Novel Porcine *Actinobacillus* species – diagnostic tools and pathogenicity evaluation

2A -115

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

By

Conny Turni

The University of Queensland
Queensland Alliance Agriculture and Food Inovation
Ecosciences Precinct
41 Boggo Road
Dutton Park QLD 4102

Collaborators:
Pat Blackall and Youssef Abs El-Osta

June 2017

Executive Summary

This project arose because of an influx of *Actinobacillus* species being submitted to our specialized reference laboratory which could not be identified with 99% accuracy and seem to be associated with disease. It was known that the 16S rDNA sequencing cannot differentiate between the closely related species in the *Pasteurellaceae* family. Hence we set out to determine the identification of these isolates and which of the identified species were associated with disease.

A total of 37 isolates, which had already been analysed by sequencing of the *recN* gene, were analysed by sequencing two further genes, the *rpoA* and *thdF* genes. These genes have been established in previous research as ideal to distinguish between very closely related species in the *Pasteurellaceae* family. The strains were revived and cultured to extract genomic DNA, which was then subjected to PCR amplification with primer sets (total of 13 primers) that catered for variability in the genes. The amplified product was then sequenced. Sequences were aligned and compared to the sequences of reference strains. From the alignment analysis distance value matrixes of sequence similarity were constructed.

Of the 37 isolates, 17 form a new species (temporarily called *Actinobacillus* Taxon C1), three were identified as *A. porcinus*, eight could not be aligned to a species, but were closely associated with *A. minor*, and nine belonged to the *H. parasuis/A. indolicus* complex. While it is interesting that the *H. parasuis* and *A. indolicus* species obviously have more variation than is currently defined in these species, in this report we have concentrated on the new species.

The pathology associated with the 17 isolates of *Actinobacillus* Taxon C1 were mainly abscesses and pleurisy detected at the abattoir. Typically, on the farm, nothing was pointing to a problem and no obvious respiratory symptoms were noticeable. The lesions seen at slaughter were very similar to those associated with *A. pleuropneumoniae* and the potential for misdiagnosis is present. Death has also been observed on one farm with the pigs dying overnight without any forewarning. In this case, only *Actinobacillus* Taxon C1 has been isolated from the diseased lungs, which was diagnosed as severe necrosuppurative and haemorrhagic bronchopneumonia.

The fact that antibiotic treatment lowered the occurrence of lesions, abscesses and pleurisy points to bacterial cause of these lesions. As well, the *apxIBD* operon, an operon associated with toxin transport in *A. pleuropneumoniae* was found in isolates of *Actinobacillus* Taxon C1, suggesting the possible presence of an Apx toxin.

We will present these findings at the next Australian Pig Veterinarians meeting to create awareness of *Actinobacillus* Taxon C1. The next step is to develop a PCR that can identify *Actinobacillus* Taxon C1. Such a tool would allow pig veterinarians to investigate if the organism is a problem on farms and whether pleuropneumonia lesions are really due to *A. pleuropneumoniae* or if *Actinobacillus* Taxon C1 is contributing to the disease.

Table of Contents

1. Introduction

Background

In recent years, the submission of *Actinobacillus*-like strains sourced from the respiratory tract of pigs to the reference service provided by our laboratory has increased. Our usual identification method involves 16S rDNA sequencing. However, this method has proven to lack the discriminatory power to separate very closely related species in the *Actinobacillus* complex. This situation prompted a preliminary study to improve the identification of *Actinobacillus*-like strains by using the *recN* housekeeping gene, chosen due to an ability to use this gene sequence to predict whole-genome DNA-DNA similarity (Kuhnert and Korczak 2006). A total of 37 field isolates, identified by the 16S rDNA sequencing as *A. porcitonsillarum, A. minor, A. porcitonsillarum/minor* complex*, A. porcinus* or *A. indolicus*, were sequenced and aligned to publicly available data of the type strains within the genus *Actinobacillus* to make multiple alignments. The score of the *recN* sequence similarity identified *Actinobacillus* species based on the threshold similarity values defined for *recN* alone. A phylogenetic tree was created for the *recN* sequences of the field strains and the type strains for the currently recognized species. Analysis of the *recN* sequence confirmed the 16S rDNA identification of *A. minor* and *A. porcinus* (3 and 2 isolates, respectively). The interesting observation was that none of the *A. porcitonsillarum* species identified by the 16S rDNA sequencing could be assigned to any recognised species by the *recN* gene method. Indeed, 23 out of the 37 isolates could not be assigned to an *Actinobacillus* species by the *recN* method. This observation was strengthened by the phylogenetic tree obtained from the *recN* sequences which indicated the presence of at least one novel species. The hint of new species among the *Actinobacillus* species that are possibly pathogenic was raised as early as 2001 (Kielstein et al 2001).

