

Antibiotic sensitivity of *Haemophilus parasuis* plus *Actinobacillus pleuropneumoniae* and other respiratory pathogens 2A-107

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Pork

By

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Executive Summary

In the last decade, the emergence of antimicrobial resistant forms of bacterial pathogens has become a problem in the treatment of bacterial diseases of livestock. Resistance to commonly used antimicrobials has been reported all over the world. A key emerging bacterial pathogen in the pig industries of the world is *Haemophilus parasuis*. This pathogen is a significant cause of economic loss as a primary pathogen. Despite the key role this organism plays, there was no accepted, validated, standardized methodology for testing for antimicrobial resistance. This lack of a standardized, validated methodology meant that veterinarians around the world lacked the critical laboratory tool to design targeted, sustainable and effective research programs. This first aim of this project was to develop a method to test for *H. parasuis* antimicrobial sensitivity. This has been successfully achieved and two methods have been published and made available to the industry.

Once validated methods for the testing of *H. parasuis* were developed, the project set out to look at antimicrobial resistance in key respiratory pathogens and then further investigate possible genes linked to the observed resistance. These two objectives have been accomplished. We have looked at antimicrobial resistance in *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, *H. parasuis* and *Pasteurella multocida*. The resistance to some of the older antibiotics, such as erythromycin and tetracycline, was marked in all species, for example 89% and 75% of *A. pleuropneumoniae* isolates showing resistance to these two agents, respectively. A worrying outcome was the resistance to the newer antimicrobial agents, such as tilmicosin where resistance was found in 25% of the *A. pleuropneumoniae* isolates and 22% in isolates of *B. bronchiseptica*. As well, an elevated minimal inhibitory concentration for tilmicosin was found in 22% of the *H. parasuis* isolates tested. There were also isolates observed with multiple drug resistance. This work has been published in two-peer reviewed articles in an international journal. Overall, while antimicrobial resistance is less of a problem in Australia than in many other countries, resistance is present in key pathogens and the industry and associated health professionals need to address the issue.

The next objective of the project was to find the underlying genetic cause for the phenotypic resistance observed. A wide variety of genes that have been reported in the literature to cause the kind of resistance observed for the Australian isolates were screened. Isolate resistance to beta-lactams and tetracyclines could be explained to a large extent by resistance genes detected, *bla_{ROB-1}* and *tetB*, respectively. However, the screening for genes reported to be associated with macrolide resistance (eight in total) gave only negative results. So at this point the resistance to macrolides cannot be explained by any of resistance genes examined in this study. Further studies are needed to explain the resistance mechanism of the Australian isolates resistant to macrolides. This study is written up as a manuscript and currently in the process of being submitted to a peer-review international journal.

The last part of this project looked at the resistance of respiratory bacteria within a pig, across a batch and between batches (all from the same farm) to determine whether resistance is evenly distributed in a bacterial population on a farm. For this study isolates of *A. pleuropneumoniae* were collected from pigs (from a single farm) at slaughter at three different occasions (ten isolates from the primary isolation plate of each lung). The three antimicrobial agents examined were tilmicosin, tetracycline and amoxicillin. Genetic fingerprinting analysis established that the isolates collected were clonal. Among the 367 clonal isolates from three different batches of pigs isolates that were either resistant or susceptible to tilmicosin were found. In some isolates, there was also statistically significant evidence of two populations in the second sampling for tilmicosin and amoxicillin, with one population showing a lower zone diameter (that is tending

towards resistance) and the other showing a higher zone diameter (tending towards more susceptible). The results suggest that a single clone of a porcine respiratory pathogen on a farm can consist of both susceptible and resistant types, with variation in zone diameters of multiple isolates varying across time. This study is currently being prepared for publication a peer-review international journal.

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1. Introduction

Antimicrobial resistance in respiratory pathogens has been reported from several countries (Vicca et al., 2004; de la Fuente et al., 2007; San Millan et al., 2009; Tang et al., 2009; Kucerova et al., 2011; Archambault et al., 2012, Vanni et al., 2012) over the last decade, however there are no such studies from Australia. A key issue in ensuring that the Australian pig industry recognized as producing a safe, quality food product via sustainable production systems is the sustainable use of antimicrobials in treatment and control programs. Antimicrobials will continue to be used in most production systems - the key is to ensure that the antimicrobials are used in a targeted and effective manner. This can only be achieved when laboratories have the capacity to test for antimicrobial resistance. As well, there is a need for a baseline of knowledge of the typical resistance patterns present in key pathogens so that treatment programs can be designed with both a farm- specific knowledge of resistance patterns, as well as, a broad industry-wide picture being available. Currently, the Australian pig industry has limited capacity in both areas.

Most laboratories currently use the American guidelines (Clinical Laboratory Standards Institute - CLSI, 2013a and 2013b) to perform antimicrobial sensitivity testing. This methodology lacks a validated method for *Haemophilus parasuis* - meaning that laboratories either do not test or simply make do with best guess methods.

There has been no organised systematic study of antimicrobial resistance patterns in key bacterial respiratory pathogens in Australia since the industry funded studies undertaken in the late 1980s in this laboratory. This is a key weakness in the ability of the industry to provide overall guidance for treatment programs as well as evidence to regulators and consumers of the capacity of the industry to deliver safe, quality meat in a sustainable manner.

This study addresses both these areas of deficiency. Phenotypic methods (both disc diffusion and Minimal Inhibitory Concentration methods) for testing the antimicrobial sensitivity of *H. parasuis* strains will be developed and validated. The developed/validated method along with existing CLSI methods for the other key pathogens will be used to test a collection of current representative isolates of *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, *H. parasuis*, and *Pasteurella multocida*. Using the results of the phenotypic testing, a set of PCR tests to detect the main resistance patterns seen in *A. pleuropneumoniae*, *H. parasuis* and *P. multocida* will be validated.

In recent times, field veterinarians have commented that the field situation is suggesting the existence within a farm of respiratory pathogens with differing levels of resistance to key antimicrobials. There is no available knowledge on this topic as there are no definitive studies looking at multiple isolates of respiratory pathogens within and across pigs. This study will address this deficiency.

Overall, this study will produce a suite of methods (phenotypic and genotypic) that will allow diagnostic laboratories to more accurately guide the implementation of antimicrobial treatment and prevention programs.

2. Methodology

The first step was to validation of Minimal Inhibitory Concentration (MIC) and Disc Diffusion techniques for determining antimicrobial susceptibility in *H. parasuis*.

