Journal of Microbiological Methods 191 (2021) 106360

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

An improved multiplex PCR for *Actinobacillus pleuropneumoniae*, Glaesserella australis and *Pasteurella multocida*

Xiaochen Sun [a], P.J. Blackall [a], Patrick Daniel [b], Kerri Chandra [c], Sarah Jenkin [d], Conny Turni [a,*]

[a] Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, Queensland 4067, Australia
[b] Pig Services Centre, Agriculture, Food and Fibre Division, Department of Jobs, Precincts and Regions, Epsom, Victoria 3551, Australia
[c] Department of Agriculture and Fisheries, Ecosciences Precinct, Dutton Park, Queensland 4102, Australia
[d] Apiam Animal Health, Bendigo, Victoria 3550, Australia

ARTICLE INFO

Keywords:
Multiplex PCR
Glaesserella australis
Actinobacillus pleuropneuominae
Pasteurella multocida
Prevalence

ABSTRACT

Glaesserella australis, a newly described bacterial species, has been isolated from pig lungs that displayed lesions very similar to those caused by *Actinobacillus pleuropneumoniae*, prompting the need for a validated diagnostic tool. In this work, we have altered a multiplex PCR used for the identification of cultures of *G. australis*, *A. pleuropneumoniae* and *Pasteurella multocida* to be more sensitive and then evaluated the use of the altered diagnostic tool on cultures and directly on tissues. The altered multiplex PCR was validated using 47 related species, both type/reference strains and field isolates. The sensitivity was assessed by serial dilutions and used a mixture of target bacteria in different concentrations. Further, 166 lung samples from 54 farms from four Australian States were used to validate the ability of the multiplex PCR to detect bacteria in lung swabs. The multiplex PCR was specific for the three target species. The assay could detect a minimum of 40 colony forming units (CFU) of *G. australis*, 786 CFU of *A. pleuropneumoniae* and 238 CFU of *P. multocida*. The multiplex PCR yielded more positives than coventional bacteriological examination. From a total of 166 lung samples, 51.9%, 51.9% and 5.6% of farms were PCR positive for *P. multocida*, *A. pleuropneumoniae* and *G. australis*, respectively. The results suggested that the new multiplex PCR was specific, sensitive and out performed traditional culture. The prevalence of *G. australis* was not very high, but it was the dominant pathogen in infected pigs.

1. Introduction

Glaesserella australis, a newly recognised species of the genus *Glaesserella,* is associated with severe clinical disease with pig mortality occurring at 12 to 20 weeks of age (Turni et al., 2020). These cases showed severe necro-suppurative and haemorrhagic bronchopneumonia that resembled lesions typically associated with *Actinobacillus pleuropneumoniae*, except that pleurisy was not observed. In some of these cases, *G. australis* was isolated in pure culture from the affected lungs while in other cases other bacteria (*P. multocida* and *Trueperella pyogenes*) as well as *G. australis* were isolated (Turni et al., 2017). In addition, *G. australis* was isolated from lung lesions, lung abscesses and pleurisy detected at slaughter in pigs that had shown little or no clinical signs on the farm (Turni et al., 2017).

As the gross lesions observed with *G. australis* infections are quite similar to those associated with *A. pleuropneumoniae*, isolates of *G. australis* might have possibly been wrongly identified. Therefore, a differential diagnostic tool is essential. In the age range where *G. australis* infections have been detected i.e. 12 to 20 weeks of age (Turni et al., 2017) the other bacterial pathogens commonly observed are *A. pleuropneumoniae* and *P. multocida* (Gottschalk and Broes, 2019; Register and Brockmeier, 2019). Furthermore, *P. multocida* and *A. pleuropneumoniae* were identified as potentially causative pathogens of pleurisy at an abattoir in Australia, with 24 out of 46 farms positive for *P. multocida* and 7 farms positive for *A. pleuropneumoniae* (Turni et al., 2021). Hence a multiplex PCR (Multiplex Ver 1) was developed to identify pure cultures of *G. australis*, *A. pleuropneumoniae* and *P. multocida* (Yee et al., 2019) but the assay proved unsuitable for direct use on lung samples because it was not sensitive enough (unpublished data). In the current study, we have modified the multiplex assay (Multiplex Ver 1) for *G. australis*, *A. pleuropneumoniae* and *P. multocida* to enhance the sensitivity and have validated the modified assay (Multiplex Ver 2) on both pure cultures of reference strains for the serovars of the species targeted and on related species for specificity, as well as

* Corresponding author at: St Lucia, Queensland 4067, Australia.
E-mail address: c.turni1@uq.edu.au (C. Turni).

https://doi.org/10.1016/j.mimet.2021.106360
Received 31 August 2021; Received in revised form 7 October 2021; Accepted 27 October 2021
Available online 2 November 2021

establishing the sensitivity. The modified assay was further evaluated directly on lung samples comparing it to conventional culturing of the samples.