A. porcitonsillarum (as identified by 16S rDNA based methods) has so far not been believed to be a pathogen. However, recently not only did we have an increase in submission of *A. porcitonsillarum*-like isolates to our laboratory, but we have also seen these organisms in the high frequency in lungs with pleurisy at the abattoir. *Actinobacillus*-like organisms were found on 28 out of 46 farms recently examined in a pleurisy study funded by the Pork CRC. To further indicate a problem, one isolate identified as *A. porcitonsillarum* by 16S rDNA sequencing was isolated from severe

lung lesions at slaughter. These *Actinobacillus*-like isolates were arising from farms that have a problem with respiratory disease and the isolates were being retrieved from the lungs of sick pigs. Observations of granulomatous lymphadenitis and pneumonia associated with *A. porcitonsillarum* in slaughter pigs in Japan have been documented (Ohba et al. 2007). In 2005, a publication by Swiss researchers revealed that *A. porcitonsillarum* has the entire *apxII* operon, which means that the ApxII toxin could be secreted (Kuhnert et al. 2005). This is similar to *A. pleuropneumoniae* serovar 7, which only secretes the ApxII toxin. Another study reported haemolytic activity associated with ApxII in strains of the *A. minor/porcitonsillarum* complex (Arya and Niven 2010).

To resolve this issue of identification we proposed to build on the *recN* knowledge by determining the sequence of two additional genes - *rpoB* and *thdF*. The use of multilocus sequence analysis (MLSA) of these three genes has been proven to be a useful taxonomic tool and is suitable for the *Pasteurellaceae* family (Kuhnert and Korczak 2006), which includes the genus *Actinobacillus* and should therefore be ideal to identify these problematic *Actinobacillus* isolates.

2. Methodology

Bacteria

A total of 37 isolates obtained from the respiratory tract of diseased pigs were used. All the isolates came from diagnostic submission and were sent to our reference laboratory at the Microbiology Laboratory, Ecosciences Precinct, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Queensland, Australia for identification.

Culture Conditions and Initial Identification

After revival from storage on BA/SN plates consisting of BBL™ Blood Agar Base (Becton Dickinson, Sparks, MD, USA) and 0.0025% NADH, 0.0005% thiamine HCL, 1% heat inactivated horse serum and 5% oleic acid bovine albumin complex (which consists of 4.75% bovine serum albumin) (Turni and Blackall, 2007). The isolates were

also plated on blood agar plate with a nurse streak of *Staphylococcus hyicus* (Turni and Blackall 2007) to check for NAD dependence and purity. The plates were incubated overnight at 37°C.

DNA Extraction

DNA was extracted using the Prepman Ultra Sample Preparation Reagent (Applied Biosystems, Austin, Texas, USA) according to manufacturer's instruction. Some samples did not yield the quantity and quality desired and these were extracted with the Generation Capture Column Kit (QIAGEN, Inc., Germany) according to manufacturer's instructions. The extracted DNA was stored at -20°C for PCR amplification.

Amplification of *recN, rpoA* and *thdF*

The three housekeeping genes (*recN, rpoA* and *thdF*) were amplified according to the method of Kuhnert and Korczak (2006) with slight variations. A wide range of primers, as previously reported (Kuhnert and Korczak 2006), was used (Table 1). The DNA polymerase and buffer used were from Roche (Penzberg, Germany) instead of the FIREPol Polymerase (Solis BioDyne, Tartu, Estonia) used in the original study. The total volume per reaction was 50 µl, which comprised of 1X PCR Buffer, 35.5 µl H_2O, 1.5 mM $MgCl_2$, 1 mM dNTPs, 0.4 µM of each forward and reverse primer, 2.5 U *Taq* DNA polymerase and 1 µl DNA template (100 ng/µl DNA template).

For the PCR cycle conditions, the first cycle was at 94°C for 3 min, then followed by 35 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 1 min. The extension cycle was at 72°C for 7 min. Electrophoresis in a 1% agarose gel for 2 hr at 70V was performed and the size of the amplified gene visualized under UV light by staining with EZ-Vision Three (Ameresco, Inc., Solon, Ohio, USA). Before sequencing the amplicon, the product was cleaned with ExoSAP-IT (Affymetrix, Inc., Santa Clara, California, USA). If non-specific bands were observed, the band of interest was cut out of the gel and a gel purification process was applied using a gel extraction kit (QIAGEN Inc., Hilden, Germany). Two species-specific PCR assays for *Haemophilus parasuis* were used to exclude this species from the study.

Table 1. Primer pairs designed for target gene amplification (Kuhnert and Korczak, 2006).