This was done by using the optimal growth media for these strains (TM/SN and TMB - an agar and a broth respectively) to develop both MIC (a broth method) and disc diffusion (an agar method) for the testing of *H. parasuis*. The work was performed using international reference strains - one each of *Escherichia coli*, and *Staphylococcus aureus*, allowing validation of the methodology as these strains have defined and recognised results (using other media) for both the MIC and disk diffusion methods.

The second part was then to determine, by the MIC method, the resistance patterns of *A. pleuropneumoniae*, *B. bronchiseptica*, *H. parasuis* and *Pasteurella multocida* isolates from Australian pigs. For the *A. pleuropneumoniae*, *B. bronchiseptica* and *P. multocida* isolates, the MIC method was the standardised method recommended by the Clinical Laboratory Standards Institute (CLSI 2013a and 2013b). For the *H. parasuis* isolates, the method used was the one validated in the current study. The isolates examined were selected from the culture collection held by our laboratory and were selected to be representative of the national herd.

The next step was to determine the genotypic characterisation of the key phenotypic resistance patterns detected in the isolates of *A. pleuropneumoniae*, *H. parasuis* and *P. multocida*. On the basis of existing literature knowledge, a bank of possible genes that could explain the phenotypic resistance were assembled and suitable polymerase chain reaction (PCR) tests developed and validated. A total of 68 *A. pleuropneumoniae*, 62 *H. parasuis* and 20 *P. multocida* isolates exhibiting phenotypic antimicrobial resistance to any of the following antimicrobial agents - ampicillin, erythromycin, penicillin, tetracycline, tilmicosin and tulathromycin - were screened for the presence of a total of 19 associated antimicrobial resistance genes by PCR.

The last step was to look at the variance in antimicrobial sensitivity of one strain of *A. pleuropneumoniae* on one farm in Australia. This was done by isolating *A. pleuropneumoniae* from lung samples collected at the abattoir from three batches of pigs from one farm. From 11 to 14 lungs *A. pleuropneumoniae* isolates were harvested per batch with ten isolates collected from each lung with the exception of one lung that yielded only seven isolates. A total of 367 isolates were identified, serotyped and genotyped to determine whether they were the same strain. All isolates were tested by disc diffusion for their antimicrobial sensitivity to tilmicosin, tetracycline and amoxicillin.

3. Outcomes

In the first component of this project, methods for antimicrobial sensitivity testing of *H. parasuis* isolates had to be established first and validated. This was successfully done for both a disc diffusion method as well as an MIC method. This means that the Australian (and indeed world) pig industry now has the choice of two validated methods for performing antimicrobial sensitivity testing for *H. parasuis* - a significant step forward. Until, there is more intensive testing performed, it is not possible to interpret the results of these assays as “sensitive” or “resistant”. However, laboratories will be able to recognize lower or higher MIC values (or smaller or larger zone diameters in the disc diffusion test) and use these results for qualitative interpretation.

The aim of the antimicrobial study was to determine the antimicrobial profiles of 71 *Actinobacillus pleuropneumoniae*, 97 *Haemophilus parasuis*, 51 *Pasteurella multocida* and 18 *Bordetella bronchiseptica* cultured from Australian pigs. The majority of *A. pleuropneumoniae* isolates were resistant to erythromycin (89%)

and tetracycline (75%). Resistance to ampicillin (8.5%), penicillin (8.5%) and tilmicosin (25%) was also identified. The *H. parasuis* isolates showed elevated minimum inhibitory concentrations for ampicillin (1%), penicillin (2%), erythromycin (7%), tulathromycin (9%), tilmicosin (22%), tetracycline (31%) and co-trimoxazole (40%). The *P. multocida* isolates exhibited a low frequency of resistance to florfenicol (2%), co-trimoxazole (2%), ampicillin (4%), penicillin (4%), erythromycin (14%), and tetracycline (28%). All the *B. bronchiseptica* isolates showed resistance to beta-lactams (ampicillin, ceftiofur and penicillin). A majority of the *B. bronchiseptica* isolates were resistant to erythromycin (94%) and resistance to florfenicol (6%), tilmicosin (22%), and tetracycline (39%) was present in some isolates. The incidence of multiple drug resistance (MDR), defined as resistance to three or more antimicrobial classes, varied across the species - in *B. bronchiseptica*, 27.8% of resistant isolates showed MDR, while lower levels were found in *A. pleuropneumoniae* (9.1%) and *P. multocida* (4.8%). These results provide evidence that antimicrobial resistance is a problem in Australia as well and suggests that isolates should be tested for their antimicrobial sensitivity profile before treatments are implemented.

The most common antibiotics used against respiratory bacterial infection in pigs are β -lactams, macrolides, phenicols, potentiated sulphonamides and tetracyclines. Overseas studies have identified a whole array of resistance genes responsible for some of the phenotypic antimicrobial resistance observed. As an example, one gene responsible for β -lactam (ampicillin and penicillin) resistance that has been discovered is the *bla*_{ROB-1} for *A. pleuropneumoniae* (Matter, et al., 2007) and *H. parasuis* (San Millan, et al., 2007; Guo et al., 2012). The β -lactam resistance in the current study could be explained with the finding of the gene for *bla*_{ROB-1} gene, found in all 10 β -lactam resistant isolates (six *A. pleuropneumoniae* and two of each of *H. parasuis* and *P. multocida*). The tetracycline resistance could be explained with the finding of the *tetB* gene in 74 out of 97 isolates (49/53 *A. pleuropneumoniae*, 17/30 *H. parasuis* and 8/14 *P. multocida*). The gene *tetH* was also discovered in one isolate of *A. pleuropneumoniae*. However, the macrolide resistance could not be explained as none of the eight genes screened in this study (*ermA*, *ermB*, *ermC*, *erm42*, *mphE*, *mefA*, *msrA* and *msrE*) were detected in 92 resistant isolates examined. The explanation of the macrolide resistance might not be the presence of a specific gene but rather a mutation in the sequences of the bacterial 23S ribosomal subunit, which has been observed in other bacterial species, such as in *Mycoplasma hyopneumoniae* and *Campylobacter* species isolated from pigs and chicken, respectively (Sakenbor et al., 2005; Ladely et al., 2009) that have shown resistance. This means that β -lactam resistance can be genetically detected by testing for the presence of *bla*_{ROB-1} and that the genes *tetB* and *tetH* are useful for the genetic tests for tetracycline resistance. Further studies are needed to determine the resistance mechanism employed by Australian isolates to macrolides.