2. Materials and methods

2.1. PCR development

In our previous study (Yee et al., 2019), a multiplex PCR for *A. pleuropneumoniae*, *P. multocida* and *G. australis* was developed and validated on pure cultures. In the current study, the original PCR was improved and then evaluated using both pure cultures as well as swabs collected from lung tissues. The assay developed in the Yee et al. (2019) study is termed Multiplex Ver 1. In the current study, a modification of the Multiplex Ver 1 assay was developed, validated and evaluated (termed the Multiplex Ver 2 assay).

The Multiplex Ver 1 assay was performed essentially as described previously (Yee et al., 2019). The primers are described in Table 1. The 25 μL reaction mix consisted of 1.5 mM $MgCl_2$, 10 mM Tris (pH 8.3), 50 mM KCl, 0.24 mM dNTPs, 0.3 μM of each primer, 0.5 U of *Taq* (Roche, Mannheim, Germany) and 1 μL of template. The reaction mix was run for one cycle at 94 °C for 2 min and then for 30 cycles at 94 °C for 30 s, 65 °C for 30 s, 72 °C for 2 min. Next, a final cycle at 72 °C for 10 min was performed. The PCR product was run on a 1% agarose gel in SB buffer (36.39 mM boric acid and 8.75 mM NaOH) for 35 min at 200 V by loading 10 μL of amplified product with 2 μL of EZ vision dye (Amresco Inc. Solon, Ohio, USA) and photographed under UV illumination.

The primers used in the Multiplex Ver 2 assay were the same as those used by Yee et al. (2019) (Table 1). The essential changes were a different polymerase and thus the concentration of $MgCl_2$ and dNTPs for this polymerase. With this change, it was necessary to undertake optimisation for the primers and polymerase as well as a new validation with target and non-target bacteria. The PCR reaction mix consisted of 1 x GoTaq buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3–0.9 μM of each primer (Table 1) and 0.625 - 2 U GoTaq (Promega Corporation, Wisconsin US). The volume of DNA template was optimized using from 1 to 4 μL of DNA from tissue samples per 25 μL reaction and dilutions of 4 μL of pure culture extracted DNA (dilutions tested being neat, 1/10, 1/100 and 1/1000). The running conditions were one cycle at 95 °C for 2 min, 30 cycles with 95 °C for 30 s, 62 to 65 °C for 30 s and extension for 72 °C for 2 min. This was followed by one extension cycle at 72 °C for 10 min. The PCR products were visualized as described above. The differences between Multiplex Ver 1 and 2 are listed in Table 2.

Table 1
Details of the primers used in the multiplex PCR for *Glaesserella australis*, *Actinobacillus pleuropneumoniae* and *Pasteurella multocida* with amplicon size of the final product.

Species	Gene	Primers	Amplicon Size (bp)	Reference
A. pleuropneumoniae	LPF	5′ - AAG GTT GAT ATG TCC GCA CC -3′	951	Gram and Ahrens (1998)
	LPR	5′ - CAC CGA TTA CGC CTT GCC A – 3′		
P. multocida	PM1231F	5′ - AGA AAG CAC ATG ACC AAA GGG – 3′	601	Liu et al. (2004)
	PM1231R	5′ - GCA GCT ACT CGC AGA AGG TT – 3′		
G. australis	GA F	5′ - AAG ATG ATG ATC GCC CAA TCG 3′	449	Yee et al. (2019)
	GAR1	5′ - CCA CGA GAA GCA AGA ACA TCT TTG ATC – 3′		

2.2. DNA extraction

Lung samples with lesions typically of *G. australis* were sent to our laboratory from an abattoir collection of two farms with 10 samples collected from one farm and 5 samples collected from the other (Table 3). The farms were located in Victoria and the samples sent to our laboratory on ice. At our laboratory, lung lesions were swabbed with one swab for culturing, and a dry swab and a FLOQ swab in 1 mL UTM media (Copan Diagnostics Inc., Brescia BS, Italy) to evaluate which swab was optimal for collecting samples for DNA processing directly from swab. Two methods of DNA extraction were validated for the PCR: the PrepMan Ultra Kit (Thermofisher, Warrington, UK) and the Qiagen DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) (Fig. 1).

For extraction via the PrepMan Ultra Kit, the dry swab was cut off and placed into a 1.5-mL tube containing 500 μL of PBS. Both the dry swab and the FLOQ swab were then processed. The tube was vortexed for 15 s and then left standing for 10 min. After vortexing for another 15 s the swab was removed. The sample was then centrifuged for 5 min at 18,320 x *g* and the supernatant removed. The pellet was then re-suspended into 200 μL PrepMan Ultra solution and vortexed. The sample was heated at 100 °C for 10 min, then kept on ice for 3 min and spun at 18,320 x*g* for 5 min. The sample was stored frozen at −20 °C.

