________

Trader Name: ____________________

Trader Code: ____________________

Producer Name: ____________________

Producer Address: ____________________

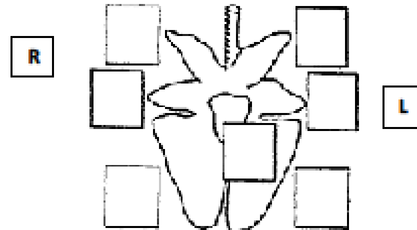
Farm Type: ☐ Commercial ☐ Backyard ☐ Semicommercial ☐ Contract

Sex: ☐ Male ☐ Female

Breed: ____________________

Notes: ____________________

Complete Score: ☐ Y ☐ N



Cranioventral Pneumonia: ☐ Acute ☐ Chronic

Pleurisy: ☐ 0 ☐ 1 ☐ 2 ☐ 3

APP: ☐ Y ☐ N

Pericarditis: ☐ Y ☐ N

Lung Number: ____________________

Trader Name: ____________________

Trader Code: ____________________

Producer Name: ____________________

Producer Address: ____________________

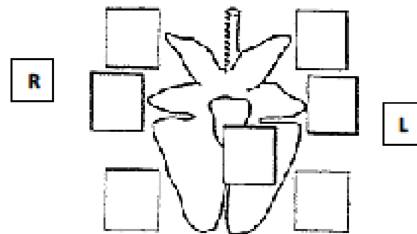
Farm Type: ☐ Commercial ☐ Backyard ☐ Semicommercial ☐ Contract

Sex: ☐ Male ☐ Female

Breed: ____________________

Notes: ____________________

Complete Score: ☐ Y ☐ N



Cranioventral Pneumonia: ☐ Acute ☐ Chronic

Pleurisy: ☐ 0 ☐ 1 ☐ 2 ☐ 3

APP: ☐ Y ☐ N

Pericarditis: ☐ Y ☐ N

Lung Number: ____________________

Trader Name: ____________________

Trader Code: ____________________

Producer Name: ____________________

Producer Address: ____________________

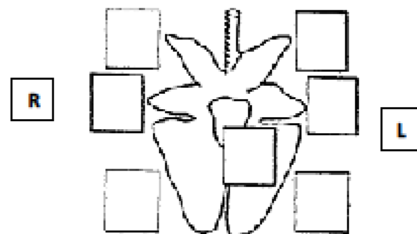
Farm Type: ☐ Commercial ☐ Backyard ☐ Semicommercial ☐ Contract

Sex: ☐ Male ☐ Female

Breed: ____________________

Notes: ____________________

Complete Score: ☐ Y ☐ N



Cranioventral Pneumonia: ☐ Acute ☐ Chronic

Pleurisy: ☐ 0 ☐ 1 ☐ 2 ☐ 3

APP: ☐ Y ☐ N

Pericarditis: ☐ Y ☐ N

Appendix 11.11: Summary of histological lesions of bronchopneumonia and peribronchial cuffing for dorsocaudal and cranioventral lobes for each category of pig (healthy, suspect *Mycoplasma hyopneumoniae* and suspect *Actinobaciullus pleuropneumoniae*) in the validation study

Frequency distribution of histological lesions of bronchopneumonia and peribronchial cuffing for dorsocaudal and cranioventral lobes for each category of pig (healthy, suspect *Mycoplasma hyopneumoniae* and suspect *Actinobaciullus pleuropneumoniae*).