Name	Primer Sequence 5' to 3'	Position
rpoA-L	TCT GTR ACA GAA TTT TTA AAR CC	10-32
rpoA-R	TTG CNG GNG GCC ART TTT CAA GG	973-951
recN-L	CAA CTY ACT ATY AAT MAT TTT GC	10-32
recN-R	CTA ATG CCY ACR TCY ACT TCA TC	1397-1375
recN-L2	CAT TTA ACG GTT AAT AAT TTT GC	10-32
recN-R2	CTA ATY CCM ACA TCN ACY TCA TC	1397-1375
thdF-first-L2	ATG AAA GAN CAN ATY GTN GCW CAR GC	7-32
thdF-first-R2	TGT TTA TTT NCC RAT RCA RAA NGA RC	1368-1343
thdF-MP-L	AAA GAM ACC ATT GTT GCW CAA GC	10-32
thdF-L2	AAA GAN ACC ATT GTT GCW CAR GC	10-32
thdF-R2	TTA TTT NCC RAT RCA RAA NGA RC	1365-1343
thdF-1	GGA ATG AAA GTC GTG ATT GCA GG	646-668
thdF-2	CCT GCA ATC ACG ACT TTC ATT CC	668-646

Sequencing

Sequencing was done by commercial companies, Macrogen in South Korean or the Australian Genome Research Facility Ltd, Brisbane, Australia. The sequences were aligned with the program Sequencher 4.9 (Gene Codes Inc., Michigan, USA). The sequences of the *rpoA*, *thdF* and *recN* genes of the relevant recognized species within the *Actinobacillus* genus, as well as *Haemophilus parasuis* and *Pasteurella multocida,* were acquired from the data bank of the National Center for Biotechnology Information (NCBI) for phylogenetic comparison. The details of the species and their accession number are shown in Table 2.

Table 2. Reference strains with accession number for three housekeeping genes *rpoA*, *thdF* and *recN*. (Kuhnert and Korczak, 2006).

Type strains	*recN*	*rpoA*	*thdF*
Actinobacillus minor NM305[T]	DQ410900	DQ410938	DQ410976
'Actinobacillus porcitonsillarum' CCUG 44996	DQ410901	DQ410939	DQ410977
Actinobacillus genomospecies 2 CCUG 15571	DQ410902	DQ410940	DQ410978
Actinobacillus hominis CCUG 19800	DQ410903	DQ410941	DQ410979
Actinobacillus ureae CCUG 2193[T]	DQ410904	DQ410942	DQ410980
Actinobacillus athritidis CCUG 24862	DQ410905	DQ410943	DQ410981
Actinobacillus suis ATCC 33415[T]	DQ410906	DQ410944	DQ410982
Actinobacillus genomospecies 1 CCUG 22229 *Actinobacillus capsulatus* CCUG 12396[T]	DQ410907 DQ410908	DQ410945 DQ410906	DQ410983 DQ410984
Actinobacillus equuli subsp.equuli ATTCC 19392[T]	DQ410909	DQ410947	DQ410985
Actinobacillus equuli subsp. haemolyticus CCUG 19799[T]	DQ410910	DQ410948	DQ410986
Actinobacillus lignieresii NCTC 4189[T]	DQ410911	DQ410949	DQ410987
Actinobacillus pleuropneumoniae serovar 1 S4074[T]	DQ410912	DQ410950	DQ410988
Actinobacillus pleuropneumoniae serovar 2 S 1536	DQ410913	DQ410951	DQ410989

Actinobacillus pleuropneumoniae serovar 3 S1421	DQ410914	DQ410952	DQ410990
Actinobacillus pleuropneumoniae serovar 4 M62	DQ410915	DQ410953	DQ410991
Actinobacillus pleuropneumoniae serovar 6 FemO	DQ410916	DQ410954	DQ410992
Actinobacillus pleuropneumoniae serovar 8 405	DQ410917	DQ410955	DQ410993
Actinobacillus indolicus 46KC2[T]	DQ410918	DQ410956	DQ410994
Actinobacillus porcinus NM319[T]	DQ410919	DQ410957	DQ410995
Haemophilus parasuis CCUG 3712[T]	DQ410920	DQ410958	DQ410996
Pasteurella multocida CCUG 43504[T]	DQ410890	DQ410928	DQ410966

Data Analysis

Multiple alignments of DNA sequences were constructed by ClustalX2 (Larkin et al. 2007). Columns with gaps were trimmed out of the multiple alignment by use of Bioedit (Hall 1999). Phylogenetic analysis of the 16S rDNA and *rpoB* gene sequences were carried out by neighbour joining, using Jukes-Cantor correction and included calculation of bootstrap support. MEGA6 (Tamora et al. 2011) was used for graphical presentation of trees. The genome similarity index was calculated according to Kuhnert & Korczak (2006).

Haemophilus parasuis **PCRs**

To exclude *H. parasuis* from the identification two species specific PCRs were used. Isolates were confirmed not to be *H. parasuis* by the PCR of Oliveira *et al*. (2002) and by the PCR of Angen et al. (2007).

Apx toxin PCR

The new species Selected isolates were analysed for the three Apx toxins. Five separate PCRs were used to identify the presence of the following operons - *apxI*CA, *apxII*CA, *apxIII*CA, *apxI*BD and *apxIII*BD. As previously described (Frey 2003), each 50 µL reaction consisted of 10 x buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3 (Roche, Mannheim, Germany)), 0.16 mM of dNTPs, 0.235 µM of each relevant forward and reverse primer (XICA-L and XICA-R, XIICA-L and XIICA-R, XIIICA-L and XIIICA-R, XID-L and XID-R, XIIID-L and XIIID2-R) (Frey, 2003), 1.25 units of *Taq* DNA polymerase (Roche, Mannheim, Germany) and 2 µL of DNA template. The PCR cycles were performed as previously described (Satran and Nedbalcova 2002) using an Eppendorf cycler. To visualize the amplified products, a 10 µL aliquot of the amplified product was run on a 1% agarose gel at 70 V for 1.5 hrs and the gel then photographed under UV illumination.