The testing of the variability of antimicrobial sensitivity of isolates from one pig and from different pigs in the same batch and across batches all isolates were identified and genotyped to ascertain whether it was the same strain causing pleuropneumonia of the pigs involved in this study. All isolates were identified as *A. pleuropneumoniae* serovar 1 with the same genetic profile. All 367 isolates were then tested for antimicrobial susceptibility to amoxicillin, tetracycline and tilmicosin. Tilmicosin was the only antimicrobial in this study for which there are CLSI interpretative breakpoints. Some, but not all, of the ten isolates selected from some pigs were resistant (Table 1). Overall, of the ten isolates tested per lung, some lungs gave only isolates that were sensitive to tilmicosin while for

other lungs, up to five of the 10 isolates were resistant. All three batches at slaughter had some pigs with all ten isolates being sensitive as well as pigs with a mixture of resistant and sensitive isolates.

When looking at the zone diameter for all three batches a considerable variability in batch 2 compared to the other batches for all the antimicrobials examined was noted (Fig 1). This pattern corresponded with the larger difference between lowest and highest measured zone diameter and the slightly higher number of resistant isolates to tilmicosin in batch 2 (Table 1).

A statistical analysis of the distribution for all antimicrobials in regards to their zone diameter revealed that the distribution for ampicillin and tilmicosin for batch 2 provided evidence of two populations (Fig 2). No evidence of bimodal distribution was detected for these two antimicrobials from the other batches of pigs or for the distribution of zone diameters of all isolates from the three batches for tetracycline.

The conclusion from this observed variability not only among batches of pigs, but also the variability between the batches has considerable implications on the testing for antimicrobial sensitivity but also for the treatment with antibiotics.

Table 1 - Tilmicosin resistance was allocated the number 2 and sensitivity the number 1. The average from each pig lung (10 isolates per lung, except lung eleven in batch 1, which had 7 isolates) was then calculated and represented in the table. As an example, the results for lung 9 in batch 2 show an average of 1.5. This means that the ten isolates (which were scored as 1 for sensitive and 2 for resistant) gave a total score of 15 - meaning that five isolates were sensitive (total score of 5) and five isolates were resistant (total score of 10) to give a score of 15 for the ten isolates and an average of 1.5. In contrast, for lung 9 in batch 3, the average score is 1, meaning that all ten isolates were sensitive (score of 1 each). In essence, the higher the average is above 1, the more isolates within the lung were resistant.

Lung	batch 1	batch 2	batch 3
1	1	1	1
2	1	1	1.1
3	1	1.1	1
4	1.4	1.3	1
5	1.3	1.4	1
6	1.2	1	1.2
7	1.1	1.2	1
8	1.1	1.3	1.2
9	1.2	1.5	1
10	1	1	1
11	1	1.1	1
12		1.3	1.3
13			1.1
14			1.1
Average	1.12	1.18	1.07

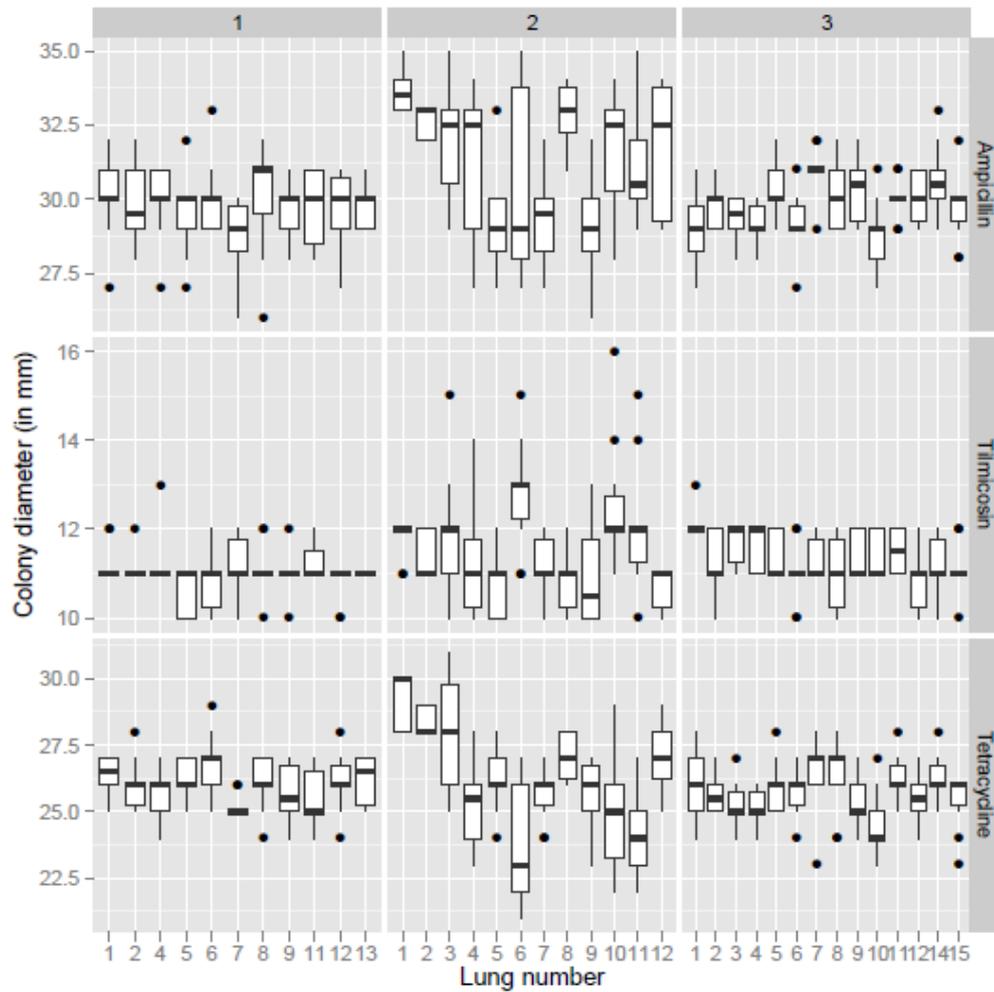


Figure 1 - The variance in zone diameter for the different lungs and batches are represented. Each box plot represents the zone diameter measured for 10 samples from one lung, with the mean of the measurements represented by a line in the box.