For extraction via the DNeasy Blood & Tissue Kit (Qiagen), two duplicate sets of dry swabs and FLOQ swabs were collected. The tip of both types of swabs was cut off and placed into a 1.5-mL tube containing 500 μL of PBS or in a tube with 1 mL UTM media. The tube was vortexed for 15 s and then left standing for 10 min. After vortexing for another 15 s, the swab was removed. For the FLOQ swab in UTM media, only 200 μL were processed further, while all 500 μL were processed for the dry swab. The suspension was centrifuged for 5 min at 18,320 x *g* and the supernatant removed. Aliquots of 180 μL of Buffer ATL (DNeasy Blood & Tissue Kit) and 20 μL proteinase K were added to the tube. The tubes were thoroughly vortexed and incubated for 1.5 h in a 56 °C water bath with occasional vortexing. The sample was then processed according to the manufacturer's instructions. The sample was eluted from the DNeasy Mini spin column into 200 μL of elution buffer. The sample was also stored frozen at −20 °C.

The improved multiplex PCR was validated and optimized first, before the stored samples of the extraction methods were analyzed with the final improved multiplex PCR. This DNA extraction optimization experiment was done on the 15 samples from two farms, as large lung samples were sent from which several swabs could be taken to allow for the experiment to be done at the same time, under the same conditions and from the same samples. The optimized extraction method was determined by the number of positive results.

2.3. Culture

Nicotinamide adenine dinucleotide (NAD) dependent bacteria for the validation of the assay were revived and grown on BA/SN, which consisted of BBL™ Blood Agar Base (Becton Dickinson, Sparks, MD, USA), supplemented with 0.0025% of NADH, 0.0005% of thiamine HCl, 1% of heat-inactivated horse serum and 5% of oleic acid bovine albumin complex (consisting of 4.75% bovine serum albumin in normal saline (containing 0.06% oleic acid and 5% 0.05 N NaOH), and on 5% sheep blood agar plate (the latter with a nurse culture of *Staphylococcus hyicus*). NAD independent bacteria were grown on 5% sheep blood agar. The plates were incubated for 24 h at 37 °C in air.

2.4. Bacteria for specificity testing

For the validation and sensitivity testing of the Multiplex Ver 2 assay, a total of 36 isolates of *G. australis* were used. These isolates included the formal type strain, HS4635^T and the other 28 isolates used in the naming

Table 2
Detailed differences between PCR Ver 1 and PCR Ver 2.

PCR Version	Primers	Sample Type	Sampling	DNA extraction	Annealing Temperature	DNA Template Volume
Multiplex Ver 1	Listed in Table 1	Pure culture	Bacteria harvested from plate	Heating cooling method	65	1 uL
Multiplex Ver 2	Listed in Table 1	Pure culture	Bacteria harvested from plate	Heating cooling method	65	4 uL of 1/1000 diluted template
Multiplex Ver 2	Listed in Table 1	FLOQ swab from tissue	FLOQ swab	DNeasy Kit	65	4 uL

Table 3
Lung samples from abattoirs used for this study.[a]

Sample Type	Farms	Number of lungs	State	Culture	PCR on tissue	Research section
Lungs (Farm known to be positive for *G. australis*)	2	5 and 10	VIC	Yes	Yes	DNA extraction testing
Lungs	3	9, 13, 12	QLD	Yes	Yes	Culture/ PCR Comparison, Prevalence
Lungs lesions	51	1 to 3 per farm	VIC, NSW, SA	Yes	Yes	Culture/ PCR Comparison, Prevalence

[a] NSW = New South Wales, QLD = Queensland, SA = South Australia, VIC = Victoria.