3. Outcomes

Research results

None of the 37 isolates was positive in the two species-specific *H. parasuis* PCR assays.

Table 3 and supplementary Table S1 show the result of the phylogenetic analysis. The supplementary table shows the results for all three genes for each isolate, while table 3 presents a summary.

A new species, consisting of 17 isolates, was identified with a genome similarity index of 0.56 to the closest related type strain - *A. indolicus.* This allocation to a new species is based on the published guidelines (Kuhnert and Korczak, 2006) that a similarity index of >0.4 and one of >0.9 indicate the same genus and species, respectively. For the purpose of this report, this group is *Actinobacillus* Taxon C1.

The type strains of *Haemophilus parasuis* and *A. indolicus* formed a group with nine isolates. A further eight isolates did not fit into a group due to lack of congruence of

7

the *thdF* gene phylogeny with *recN* and *rpoA* and their identity remains uncertain. Three isolates were identified as *A. porcinus* (Table 3).

Some of the *Actinobacillus* Taxon C1 isolates were associated with pathology (Table 4). These isolates seem to be associated with pleurisy, lung abscesses and lung lesions at the abattoir with reports of up to 13.8% pleurisy, 40% of lung lesions and 22.5 % abscesses linked with isolate 35 and 30% lung abscesses at slaughter linked with isolate 37. The lung lesions are reported to look very similar to a pleuropneumonia lesion and there could be potential misdiagnosis at slaughter checks (Table 4).

From one farm, samples were collected over several years starting in 2011. The more recent samples were just compared with the *recN* and *rpoA* genes and if the sequences were the same as of previous isolates, they were regarded as the same strain. Figure 1 relate to a more recent occurrence on this farm (2016). Samples from two pigs (20 and 12 weeks old) were submitted and were from an acute consolidated dorsal lung lesion affecting approximately 50% of the lung with no gross abscesses or pleurisy but rapid necrosis or autolysis of affected lung area. From the lung of the 20-week-old pig, *Pasteurella multocida* and *Trueperella pyogenes* were retrieved. For the 12-week-old pig, only *Actinobacillus* Taxon C1 was retrieved from the lung. In 2017, a post mortem of two-16-week-old pigs from this farm was done. The pigs had suddenly died and showed similar lesion to Figure 1. The lungs were sampled and cultured. Both lungs yielded *Actinobacillus* Taxon C1 and no other bacterial species. The pathology report described the lesions as severe necrosuppurative and haemorrhagic bronchopneumonia containing occasional intra-lesional bacilli. The lesions were confirmed as bacterial in origin and were acute, severe and haemorrhagic without any pleurisy. It was concluded that the presence of intralesional thrombi suggests *Actinobacillus pleuropneumoniae* as the most likely cause. Macroscopic investigation revealed extensive haemorrhage and necrosis through large area of lung associated with oedema, vascular congestion and multiple pockets of neutrophils. The other areas of the lung also had extensive alveolar and interlobular oedema with intense neutrophilic inflammation.

Figure 2 relates to isolate 32, which was collected at slaughter and was associated with 30% of lungs having lesions. As well, pleurisy and abscesses were observed. On the farm, there are hardly any signs of respiratory disease, such as coughing.

One interesting point was that once the *Actinobacillus* Taxon C1 was targeted with antibiotic treatment the pleurisy, lung lesion and abscess rate was notably reduced at slaughter observations (Table 4, isolate 35). A couple of isolates have been tested for antimicrobial sensitivity and one was sensitive to ampicillin, ceftiofur, trimethoprim/sulphamethoxazole, florfenicol, tetracycline, tilmicosin and tulathromycin, while another was resistant to tetracycline.

Actinobacillus Taxon C1 possesses the *apxIB* and *apxID* genes. In *A. pleuropneumoniae,* these genes are associated with the secretion of both ApxI and ApxII toxins. No other positive signal occurred for any of the other Apx associated genes. This may mean that the *Actinobacillus* Taxon C1 isolates have toxin-associated genes that have significant sequence variation in the primer region used for the *A. pleuroponeumoniae apx* PCR assays. Alternatively, it is possible that no such toxin linked genes are present. The similarity in the lesions associated with the *Actinobacillus* Taxon C1 isolates and *A. pleuropneumoniae* suggests that it seems more likely that the *Actinobacillus* Taxon C1 isolates do indeed produce an Apx-like toxin.

Table 3. Sequence of isolates and type strains were aligned with CLUSTAL. Values of greater than 96% belonged to the same species, values between 84 – 96% were uncertain and values below 84% had no similarity.