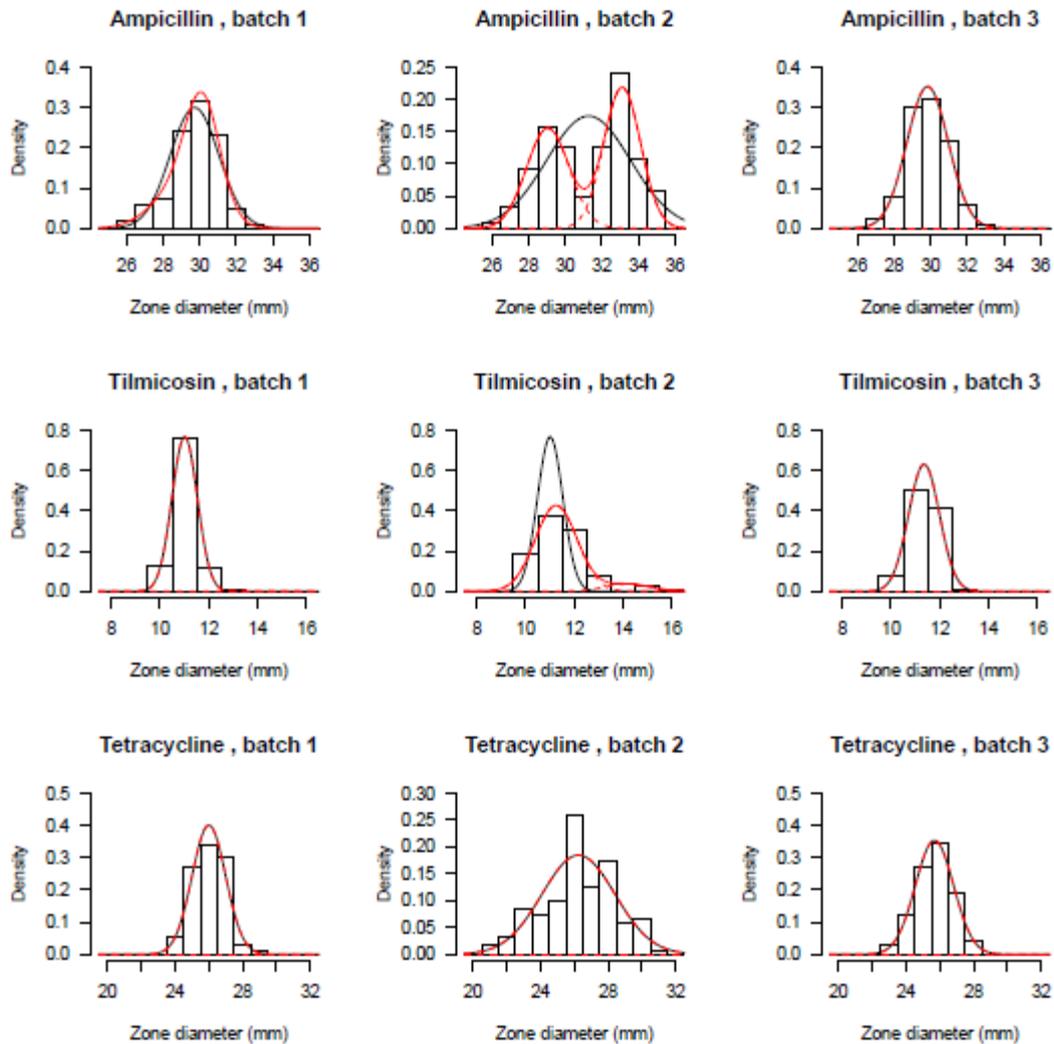


Figure 2 - An illustration of zone diameter distributions of antimicrobial agents to 367 isolates of *A. pleuropneumoniae* from 37 lungs (11, 12 and 14 lungs from batch 1, 2 and 3 respectively) that were best fitted by a mixture of Gaussian distributions. The black lines show the unimodal distribution, while the red lines show a mix of Gaussian distributions. The dotted line show the bimodal distribution, which is the best fit for the data of the antibiotics amoxicillin and tilmicosin of batch 2.

Olofsson and Cars (2007) hypothesised that during antimicrobial therapy, different gradients of antimicrobial concentration exist in the body, which creates different environments with different selective pressures. This leads to a selection of sub-populations of resistant bacteria by certain drug concentration, which will in turn promote the survival of resistant bacteria during treatment.

In the current study, the finding of apparently clonal isolates that exhibit considerable variability in their antimicrobial sensitivity patterns within and between pigs and batches supports this hypothesis. The pigs in this study had been on in-feed medication (400 ppm tilmicosin) for 2 weeks after weaning, followed by 3 weeks in-feed tylosin (100 ppm). The pigs were then fed a grower feed supplemented with chlortetracycline (200 ppm) until they were sent to the

study (grow-out) farm. At the study farm, the grower feed was supplemented with chlortetracycline (400 ppm) for 5 weeks, tilmicosin (200 ppm) for 2 weeks and chlortetracycline (400 ppm) for a further two weeks. The pigs were on a continuous cycle of medication to reduce the clinical signs associated with *A. pleuropneumoniae*. Considering the level of antimicrobial exposure this bacterial species has encountered the observation of resistant isolates being present within a susceptible population on this farm is not surprising. There seems no doubt in the literature that the level of antimicrobial treatment has an effect on the selection of resistance. The fact that resistant and susceptible bacteria were isolated from lungs collected from pigs at slaughter suggests that the antimicrobial levels in the pig feed were not sufficient to inhibit the growth or survival of *A. pleuropneumoniae* in the lungs. It has been noted by Olofsson and Cars (2007) that if the antimicrobial dosage is not optimal to inhibit all wild type and mutant bacteria, that exhibit different degrees of resistance, then there is a selection for the resistant bacteria. As well, Pyörälä-Baptiste, Catry *et al.* (2014) have recommended that, as even very low antibiotic concentrations have been shown to select for resistance, there is a need for studies looking at the impact of long acting and short acting macrolides in feed on the development of antimicrobial resistance.

From a diagnostic viewpoint, the current study emphasises the importance of multiple samples from one animal to determine the antimicrobial sensitivity of one animal. However, the detection of variability between animals and the variability between batches suggests that multiple animals also need to be examined. Provided resources are sufficient, the current study shows that multiple isolates within a pig and multiple pigs need to be tested to determine the status of antimicrobial resistance for at least two of the three key agents used in the Australian pig industry.