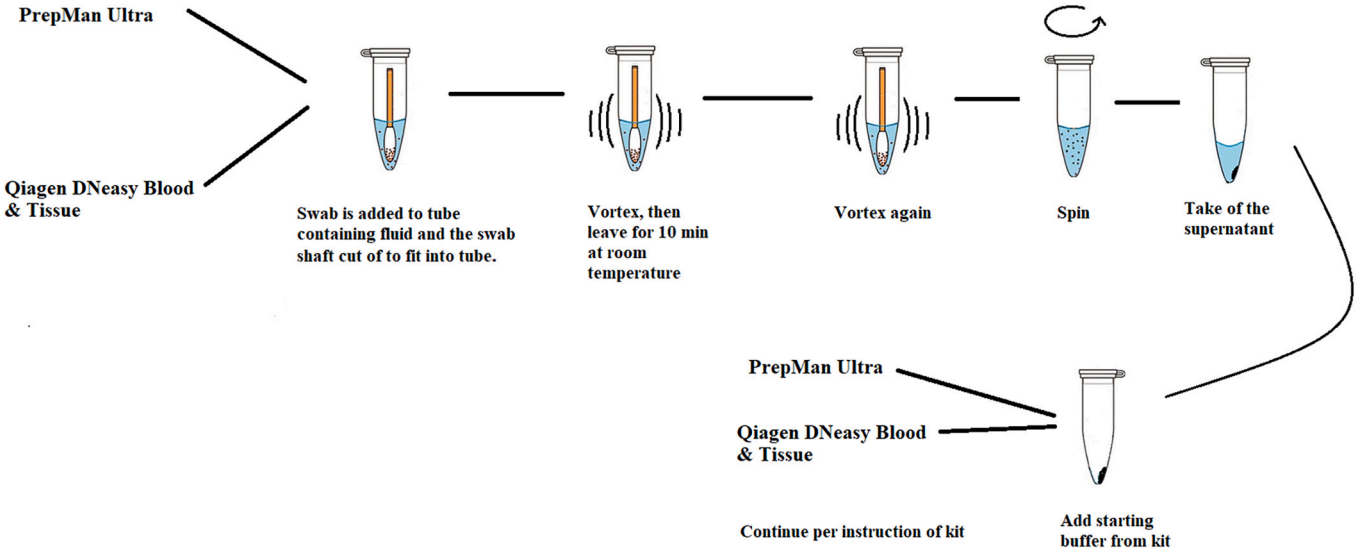

Fig. 1. Flow diagram of the two extraction methods used in this study.

of the species including three additional reference strains HS4420 (= LMG 30594), HS4509 (= LMG 30593) and HS4607 (= LMG 30592). A further seven isolates of *G. australis* identified by the phenotypic characteristics detailed by Turni et al. (2020) were used. The formal reference strains for serovars 1 to 15 and one field strain of *A. pleuropneumoniae* and the 16 Heddleston serovar reference strains and the formal type strain of *P. multocida* were used to validate specificity together with 44 isolates/strains that represented closely related species including 18 type strains, two reference strains and 24 field strains covering 12 genera, 25 species and two taxa of uncertain allocation (Supplementary Table 1).

2.5. Sensitivity testing

Glaesserella australis HS4635^T, *A. pleuropneumoniae* HS 7 and HS 143 and *P. multocida* PM 486 and PM 499 were harvested from an overnight culture into 6 mL PBS and the concentration of the suspensions adjusted to McFarland No 5 (approximately 1.5×10^9 CFU/mL) and a 10-fold dilution series was set up. The concentration of the dilutions was confirmed by viable counts performed by spreading 100 µL of dilutions in the range of approximately 10^2 to 10^4 CFU/mL on either BA/SN or sheep blood agar that were incubated for 24 h. From this viable count the concentration of the original suspension plus all dilutions were calculated. Dilutions in the ten-fold series in PBS were amplified by PCR after extraction of the DNA with a heating cooling method. A 200 µL aliquot of the suspension and dilutions was added to a 1.5-mL tube. The suspension was left on ice for 5 min and then held on a heating block at 98 °C for 5 min. The heating and cooling steps were repeated and the sample was then centrifuged for 2 min at 18,320 x *g*. The supernatant was stored frozen at −20 °C if not used for a PCR directly. To evaluate the sensitivity of the Multiplex Ver 2 assay for all three species, especially for *G. australis*, a low concentration (10^4 CFU/mL for *G. australis* and 10^5 CFU/mL for *P. multocida* and *A. pleuropneumoniae*) and a high concentration (10^7 CFU/mL for *G. australis* and 10^8 CFU/mL for *P. multocida* and *A. pleuropneumoniae*) of the target bacteria were mixed.

2.6. Abattoir sampling

A total of 166 lungs from 54 farms were sampled at slaughter. Lungs from pigs from four States were collected (23 farms in New South Wales, 26 in Victoria, one from South Australia, three from Queensland and one unknown). From most farms, 3 lungs were sampled, except for the three

Queensland samples where up to 13 lungs were sampled (Table 3).

The sampling was targeted to collect from lesions similar to *A. pleuropneumoniae* lesions – i.e. lesions located in the dorso-caudal lobe that were typically a haemorrhagic lump or a palpable mass indicating a regressing lesion (Gottschalk and Broes, 2019). Areas of lung with these lesions were collected and sent on wet ice to the laboratory. Samples were collected every second week. For each farm, up to three lung lesions (one lesion per lung) were collected. Once the lung samples arrived in the laboratory, the tissues were sampled with two swabs. One swab was used for culture while the second swab was used for the novel multiplex PCR assay. The surface of the lesion was heat seared and then a fresh cut made into the lesion. The two swabs were then sequentially inserted into the lesion area.