No of isolates	16S idenification	*recN*	%	*rpoA*	%	*thdF*	%	Group with
8	A. indolicus / H.parasuis / A. rossi	H. parasuis / A. indolicus	> 96.05	A. indolicus / H. parasuis	> 97.44	uncertain	> 92.20	**H. parasuis / A. indolicus**
1	A. porcitonsillarum/minor	uncertain	84.96	A. indolicus / H. parasuis	98.57 / 97.70	uncertain	94.44	
3	A. minor	A. minor	100	A. minor	99.62	uncertain	94.64	**?**
1	A. minor	no similarity	< 84	no similarity	< 84	A. minor	97.92	
4	A. porcitonsillarum/minor	no similarity	< 84	no similarity / A. minor	< 84	A. minor / uncertain	< 97.92	
2	A. porcinus	A. porcinus	< 99.2	A. porcinus	99.87	A. porcinus	< 99.71	**A. porcinus**
1	A. porcinus	uncertain	84.96	A. porcinus	99.87	uncertain	87.29	
7	A. indolicus / H. parasuis	no similarity	< 84	A. indolicus / H. parasuis	97.18 / < 97.50	uncertain	85.22	**A. Taxon C1 (new species)**
10	A. porcitonsillarum/minor	no similarity	< 84	A. indolicus / H. parasuis	97.18 / 97.44	uncertain	> 85.14	

Table 4. Summary of available information on the *Actinobacillus* Taxon C1 isolates.

Sample no	Year of sub-mission	*recN*	*rpoA*	*thdF*	Farm	Tissue	Clinical signs/pathology	Farm background
22*	2011	*no similarity*	*A. indolicus/ H. parasuis*	*uncertain*	1	Lung	Clinical signs not usually present and there is no overt respiratory disease, such as coughing or ill-thrift. There are 15 to 20% pleurisy lesion at slaughter with 5 - 10% severe and about 5% lung abscesses. Abscesses are usually in dorsal lobes 2 to 5 cm diameter being resolved single, circumscribed firm lumps. The isolate is now being observed in 12 to 18 weeks old pigs, which are found dead. The pigs have purple extremities and the only lesions found are a dark, circumscribed area of dorsal lung with no gross pleurisy. Lesions are rapidly necrotising or autolysin, fulminant pneumonia, no obvious abscesses or adhesions (Picture 1). From pigs with mortalities at farm only 1 in 10 shows these lesions.	*Pasteurella* and *Trueperella* also reported. The farm is positive for *A. pleuropneumiae* serovar 12. There are some mild signs of respiratory disease in newly weaned pigs, which is assumed to be associated with *Haemophilus parasuis* or *Streptococcus suis*.
23*	2011	*no similarity*	*A. indolicus/ H. parasuis*	*uncertain*	1	Lung		
24*	2011	*no similarity*	*A. indolicus/ H. parasuis*	*uncertain*	1	Lung		

2	2012	*no similarity*	*A. indolicus/ H. parasuis*	*uncertain*	2	Lung		Found at slaughter check
25	2012	*no similarity*	*A. indolicus/ H. parasuis*	*uncertain*	3	Lung		
26	2012	*no similarity*	*A. indolicus/ H. parasuis*	*uncertain*	3	Lung		
28	2012	*no similarity*	*A. indolicus/ H. parasuis*	*uncertain*	4	Lung Abscess		Has *A. pleuropneumoniae* serovar 7
9	2012	*no similarity*	*A. indolicus/ H. parasuis*	*uncertain*	4	Lung Abscess		
10	2012	*no similarity*	*A. indolicus/ H. parasuis*	*uncertain*	4	Lung Abscess		
11	2012	*no similarity*	*A. indolicus/ H. parasuis*	*uncertain*	5	Lung		

12	2012	*no similarity*	*A. indolicus/ H. parasuis*	*uncertain*	5	Lung	
13	2012	*no similarity*	*A. indolicus/ H. parasuis*	*uncertain*	5	Lung	
16	2013	*no similarity*	*A. indolicus/ H. parasuis*	*uncertain*	6	Lung	Disease investigation
37	2015	*no similarity*	*A. indolicus/ H. parasuis*	*uncertain*	7	Lung	30% lungs with abscess at slaughter that look exactly like *A. pleuropneumoniae*. Abscesses 3 - 10 cm in diameter on dorsal lobe. Pigs are fine and growing well.	
35	2015	*no similarity*	*A. indolicus/ H. parasuis*	*uncertain*	8	Lung	At abattoir: prevalence of lung lesions was 40%, 22.5% lung abscesses (1-5 cm diameter) and pleurisy prevalence 13.8% with 2% heart disease associated with lung abscess and pleurisy	Outdoor sow farm, good air quality, progeny raised in straw based shelters, herd free from *A. pleuropneumoniae* and *M. hyopneumoniae,* herd free of internal and external parasites. The farm was medicated to treat the new species and lung lesions went down to 23%, pleurisy to 11.5% and lung

								abscesses to 13.5% observed at slaughter after treated batch went to abattoir.
33	2015	*no similarity*	*A. indolicus/ H. parasuis*	*uncertain*	9	Lung	Herd has increase in abscesses in lungs at slaughter	Herd is *A. pleuropneumoniae* serovar 15 positive
32	2015	*no similarity*	*A. indolicus/ H. parasuis*	*uncertain*	10	Lung	Signs seen only at slaughter, 30% of lungs had lesions and pleurisy and abscesses are observed, yet rarely any coughing at farm level (Picture 2)	*M. hyopneumoniae* and *A. pleuropneumoniae* free