4. Application of Research

This project has the following outcome and deliveries:

- Validated disk diffusion and MIC antibiotic sensitivity method for testing *H. parasuis* isolates
- Knowledge of the current resistance patterns in the key pathogens - *A. pleuropneumoniae*, *B. bronchiseptica*, *H. parasuis* and *P. multocida*
- A series of PCR assays have been used to identify genes which are responsible for the phenotypic resistance patterns observed for Australian isolates
- Establishment of occurrence of variation in antimicrobial sensitivity of one clonal bacterial population on a farm and thus the need for diagnostic laboratories to consider testing multiple animals and multiple isolates within an animal to determine antimicrobial sensitivity patterns and provide sound advice to clinicians seeking effective antimicrobial treatment strategies on a farm.

Overall, the information obtained in this project will help the pig industry to establish effective, sustainable antimicrobial treatment and prevention programs.

5. Conclusion

There has been no organised systematic study of antimicrobial resistance patterns in key porcine bacterial respiratory pathogen in Australia in the last two decades. The establishment of a sustainable disease treatment/prevention program that

includes antimicrobials needs knowledge of the resistance patterns for respiratory bacterial pathogens. This knowledge was very limited for Australian strains, with the additional problem of a lack of validated methods for antibiotic sensitivity testing for *H. parasuis*

This study has generated current data on the antimicrobial susceptibility/resistance patterns of key respiratory bacterial pathogens in Australia. This work included the development and validation of a methodology suitable for use with *H. parasuis*. Moreover, a genetic picture of reasons for the antimicrobial resistance detected in *A. pleuropneumoniae*, *H. parasuis* and *P. multocida* was also determined. This project filled significant gaps in our knowledge of antimicrobial resistance patterns in Australia and has provided information to veterinarians for guidance in relation to antimicrobial susceptibility and resistance of four respiratory bacterial pathogens.

The finding of observed resistance in all four species of bacteria highlights the importance of antimicrobial sensitivity testing to optimize treatment regimes.

This work has been published as two peer-reviewed articles and the publications are attached to the report.

Unfortunately, the genetic basis for the macrolide resistance could not be explained by the genes screening performed in the current study, even though the genes have been indicated by overseas studies to be involved in macrolide resistance. Further studies are needed to explore the mechanism responsible for the phenotypic resistance of macrolides. The β -lactam and tetracycline resistance could be explained by the presence of *bla*_{ROB-1} and *tetB/tetH*, respectively.

The variability of antimicrobial sensitivity between batches as well as in the same pig has highlighted that just testing one isolate per pig is not sufficient to make predictions about the antimicrobial sensitivity patterns of *A. pleuropneumoniae* on a farm. It also highlights the need for recognizing this variability when developing specific treatment and prevention regimes for a farm.

6. Limitations/Risks

The main limitation to this study is that the collection of isolates used to perform the study may not necessarily reflect the total national pig herd. The isolates were selected from the largest collection available in Australia and were selected to be as representative of known geographical diversity as possible. Hence, the risk of a non-representative collection does exist but it has been minimized as much as possible.

The evidence of variation in antimicrobial resistance patterns has been generated by examining one pathogen (*A. pleuropneumoniae*) and one farm. Additional studies are needed to confirm if the results are also relevant for other pathogens as well as other antimicrobial agents.

7. Recommendations

Diagnostic laboratories should consider adopting the disc diffusion or MIC method established in the current study when required to test for antimicrobial resistance in *H. parasuis* isolates.

The pig industry and the associated health professionals need to remain vigilant on the issue of antimicrobial resistance. The current study has shown low levels of resistance to most agents. However, there is evidence of a low level of multi-drug resistance and of resistance to the more recently available agents.

Diagnostic laboratories need to consider testing more than one isolate of *A. pleuropneumoniae* per submission, particularly when testing for tilmicosin and ampicillin resistance. At a minimum, one isolate from multiple lungs need to be examined. Preferably, multiple isolates per lung and multiple lungs need to be tested.

8. Acknowledgement

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Appendix

Variation of antimicrobial sensitivity of *Actinobacillus pleuropneumoniae* in a pig, within a batch of pigs and among batches of pigs from one farm

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ABSTRACT

Antimicrobial resistance in porcine respiratory pathogens has been shown to exist in many countries. However little is known about the variability in antimicrobial resistance within a population of one bacterial respiratory pathogen on a pig farm. This study examined the antimicrobial sensitivity of *Actinobacillus pleuropneumoniae* using multiple isolates within a pig and across the pigs at three different sampling times. Three antimicrobial agents examined were tilmicosin, tetracycline and amoxicillin. A total of 367 isolates of *A. pleuropneumoniae* were tested. The isolates were identified, serotyped and a subsample genotyped. All isolates were tested for their antimicrobial sensitivity. All isolates were identified as *A. pleuropneumoniae* serovar 1 with the same genetic profile. The three batches were found to consist of a mix of resistant and susceptible isolates against tilmicosin and there was a shift from the susceptible wild type towards a resistant type observed that was statistically significant in the second sampling for tilmicosin and amoxicillin. There was considerable variance in the second sampling period and the zone diameter across the isolates towards the three antimicrobials varied considerably. The results support the hypothesis that one population of a porcine respiratory pathogen can consist of both susceptible and resistant types, with the prevalence of resistant types varying across time.

INTRODUCTION

There are several mechanisms by which a bacteria species can acquire antimicrobial resistance, horizontal acquisition of resistance genes, recombination of foreign DNA into the chromosome or mutation.¹⁸ The development of antimicrobial resistance depends on many factors such as the mutation frequency, resistance genes on mobile genetic elements, bacterial population size, bacterial stress (competition, lack of nutrients, host defence), concentration and type of antibiotics, the biological fitness cost of

antimicrobial resistance (cost of mutation or replicating of resistance gene in absence of antibiotic) and the ability to compensate for the biological fitness cost.^{1,10,18,3,21}