2.7. Culture and identification of bacteria from lung lesions

The swabs were plated on BA/SN and 5% sheep blood agar plate. All plates were incubated for 24 h at 37 °C. After incubation, apparent NAD dependent bacteria were selected from BA/SN agar plates with a focus on detecting *A. pleuropneumoniae* and *G. australis*. In addition, *P. multocida* and other dominant bacteria were selected from the 5% sheep blood agar and/or BA/SN agar plates. Following subculture, DNA was extracted from bacteria by the heating cooling method described above.

The suspect *P. multocida* and NAD dependent species were identified with the Multiplex Ver 1 PCR. Isolates identified as *A. pleuropneumoniae* by the Multiplex Ver 1 PCR were then examined in a multiplex *A. pleuropneumoniae* PCR that confirmed the species identification and then determined if the isolate belongs to serovars 1, 5, 7, 12 and 15 (Turni et al., 2014). Isolates identified as *P. multocida* by the Multiplex Ver 1 PCR were re-confirmed at species level by a species-specific PCR (Townsend et al., 1998) and then the lipopolysaccharide (LPS) genotype established by PCR (Harper et al., 2015).

The BA/SN plates, were scored according to the following scoring system:

0 =no growth

1 = colonies growing only in directly swabbed area.

2 = colonies growing directly in swabbed area and in first streak only

3 = colonies growing in directly swabbed area and in first and second streaks.

4 = colonies growing in directly swabbed area and in first, second and third streaks.

2.8. Identification from lung swabs

All the DNA extracts from lung swabs were tested with the Multiplex Ver 2 PCR.

2.9. Data management and statistical analyses

A Chi-Square test was used to evaluate the comparison of methods of DNA extraction with culture results. A Generalised Linear Mixed Model with a binomial distribution and logit link with method, species and their interaction as fixed effects and farm as a random effect was used for the comparison of culture and PCR method for the 166 field samples from the 54 farms. Farm prevalence was calculated as the number of farms with at least one positive culture or/and PCR from a lung divided by the total number of farms sampled (54 farms).

A Kappa coefficient was calculated to test for the level of agreement and % data that are reliable using the interpretation by McHugh (2012).

3. Results

3.1. Optimized conditions and annealing temperature for the Multiplex Ver 2 PCR

The optimal primer concentration for the Multiplex Ver 2 PCR was 0.6 μM of each primer with 0.2 mM dNTPs and 2 U Taq. The optimal annealing temperature was 65 °C. The amount of DNA template was 4 μL of DNA from tissue samples per 25 μL reaction and 4 μL of pure culture extracted DNA that was diluted 1/1000.

3.2. Specificity testing of the Multiplex Ver 2 PCR

The species specificity of the Multiplex Ver 2 PCR assay was evaluated with reference and field strains of both target and non-target species. All the reference strains and all the field isolates of the three target species yielded bands of the expected size, while none of the 44 non-target taxa yielded a species-specific band in the Multiplex Ver 2 PCR.

3.3. Sensitivity testing

The analytical sensitivity of the Multiplex Ver 2 PCR was tested by amplification of serial dilutions of bacteria. The lowest detectable concentration for the Multiplex Ver 2 PCR assay was 3.97×10^4 CFU/mL (40 CFU/reaction) for *G. australis* and an average of 7.86×10^5 CFU/mL (786 CFU/reaction) for *A. pleuropneumoniae* and an average of 2.38×10^5 CFU (238 CFU/reaction) for *P. multocida* (Table 4). The limit of detection was consistent among different serovars/LPS types of *A. pleuropneumoniae* and *P. multocida* (Table 4).

To be able to evaluate the loss of sensitivity as a result of mixed high/low target bacterial DNA content, the Multiplex Ver 2 PCR was performed with different combinations of the lowest and highest DNA concentrations (Fig. 2). Interestingly, the sensitivity of the multiplex PCR was affected when 10^7 CFU/mL *G. australis* was mixed with 10^5 CFU/mL of either *A. pleuropneumoniae* or *P. multocida*. In this situation, only *G. australis* was amplified (Fig. 2). The other combinations of species did not have a negative impact. However, the band of the bacterium with the lowest sensitivity (*P. multocida* = average of 2.38×10^5 CFU/mL) was a weak band when *A. pleuropneumoniae* was mixed at 10^8 CFU/ml with *P. multocida* at 10^5 CFU/mL.

3.4. Validation of DNA extraction method for lung swabs

We tested two different swab types and two commercial extraction kits to compare which of the extraction of DNA methods from tissue

Table 4
The lowest detectable concentration for the Multiplex Ver 2 PCR assay.