* The isolate has been found throughout the years and has been identified by *rpoA* and *recN* as being the same isolate (isolates that do not have the three genes sequenced are not shown), hence all the pathology and clinical signs available is being recorded for farm 1 in general without reference to single isolate.

Picture 1 Lung at necropsy on farm 1 (photo supplied by Bernie Gleeson)

Figure 2 Lung pictures A to C from which isolate 32 was taken. Lung was collected at the abattoir (photos supplied by Trish Holyoake). The photos show chronic encapsulated abscesses within the lung lobes without apparent necrosis or haemorrhage.

Figure 2 A

Figure 2 B

Figure 2 C

4. Application of Research

This finding of a new species, named *Actinobacillus* Taxon C1 for the interim, which is associated with lesions and abscesses at the abattoir, but also with lesion and death at the farm level is an important finding for the industry. The similarity of some of the lesions/abscesses at slaughter associated with *Actinobacillus* Taxon C1 with the lesions and abscesses associated with *A. pleuropneumoniae* could indicate that these lesions could have been misidentified as *A. pleuropneumoniae*-linked lesions at slaughter check. The finding of up to 30% lungs with abscesses and 40% lungs with lesions reported on farms free of *A. pleuropneumoniae* but positive for *Actinobacillus* Taxon C1 suggests a significant impact on the industry by this organism.

The awareness of this new species is making it possible to have a closer look at farms with high pleurisy and farms, thought to be associated with *A. pleuropneumoniae,* to determine if *Actinobacillus* Taxon C1 is a problem on these farms.

Future directions would be to develop a PCR for the rapid and confident identification of *Actinobacillus* Taxon C1, so that front line laboratories can identify the species easily and therefore screen large sample numbers for this species to evaluate the impact on farms.

5. Conclusion

This work has confirmed the inadequacy of the 16S rDNA identification within the family *Pasteurellaceae.* None of the strains identified as *A. porcitonsillarum*/*minor* by 16S rDNA sequencing were confirmed as such with the multi-locus sequence analysis approach. The work has also highlighted that no single conserved gene by itself can be used for identification in this family. This would suggest that research is needed to develop a PCR to recognize *Actinobacillus* Taxon C, the new species recognized in this work. Such an assay would help generate knowledge on how common this organism, is and what kind of pathology is associated to it on different farms. Such knowledge is needed to determine if *Actinobacillus* Taxon C1 is a

significant problem and hence whether focused prevention and control programs, possibly using medication or vaccination, are needed.

Some of the *Actinobacillus* Taxon C1 isolates are apparently quite pathogenic and appear to be causing significant pleurisy, lung lesions and abscesses. Some of the farms had 40% lung lesions, 30% lung abscesses and 13.8% pleurisy. It was noted by one of the vets involved that the lesions, abscesses and pleurisy went down when this new species was targeted with antibiotics. This would further suggest that the new species is associated with pathological signs observed at slaughter.

The fact that the *apxIBD* operon was found and that this operon is normally needed for either ApxI or ApxII toxins further points to the possibility of *Actinobacillus* Taxon C causing significant pathology. Recent research has found that the same occurred for *Actinobacillus suis,* which has homologous genes that are very similar in the DNA sequence to the *apx* genes of *A. pleuropneumoniae* (Schaller *et al.* 2000). Schaller et al (2000) then designed special methods for detecting these related toxin genes for the *Actinobacillus* species and were able to show that *A. suis* has the apxICA and the apxIICA operons, meaning that it has the capacity to express ApxI and ApxII toxins.

6. Limitations/Risks

Not applicable

7. Recommendations

The finding of a new species, *Actinobacillus* Taxon C1, highlights the need for screening for this species if similar pathology is found on farms. To screen means that methods are needed to easily identify this species. The easiest would be do develop a PCR for this species, which could be done using the isolates already collected in the current project and with reference strains of related species in the family of *Pasteurellaceae* that already are present in our culture collection.

8. Acknowledgement

9. References

Angen O, Oliveira S, Ahrens P, Svensmark B and Leser, T.D: (2007). Development of an improved species specific PCR test for detection of *Haemophilus parasuis*. Veterinary Microbiology, 119, 266-276.

Arya, G. and Niven, D. F. (2010). Production of haemolysins by strains of the *Actinobacillus minor/"porcitonsillarum"* complex. Veterinary Microbiology, 141, 332-341.