	Dorsocaudal lobe				Cranioventral lobe			
Histopathology	Healthy	Suspect MHYO	Suspect APP	Total	Healthy	Suspect MHYO	Suspect APP	Total
Bronchopneumonia status								
Absent	27 (51.9)	30 (57.7)	21 (40.4)	78 (50.0)	47 (90.4)	38 (73.1)	36 (69.2)	121 (77.6)
Mild	0 (0.0)	0 (0.0)	2 (3.8)	2 (1.3)	0 (0.0)	1 (1.9)	0 (0.0)	1 (0.6)
Moderate	16 (30.8)	14 (26.9)	20 (38.5)	50 (32.1)	3 (5.8)	9 (17.3)	11 (21.2)	23 (14.7)
Severe	8 (15.4)	8 (15.4)	9 (17.3)	25 (16.0)	2 (3.8)	2 (3.8)	5 (9.6)	9 (5.8)
Missing	1 (1.9)	0 (0.0)	0 (0.0)	1 (0.6)	0 (0.0)	2 (3.8)	0 (0.0)	2 (1.3)
Bronchopneumonia stage								
Absent	27 (51.9)	30 (57.7)	21 (40.4)	78 (50.0)	47 (90.4)	38 (73.1)	36 (69.2)	122 (78.2)
Active	24 (46.2)	22 (42.3)	31 (59.6)	77 (49.4)	5 (9.6)	12 (23.1)	15 (28.8)	31 (19.9)
Resolving	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.9)	1 (0.6)
Missing	1 (1.9)	0 (0.0)	0 (0.0)	1 (0.6)	0 (0.0)	2 (3.8)	0 (0.0)	2 (1.3)
Bronchopneumonia distribution								
Absent	27 (51.9)	30 (57.7)	21 (40.4)	78 (50.0)	47 (90.4)	38 (73.1)	36 (69.2)	121 (77.6)
Focal	11 (21.2)	8 (15.4)	12 (23.1)	31 (19.9)	2 (3.8)	7 (13.5)	8 (15.4)	17 (10.9)
Generalised	13 (25.0)	14 (26.9)	19 (36.5)	46 (29.5)	3 (5.8)	5 (9.6)	8 (15.4)	16 (10.3)
Missing	1 (1.9)	0 (0.0)	0 (0.0)	1 (0.6)	0 (0.0)	2 (3.8)	0 (0.0)	2 (1.3)
Peribronchial cuffing status								
Absent	0 (0.0)	2 (3.8)	0 (0.0)	2 (1.3)	3 (5.8)	2 (3.8)	2 (3.8)	7 (4.5)
Mild	18 (34.6)	20 (38.5)	14 (26.9)	52 (33.3)	39 (75.0)	27 (51.9)	26 (50.0)	92 (59.0)
Moderate	30 (57.7)	25 (48.1)	35 (67.3)	90 (57.7)	10 (19.2)	20 (38.5)	24 (46.2)	54 (34.6)
Severe	3 (5.8)	5 (9.6)	3 (5.8)	11 (7.1)	0 (0.0)	1 (1.9)	0 (0.0)	1 (0.6)
Missing	1 (1.9)	0 (0.0)	0 (0.0)	1 (0.6)	0 (0.0)	2 (3.8)	0 (0.0)	2 (1.3)
Peribronchial cuffing stage								
Absent	0 (0.0)	2 (3.8)	0 (0.0)	2 (1.3)	3 (5.8)	2 (3.8)	2 (3.8)	7 (4.5)
Active	51 (98.1)	50 (96.2)	52 (100.0)	153 (98.1)	49 (94.2)	48 (92.3)	49 (94.2)	146 (93.6)
Resolving	(0.0)	(0.0)	(0.0)	(0.0)	0 (0.0)	0 (0.0)	1 (1.9)	1 (0.6)
Missing	1 (1.9)	0 (0.0)	0 (0.0)	1 (0.6)	0 (0.0)	2 (3.8)	0 (0.0)	2 (1.3)

Frequency distribution of histological lesions of bronchopneumonia and peribronchial cuffing for dorsocaudal and cranioventral lobes for each category of pig (healthy, suspect *Mycoplasma hyopneumoniae* and suspect *Actinobaciullus pleuropneumoniae*).

	Dorsocaudal lobe				Cranioventral lobe			
Histopathology	Healthy	Suspect MHYO	Suspect APP	Total	Healthy	Suspect MHYO	Suspect APP	Total
Peribronchial cuffing distribution								
Absent	0 (0.0)	2 (3.8)	0 (0.0)	2 (1.3)	3 (5.8)	2 (3.8)	2 (3.8)	7 (4.5)
Focal	11 (21.2)	11 (21.2)	11 (21.2)	33 (21.2)	10 (19.2)	15 (28.8)	11 (21.2)	36 (23.1)
Generalised	40 (76.9)	39 (75.0)	41 (78.8)	120 (76.9)	39 (75.0)	33 (63.5)	39 (75.0)	111 (71.2)
Missing	1 (1.9)	0 (0.0)	0 (0.0)	1 (0.6)	0 (0.0)	2 (3.8)	0 (0.0)	2 (1.3)