Strain	Serovar/LPS type	Lowest concentration detectable	
		CFU/mL	CFU/reaction
G. australis			
HS 4635		3.97×10^4	40
Actinobacillus pleuropneumoniae			
HS 7	serovar 5	6.46×10^5	646
HS 143	serovar 15	9.25×10^5	925
Pasteurella multocida			
PM 486	L3	2.61×10^5	261
PM 499	L4	2.14×10^5	214

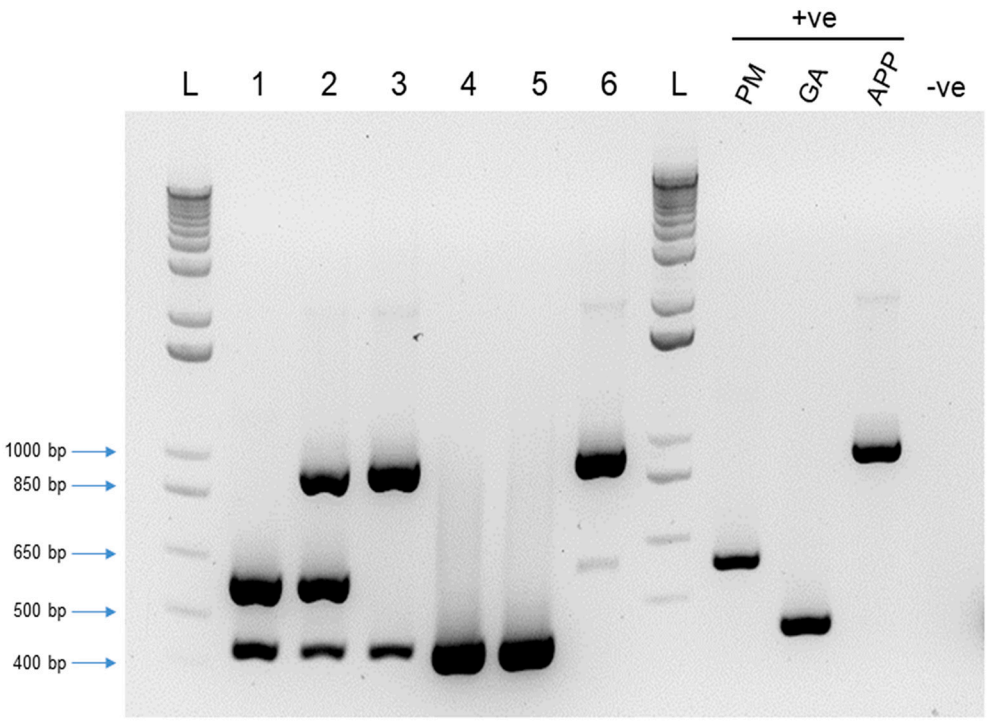

Fig. 2. Sensitivity loss due to mixed cultures. PBS was spiked with different concentration of bacterial species. The units are displayed as CFU/mL. L stands for 1 kb ladder (Invitrogen). PM for *Pasteurella multocida* (PM 499), GA for *Glaesserella multocida* (HS 4635) and APP for *Actinobacillus pleuropneumoniae* (HS 143). Lane 1 has a mixture of PM 10^8 CFU/mL and GA 10^5 CFU/mL. Lane 2 has a mixture of PM 10^8 CFU/mL, GA 10^5 CFU/mL and APP 10^8 cfu/mL. Lane 3 has a mixture of GA 10^5 CFU/mL and APP 10^8 CFU/mL. Lane 4 has a mixture of PM 10^5 CFU/mL and GA 10^7 CFU/mL. Lane 5 has a mixture of GA 10^7 CFU/mL and APP 10^5 CFU/mL. Lane 6 has a mixture of PM 10^5 CFU/mL and APP 10^8 CFU/mL.

samples were optimal (Table 5). This was done on lung samples from two farms sent to our laboratory and sampled with two commercial extraction kits – PrepMan Ultra and Qiagen DNeasy Blood & Tissue Kit – and using a dry swab and a FLOQ swab in 1 mL UTM medium to sample the lung. Every lung that was positive by culture for either *G. australis* or *P. multocida* was also positive by all four molecular assays (two swabs and two extraction methods). There were a number of swabs (a maximum of 6 with the use of normal swabs and the DNeasy Kit) which were positive in one or more the molecular assays while being negative by culture. However, Chi-square analysis demonstrated that no combination of swab or DNA extraction method resulted in a significantly greater number of positive results as compared with the gold standard of culture ($P = 0.596$). As the DNeasy kit with FLOQ swab had slightly higher number of positives this method was used in all subsequent extractions of DNA from lung swabs.