A recent study⁸ showed that antimicrobial resistance exists in Australia in some pig herds for the bacterial species *Actinobacillus pleuropneumoniae*, *P. multocida* and *Bordetella bronchiseptica*. Of the seventy-one isolates of *A. pleuropneumoniae* examined, 75% were resistant to tetracycline, 25% to tilmicosin and 8.5% to ampicillin.⁸ However, this study did not look at antimicrobial resistance within one farm. The general knowledge about spontaneous mutations and the models developed to predict the mutation rate leads to the conclusion that a bacterial population may consist of susceptible wild type bacteria and mutated bacteria with different degrees of resistance existing in low numbers.²¹ A prior study⁴ has evaluated the antimicrobial resistance variability of *Escherichia coli* from faecal samples of sows and growers from two farms in Norway. The faecal samples were collected from the rectum of each animal and ten *E. coli* isolates were tested from each animal. For the sows, the time of sampling had a great effect on the variability, while the only within-sow variation prominent was for resistance to ampicillin⁴. An even greater variation was found between the three sampling times of the grower pigs. The suggested explanation for difference in antimicrobial sensitivity in the grower pigs between the samplings was the amount of antimicrobial to which the pigs were exposed⁴. Overall, the conclusion of this study was that, when estimating the prevalence of resistant isolates in a herd, the variability within an animal or the number of isolates per sample is of minor importance⁴. However, this study was for *E. coli* in faeces, a bacterial species which includes pathogenic and non-pathogenic forms and which may not be clonal^{2,6,11,29}. No similar knowledge of the variability within pigs and between pigs of the same batch and between batches appears to exist for porcine respiratory pathogens. *Actinobacillus pleuropneumoniae* (the causative agent for porcine pleuropneumonia of pigs²⁷) is normally only present as one serovar on an infected farm²⁰. This scenario of one serovar would mean a more defined population of *A. pleuropneumoniae*, possibly just a single genotype, as compared with *E. coli*. Whether this likely scenario - a single genotype of *A. pleuropneumoniae* - means a limited variability in antimicrobial resistance is unclear. The current study was designed to examine the key question:- Can isolates of *A. pleuropneumoniae* on a single farm show variation in resistance to antimicrobial agents?

MATERIALS AND METHODS

Farm

The farm was located in Western Australia south of Perth. This farm was a continuous flow, grow-out farm rearing pigs from 10 weeks to slaughter with pigs arriving on a weekly basis. Pigs were housed in eco-shelters on straw with each age group and sex being housed in their own shelter. Pigs were sold to slaughter by weight (90 - 110 kg), which meant that pigs remained on farm if they did not meet sale weight criteria. Pigs sampled at slaughter would have been 20 to 23 weeks of age. Data on the history of pleuropneumonia, the vaccination regime, general production parameters and antimicrobial treatment programs were collected.

Sampling

Pig lungs showing typical lesions of pleuropneumonia were sampled at an abattoir in Western Australia and the samples sent to the Department of Agriculture in Western Australia, where swabs (one swab per lung) were taken from the lungs and plated onto 5% sheep blood agar plates that were cross-streaked with a nurse colony of *Staphylococcus hyicus*. After incubation, these cultures were sent to the EcoSciences Precinct laboratory, where ten suspect *A. pleuropneumoniae* colonies were picked from each blood agar plate (each plate representing one lung) and grown on BA/SN, which was prepared as previously described⁹. The incubation was under aerobic conditions incubation at 37°C overnight. The sampling was done on three occasions, the 6th March, the 18th March and the 25th March 2014.

Identification and serotyping of *A. pleuropneumoniae*

At the first sampling 13 lungs were sampled and *A. pleuropneumoniae* was isolated from 11 lungs. From one lung only seven isolates could be cultured. A total of 107 isolates were collected from this sampling event. The second sampling yielded 10 isolates from 12 lungs with a total of 120 isolates of *A. pleuropneumoniae*. The last sampling yielded 10 isolates from 14 lungs with a total of 140 isolates. Altogether a total of 37 lung and 367 isolates were investigated.

All of the isolates were identified as *A. pleuropneumoniae* serovar 1 by the multiplex PCR. The subset of samples genotyped displayed the same rep-PCR profile for all isolates, suggesting that this is one clone of *A. pleuropneumoniae*.

Antimicrobial resistance testing

For tilmicosin, the only antimicrobial in this study for which there are CLSI interpretation criteria¹, some, but not all of the ten isolates selected from some pigs were resistant (Table 1). Overall, in some pigs no resistant isolates were cultured, while others had up to 5 of the 10 isolates that displayed resistance. All three batches at slaughter had some pigs with only sensitive isolates as well as pigs with resistant and sensitive isolates.

Looking at the variability of the zone diameter measured in the three batches, there was a considerable variability in batch 2 compared to the other batches for all the antimicrobials examined (Figure 1). This pattern corresponded with the larger difference between lowest and highest measured zone diameter and the slightly higher number of resistant isolates to tilmicosin in batch 2 (Table 1).

The statistical analysis for the distribution for ampicillin and tilmicosin for batch 2 provided evidence of two populations (Figure 2). No evidence of bimodal distribution was detected for these two antimicrobials for the isolates of *A. pleuropneumoniae* from the other batches of pigs or for the distribution of zone diameters of all isolates from the three batches for tetracycline.

DISCUSSION

In 2009 a study by Jordan et al¹⁴ revealed that the most used antimicrobials in the Australian pig industry are penicillin (mainly used as an injection) and tetracycline (injection/oral use), followed by macrolides (injection/oral use) and sulfonamides (injection only use). The current study farm used tetracycline and tilmicosin, typical of the common antimicrobial practices on Australian piggeries. The farm normally has two week periods of no antibiotic use, but for these batches this did not occur. Ampicillin, tilmicosin and tetracycline were selected for antimicrobial susceptibility testing in the current study as a recent study⁸ has confirmed resistance to these antimicrobials (8.5%, 75% and 25% respectively) in *A. pleuropneumoniae* isolates from Australian pigs

According to Davies (1997)⁷ the beta-lactamase enzyme, which hydrolyse the beta-lactam ring of beta-lactam antibiotics, such as ampicillin, exists in most bacterial species. The recognised number and varieties of beta-lactamases have increased due to the newer antimicrobials in this group and the fact that a single base change in the gene for beta-lactamase is able to change the substrate specificity of beta-lactamase.⁷ It is also known that genes encoding these enzymes can transfer from the chromosome onto plasmids and other mobile elements and then back onto the chromosome.⁵ The TEM and the ROB-1 beta-lactamase and their associated *bla* genes, *bla*_{TEM} and *bla*_{ROB-1}, have been associated with resistance to beta-lactams in *A. pleuropneumoniae*¹⁹ and *Haemophilus parasuis*¹².

For macrolides, like tilmicosin, resistance is associated with methylases (*ermA*, *ermB* and *ermC*) with the corresponding gene located on transposons or plasmids. There have been other genes described in the literature for macrolide resistance, such as *erm42*, *mphE*, *mefA*, *msrA* and *msrE*^{24,25}. However, not all macrolide resistance can be explained by these genes and some other mechanism might be at play, such as mutation in the sequences of the bacterial 23S ribosomal subunit^{16,24}. Tetracycline resistance is associated with *tet*-genes and these genes have been found on the chromosome as well as on plasmids in members of the family *Pasteurellaceae*¹⁵.