Frey J. (2003) Detection, identification, and subtyping of *Actinobacillus pleuropneumoniae.* Method in Molecular Biology, 2003, 87-95.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nuclear Acids Symposium Series, 41, 95-98.

Kielstein, P., Wuthe, H.-H., Angen O, Mutters. R and Ahrens P. (2001) Phenotypic and genetic characterization of NAD-dependent *Pasteurellaceae* from the respiratory tract of pigs and their possible pathogenetic importance. Veterinary Microbiology, 81, 243–255.

Kuhnert, P., Schlatter, Y. and Frey J. (2005) Characterization of the type I secretion system of the RTX toxin ApxII in "*Actinobacillus porcitonsillarum*". Veterinary Microbiology, 107, 225–232.

Kuhnert, P. and Korczak, B. M. (2006). Prediction of whole-genome DNA–DNA similarity, determination of G+ C content and phylogenetic analysis within the family *Pasteurellaceae* by multilocus sequence analysis (MLSA). Microbiology, 152, 2537-2548.

Larkin, M.A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. and Higgins, D. G. (2007). Clustal W and Clustal X version 2.0. Bioinformatics, 23, 2947-2948.

.Satran P, Nedbalcova K. (2002) Prevalence of serotypes, production of Apx toxins, and antibiotic resistance in strains of *Actinobacillus pleuropneumoniae* isolated in the Czech Republic. *Veterinary Medicine – Czechoslovakia*; 47, 92–98.

Ohba, T., Shibahara, T., Kobayashi, H., Takashima, A., Minami, Y. and Kubo, M. (2007). Granulomatous lymphadenitis and pneumonia associated with *Actinobacillus porcitonsillarum* in a slaughter pig. Journal of Comparative Pathology, 137, 82-86.

Oliveira S, Galina L and Pijoan C: (2001). Development of a PCR test to diagnose *Haemophilus parasuis* infections. Veterinary. Diagnostic. Investigations,. 13**,** 495–501

Schaller, A. Kuhnert, P., dela Puente-Redondo, V.A., Nicolet, J. and Frey J. (2000) Apx toxins in *Pasteurellaceae* species from animals. Veterinary Microbiology, 74, 365–376.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution, 28, 2731-2739.

Turni, C. and Blackall, P.J. (2007). An evaluation of the *apx*IVA based PCR-REA method for differentiation of *Actinobacillus pleuropneumoniae*. Veterinary Microbiology, 121, 163-169

Appendices

Table S1. Sequence of isolates and type strains were aligned with CLUSTAL. Values of greater than 96% belonged to the same species, values between 84 – 96% were uncertain and values below 84% had no similarity.

Sample No*	16S identification	H. parasuis PCR Oliveira	H. parasuis PCR Oystein	recN	%	rpoA	%	thdF	%
1 (*H.par/A. ind*)	*A. indolicus/H. parasuis*	negative	negative	*H. parasuis*	97.05	*A. indolicus*	99.10	*uncertain*	92.25
2 (*A.* C1)	*A. indolicus/H. parasuis*	negative	negative	*no similarity*	< 84	*A. indolicus/H. parasuis*	97.18/96.16	*uncertain*	85.22
3 (*H.par/A. ind*)	*A. indolicus/H. parasuis*	negative	negative	*A. indolicus/H. parasuis*	96.55 / 95.96	*A. indolicus*	99.36	*uncertain*	95.23
4 (*H.par/A. ind*)	*A. indolicus/H. parasuis*	negative	negative	*H. parasuis*	97.05	*A. indolicus*	99.10	*uncertain*	92.25

5 (*H.par*/*A.ind*)	*A. indolicus*/*H. parasuis*	negative	negative	*H. parasuis*	96.38	*A. indolicus*/*H. parasuis*	98.85/97.44	*uncertain*	92.76
6 (*H.par*/*A.ind*)	*A. indolicus*/*H. parasuis*	negative	negative	*H. parasuis*	96.63	*A. indolicus*	99.10/97.18	*A. indolicus*	97.72
7 (*H.par*/*A.ind*)	*A. indolicus*/*H. parasuis*	negative	negative	*A. indolicus*/*H. parasuis*	96.55 / 96.04	*A. indolicus*/*H. parasuis*	98.98/97.57	*uncertain*	93.75
8 (*H.par*/*A.ind*)	*A. indolicus*/*H. parasuis*	negative	negative	*A. indolicus*	96.97	*A. indolicus*/*H. parasuis*	98.98/97.82	*uncertain*	94.94
9 (*A*. C1)	*A. indolicus*/*H. parasuis*	negative	negative	*no similarity*	< 84	*A. indolicus*/*H. parasuis*	97.18/97.44	*uncertain*	85.22
10 (*A*. C1)	*A. indolicus*/*H. parasuis*	negative	negative	*no similarity*	< 84	*A. indolicus*/*H. parasuis*	97.18/97.44	*uncertain*	85.22