Table 5
Results of the validation of DNA extraction methods and sample swab type. Two extraction methods (PrepMan Ultra and Qiagen DNeasy Blood & Tissue Kit) with two kinds of swabs (normal cotton swab without media – termed dry normal and FLOQ swabs in 1 ml of UTM) were evaluated.[a]

Lungs (No.)	Culture Result	Normal Swab extracted with		FLOQ Swab extracted with	
		PrepMan Ultra Kit	DNeasy Kit	PrepMan Ultra Kit	DNeasy Kit
Glaesserella australis					
11	+	+	+	+	+
2	–	+	+	+	+
1	–	–	+	–	+
1	–	–	–	–	+
Pasteurella multocida					
11	–	–	–	–	–
1	+	+	+	+	+
2	–	–	+	–	–
1	–	–	+	–	+
TOTAL[b]	12	14	18	14	16

[a] No *Actinobacillus pleuropneumoniae* was detected and therefore only results for *Glaesserella australis* and *P. multocida* are displayed. + is a positive result in the PCR or in culture and – is a negative result in the PCR or in culture.

[b] Total = total number of positive results for the relevant methodology (combining both *G. australis* and *P. multocida*). Chi-square analysis showed that none of the various PCR methods (dry swab, FLOQ swab, PrepMan kit or DNeasy Kit) gave significantly more positives than culture ($P < 0.5$).

3.5. Results of abattoir sampling

A total of 166 lungs from 54 farms were sampled with 23 farms from New South Wales (NSW), 26 farms from Victoria (VIC), 3 from Queensland (QLD), one from South Australia (SA) and one that could not be assigned to a state. From most farms, 3 lung samples were collected. Most of the BA/SN plates had a score of 1 for their plate count, which means that the bacteria were only in the base streak and not in large number. When looking at the plates with higher score (3 and 4) most of the high scores were due to the growth of *P. multocida* and *A. pleuropneumoniae*. Some of the plates had environmental bacteria on them.

3.6. Comparison of Multiplex Ver 2 PCR and culture method

The results of the culture and Multiplex Ver 2 PCR analysis of the lungs are illustrated in Fig. 3. The analysis of 166 lesions sampled via traditional culture method resulted in 77 positive cultures, consisting of 39 *A. pleuropneumoniae* isolates, 24 *P. multocida* isolates, and 14 *G. australis* isolates. The Multiplex Ver 2 PCR identified 145 positive samples, consisting of 55 *A. pleuropneumoniae* positive samples, 72 *P. multocida* positive samples and 18 *G. australis* positive samples. When comparing the results from the Multiplex Ver 2 PCR with results from culture across the three species, significantly more positive samples were identified ($P > 0.01$) with the multiplex PCR method, with the difference being statistically significant for *P. multocida* (48 more) and *A. pleuropneumoniae* (16 more) (Fig. 3). However, as there could be false negatives in what we assumed our gold standard, the culture method, the Kappa coefficient was calculated to check for the level of agreement. The calculated Kappa coefficients were 0.79 for *G. australis,* 0.71 for *A. pleuropneumoniae* 0.23 for *P. multocida*. When results of all three bacteria were combined a Kappa value of 0.53 was observed. The overall

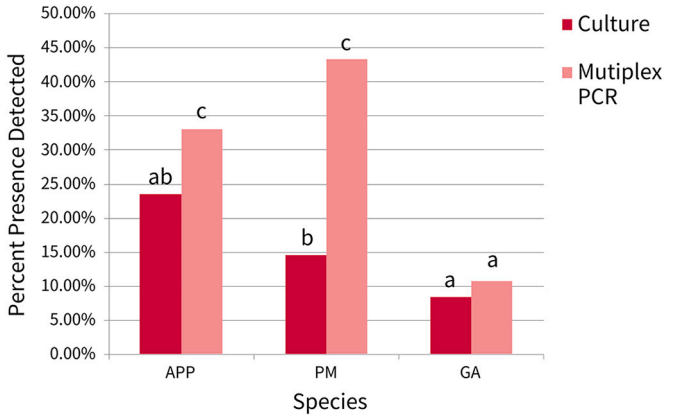

Fig. 3. Comparison of multiplex PCR analysis and culture method for three pathogens *Actinobacillus pleuropneumoniae, Pasteurella multocida* and *G. australis*. Different letters indicating significant differences ($P < 0.05$).

result of 0.53 would suggest that there is a weak level of agreement. The *P. multocida* level of agreement is minimal. For *A. pleuropneumoniae* and *G. australis* there was a moderate level of agreement (McHugh, 2012).

In the Multiplex Ver 2 PCR, some samples gave weak reactions. Most of the lung samples giving these weak reactions for *P. multocida, A. pleuropneumoniae* or *G. australis* failed to yield the relevant organism by the culture method, which indicated the species were not present in huge numbers when we sampled from the lung lesions.