The finding of two populations in batch 2 for two of the antimicrobials, ampicillin and tilmicosin, tested could mean that resistance to the two agents might be genetically linked. The farm had occasionally used ampicillin in the past for some groups but not for these batches. However, ampicillin resistant genes have been found on plasmids.⁷ Dayao et al (2014)⁸ observed multiple agent resistance in some *A. pleuropneumoniae* strains, the resistance to ampicillin seen in the current study could involve plasmid involvement or mobile gene cassettes as has been reported for other bacteria.¹³ This is a particular concern, as in cases of multi-resistance with genetically linked resistance determinants, resistance will remain in the population if antimicrobials to which the other determinants are genetically linked are used¹⁷.

Olofsson and Cars (2007)²² stated that during antimicrobial therapy different gradients of antimicrobial concentration exist in the body, which creates different environments with different selective pressures. Subpopulations of resistant bacteria will be selected by certain drug concentration, allowing these resistant bacteria to survive and multiply during treatment²². Prescott (1997) discussed this issue of multi-resistant organisms, which persist in the host or the environment in the absence of antimicrobial selection acting as a reservoir for the genes. In light of this resistance being expressed by genes on plasmids, the survival of these genes is enhanced, as bacterial populations can share genetic information.

In the current study amoxicillin and tetracycline were used in the disc diffusion method (CSLI), which does not have interpretative criteria for these antimicrobials. Tilmicosin, on the other hand, has interpretative criteria and the use of those criteria in the current study has shown that some, though not all, apparently clonal isolates are resistant isolates, within and between pigs and batches. The observation of two populations with the population with the larger zone diameter being very small compared to the population with the smaller zone diameter, means that the population with the smaller zone diameter has increased and has become dominant. For ampicillin, an antimicrobial with no interpretation, the current study has shown the existence of two populations - isolates with a bigger zone diameter and isolates with a smaller zone diameter.

The observation of resistant isolates being present within a susceptible population on this farm is not surprising considering the level of antimicrobial exposure this bacterial species have encountered. There seems no doubt in the literature that the level of antimicrobial treatment has an effect on the selection of resistance²³. The fact that resistant and sensitive bacteria were isolated from lungs collected at the abattoir, suggests that the antimicrobial levels in the pig feed were not sufficient to inhibit the *A. pleuropneumoniae* present in the lungs. The caution by Olofsson and Cars (2007)²² that if the antimicrobial dosage is not optimal to inhibit all wild type and mutant bacteria, that exhibit different degrees of resistance, then there is a selection for the resistant bacteria, needs to be kept in mind. A very recent study on the macrolide resistance echoed these warnings stating that to achieve a decrease of the risk of resistance development of an update and harmonisation of the dosing regimes of macrolides is necessary.²⁴ The biological cost of mutation to the resistance phenotype or replicating of resistance gene in absence of antibiotic is normally associated with less fitness of the isolate.^{3,22} However, it has been found that if resistant bacteria can exist in a population for a long time, even in the absence of antimicrobials, they can overcome the biological cost associated with resistance by either restoring the fitness with mutations²² or by yet unexplained mechanism as in the case of additional genes.³ If that is happening then even the restriction of the antimicrobials will not lead to the reversibility of resistance²².

The study by Brun et al (2002)⁴ suggested that if sampling for general tendencies then one isolate per animal at each repeated sampling time was optimal, while several samples per animal are needed if focus is on one animal. The current study certainly emphasises the importance of multiple samples from one animal. However, the detection of variability between animals and the variability between batches suggest that multiple animals with multiple samples from each animal need to be tested to determine the status of the batch.

In light of resistant strains emerging, which might possibly be on a plasmid, and the level of antimicrobials given in the food that does not seem sufficient to inhibit the bacterial species, selection for resistance is optimized. If this practice is continued the fitness level of these resistant strains might increase, leading to irreversible resistance. The finding - of variability in antimicrobial susceptibility of *A. pleuropneumoniae* isolates within/between pigs and between batches in the current study - agrees with the observations in the field that there seems to be variable effectiveness of antimicrobial treatments between batches. The current study highlighted the importance of finding and using an effective dose level that reduces the likelihood of the development of resistance.

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Figure 1 The variance in zone diameter for the different lungs and batches are represented. Each box plot represents the zone diameter measured for 10 samples from one lung, with the mean of the measurements represented by a line in the box.