11 (A. C1)	*A. indolicus/H. parasuis*	negative	negative	*no similarity*	< 84	*A. indolicus/H. parasuis*	97.18/97.44	*uncertain*	85.22
12 (A. C1)	*A. indolicus/H. parasuis*	negative	negative	*no similarity*	< 84	*A. indolicus/H. parasuis*	97.18/97.44	*uncertain*	85.22
13 (A. C1)	*A. indolicus/H. parasuis*	negative	negative	*no similarity*	< 84	*A. indolicus/H. parasuis*	97.18/97.44	*uncertain*	85.22
14 (?)	*A. minor*	negative	negative	*A. minor*	100	*A. minor*	99.62	*uncertain*	94.64
15 (?)	*A. minor*	negative	negative	*no similarity*	< 84	*no similarity*	< 84	*A. minor*	97.92
16 (A. C1)	*A. minor*	negative	negative	*no similarity*	< 84	*A. indolicus/H. parasuis*	97.18/97.44	*uncertain*	85.22
17 (?)	*A. minor*	negative	negative	*A. minor*	100	*A. minor*	99.62	*uncertain*	94.64
18	*A. minor*	negative	negative	*A. minor*	100	*A. minor*	99.62	*uncertain*	94.64

(?)									
19 (*A. porc*)	*A. porcinus*	negative	negative	*A. porcinus*	99.16	*A. porcinus*	99.87	*A. porcinus*	99.71
20 (*A. porc*)	*A. porcinus*	negative	negative	*uncertain*	84.96	*A. porcinus*	99.87	*uncertain*	87.29
21 (*A. porc*)	*A. porcinus*	negative	negative	*A. porcinus*	97.73	*A. porcinus*	99.87	*A. porcinus*	99.31
22 (*A.* C1)	*A. porcitonsillarum*	negative	negative	*no similarity*	< 84	*A. indolicus/H. parasuis*	97.18/97.44	*uncertain*	85.22
23 (*A.* C1)	*A. porcitonsillarum*	negative	negative	*no similarity*	< 84	*A. indolicus/H. parasuis*	97.18/97.44	*uncertain*	85.22
24 (*A.* C1)	*A. porcitonsillarum*	negative	negative	*no similarity*	< 84	*A. indolicus/H. parasuis*	97.18/97.44	*uncertain*	85.22

25 (*A.* C1)	*A. porcitonsillarum*	negative	negative	*no similarity*	< 84	*A. indolicus/H. parasuis*	97.18/97.44	*uncertain*	85.22
26 (*A.* C1)	*A. porcitonsillarum*	negative	negative	*no similarity*	< 84	*A. indolicus/H. parasuis*	97.18/97.44	*uncertain*	85.22
27 (?)	*A. porcitonsillarum*		negative	*no similarity*	< 84	*no similarity*	< 84	*A. minor*	97.92
28 (*A.* C1)	*A. porcitonsillarum /minor*	negative	negative	*no similarity*	< 84	*A. indolicus/H. parasuis*	97.18/97.44	*uncertain*	85.22
29 (*H.par/A. ind*)	*A. porcitonsillarum /minor*	negative	negative	*uncertain*	84.96	*A. indolicus/H. parasuis*	98.57/97.70	*uncertain*	94.44
30 (?)	*A. porcitonsillarum /minor*		negative	*no similarity*	< 84	*no similarity*	< 84	*uncertain*	85.22

31 (*H.par*/*A. ind*)	*A. rossi*	negative	negative	*H. parasuis*	96.63	*A. indolicus*	99.10	*A. indolicus*	97.72
32 (*A.* C1)	*A. porcitonsillarum /minor*	negative	negative	*no similarity*	< 84	*A. indolicus*/*H. parasuis*	97.18/97.44	*uncertain*	85.13
33 (*A.* C1)	*A. porcitonsillarum*	negative	negative	*no similarity*	< 84	*A. indolicus*/*H. parasuis*	97.18/97.44	*uncertain*	85.22
34 (?)	*A. porcitonsillarum /minor*		negative	*no similarity*	< 84	*A. indolicus*/*H. parasuis*	97.18/97.44	*uncertain*	94.64
35 (*A.* C1)	*A. porcitonsillarum /minor*	negative	negative	*no similarity*	< 84	*A. indolicus*/*H. parasuis*	97.18/97.44	*uncertain*	85.22
36 (?)	*A. porcitonsillarum /minor*	negative	negative	*no similarity*	< 84	*A. indolicus*/*H. parasuis*	97.18/97.44	*uncertain*	85.17

| 37 (*A.* C1) | *A. indolicus/H. parasuis* | negative | negative | *no similarity* | < 84 | *A. indolicus/H. parasuis* | 97.18/97.44 | *uncertain* | 85.22 |

* The information within the brackets indicates the final identification of the isolate – *H. para/A. ind* = identified as belonging to the *H. parasuis/A. indolicus* group; *A.* C1 = identified as belonging to *Actinobacillus* Taxon C1; *A. porc* = identified as belonging to *Actinobacillus porcinus* and? = cannot be identified.