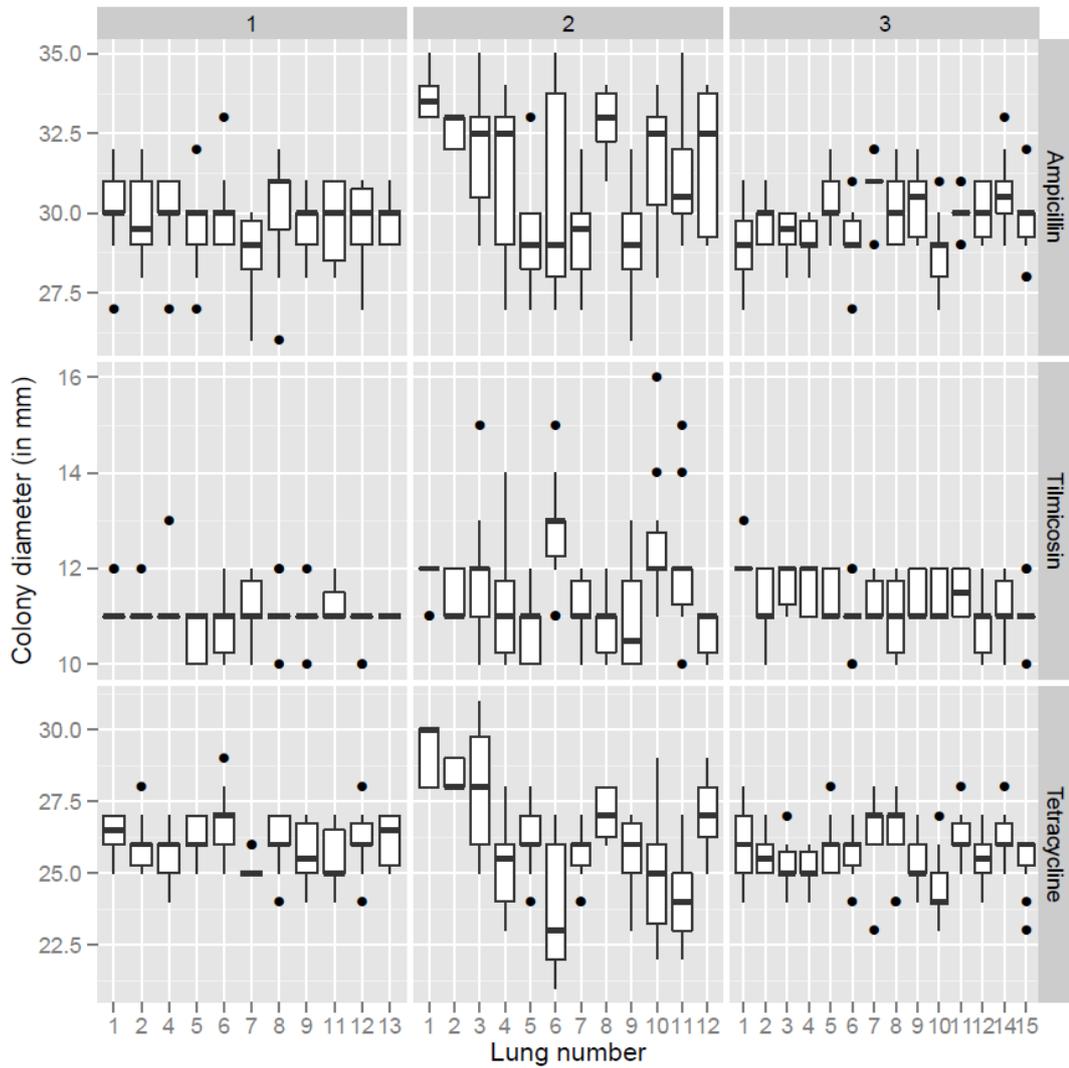


Figure 2 An illustration of zone diameter distributions of antimicrobial agents to 367 isolates of *A. pleuropneumoniae* from 37 lungs (11, 12 and 14 lungs from batch 1, 2 and 3 respectively) that were best fitted by a mixture of Gaussian distributions. The black lines show the unimodal distribution, while the red lines show a mix of Gaussian distributions. The dotted line show the bimodal distribution, which is the best fit for the data of the antimicrobials amoxicillin and tilmicosin of batch 2.

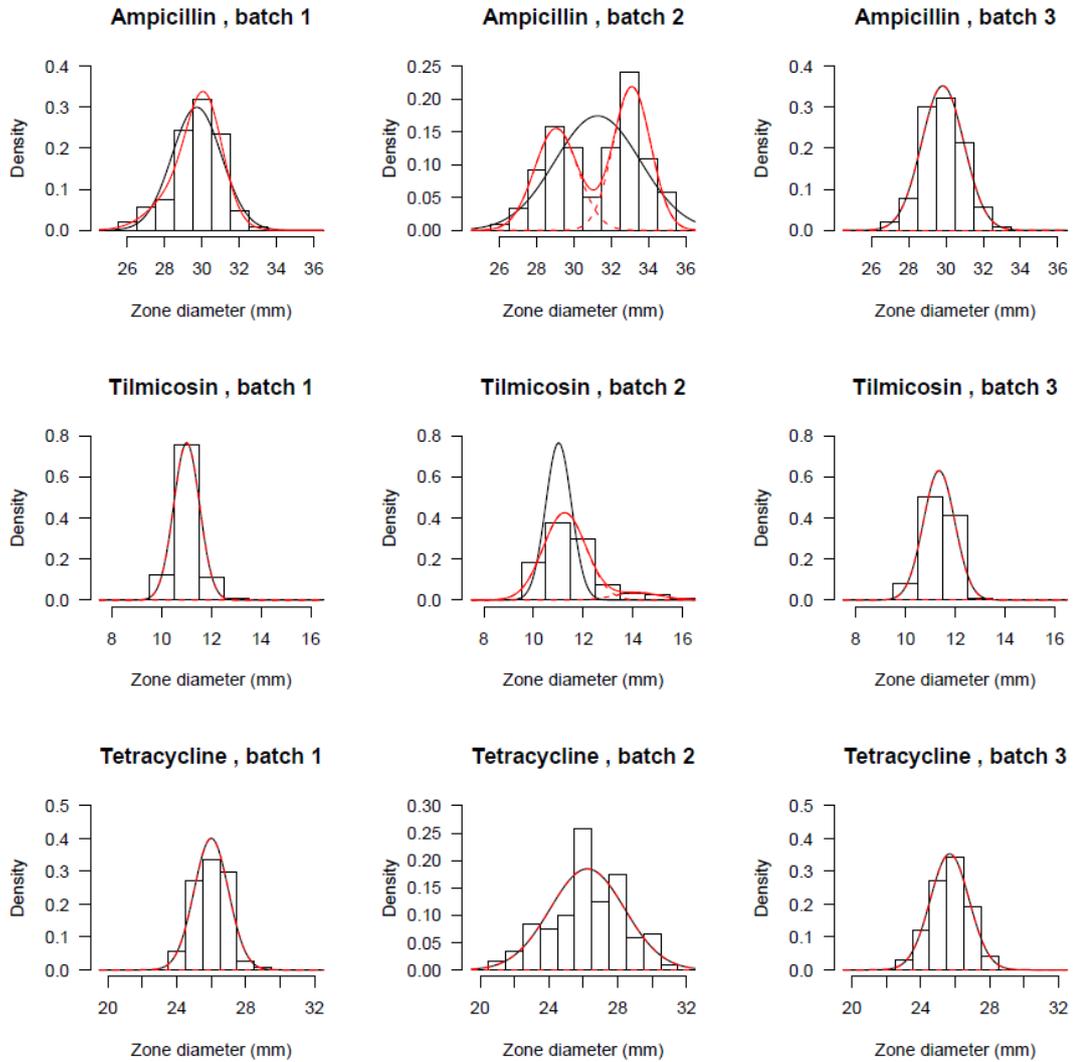


Table 1 Tilmicosin resistance was allocated the number 2 and sensitivity the number 1. The average from each pig lung (10 isolates per lung, except lung eleven batch 1, which had 7 isolates) was then calculated and represented in the table.

Lung	batch 1	batch 2	batch 3
1	1	1	1
2	1	1	1.1
3	1	1.1	1
4	1.4	1.3	1
5	1.3	1.4	1
6	1.2	1	1.2
7	1.1	1.2	1
8	1.1	1.3	1.2
9	1.2	1.5	1
10	1	1	1
11	1	1.1	1
12		1.3	1.3
13			1.1
14			1.1
Average	1.12	1.18	1.07