When the performance of the Multiplex Ver 2 PCR was compared with culture at the farm level, the PCR detected significantly more farms (28) as positive to *P. multocida* than culture (15) ($P < 0.05$). However, there was no significant difference between culture and the Multiplex Ver 2 PCR for the other two pathogens (*A. pleuropneumoniae* and *G. australis*) at the farm level (Table 6).

3.7. Prevalence

The data of prevalence study were based on 166 lungs from 54 farms from abattoir (23 farms from NSW, 26 from VIC, three from QLD, one from SA and one unknown). In order to investigate the serovar/LPS type prevalence of each species, the results of prevalence were only determined with the culture method and serotyping multiplex PCR.

The frequency distribution of infectious agents and serovars/LPS types are listed in Tables 6 and 7. Among 54 tested farms, *P. multocida* was present on 28 farms (51.9%), *A. pleuropneumoniae* was present on 28 farms (51.9%) (with PCR and culture method) (Table 6). However, *G. australis* was not commonly found, it was only isolated on 2 farms (3.7%) and detected by PCR on 3 farms (5.6%). In regard to serovar/LPS types of each species, *P. multocida* L6 and *A. pleuropneumoniae* serovar 15 were the most prevalent being present on 12.96% and 37.04%, respectively, of the sampled farms (Table 7). There were farms with multiple pathogens detected (Table 8). There were also farms where only one target species was found with pure growth, in large numbers on the plates; 17 farms yielded pure *A. pleuropneumoniae* (21 samples), 5 farms pure *P. multicides* (7 samples) and 2 farms pure *G. australis* (9 samples).

Table 6
Results of culture method and Multiplex Ver 2 PCR analysis for three pathogens *Actinobacillus pleuropneumoniae, Pasteurella multocida* and *G. australis* at the farm level for the 54 farms examined in this study.

Category of Result	Number of farms with the indicated result for:		
	A. pleuropneumoniae	*P. multocida*	*Glaesserella australis*
Culture -; PCR +	5	15	1
Culture +; PCR -	0	2	0
Culture +; PCR +	23	13	2
Culture -; PCR -	26	24	51
Total farms positive by PCR	28	28*	3
Total positive farms by culture	23	15*	2

* Significant difference at farm level for *P. multocida.*

Table 7
The frequency distribution of infectious agents and serovar from 54 farms. Bacteria were isolated via traditional culture method and analyzed via serotyping/ *G. australis* multiplex PCR.

Prevalence of serovars on 54 farms		
	Serovar	No. +ve farms (%)
Pasteurella multocida	L1	3 (5.56%)
	L3	4 (7.40%)
	L4	3 (5.56%)
	L6	7 (12.96%)
Actinobacillus pleuropneumoniae	7	6 (11.11%)
	15	20 (37.04%)
G. australis		2 (3.70%)

4. Discussion

The lack of sensitivity of the original multiplex assay and therefore an inability to function effectively when used directly on tissues forced the development of this altered PCR. The change in polymerase and the optimization of the amount of DNA added together with an optimal concentration of $MgCl_2$ and dNTPs for the polymerase contributed to the enhanced sensitivity. Indeed, samples from pure culture that were extracted by the heating and boiling methods had to be diluted 1/1000. Optimization of the extraction method specially for swabs from tissue further enhanced the sensitivity for tissue samples. DNA extracted by the Qiagen DNease Blood & Tissue kit has been shown to yield higher concentration of DNA than the combination of boiling and enzymatic lysis (Tell et al., 2003). The addition of FLOQ swabs instead of cotton swabs provided the extra absorbance and according to the manufacturer (COPAN Diagnostics Inc., Brescia BS, Italy) 90% elution of the sample, which would have added even further to the sensitivity.

The Multiplex Ver 2 PCR was specific for the species it was designed to detect and did not give false positives with any of the related species. The detection limit of the Multiplex Ver 2 PCR was an average of 786 CFU per reaction for *A. pleuropneumoniae* and 238 CFU per reaction for *P. multocida*. This value is higher than those reported for *A. pleuropneumoniae* conventional PCR which has been reported as being able to detect 100 CFU per reaction (Gram and Ahrens, 1998) and a multiplex PCR that was reported as being able to detect 100 CFU per reaction of *A. pleuropneumoniae* and 10 CFU per reaction of *P. multocida* (Hricinova et al., 2010). Definitive evidence of the comparative sensitivity of these published assays (Gram and Ahrens, 1998; Hricinova

Table 8
The frequency distribution of farms with multiple pathogens or serovars detected from 54 farms. Bacteria were isolated via traditional culture method and identified and serotyped (latter for *Actinobacillus pleuruopneumoniae* and *Pasteurella multocida* only) by PCR.

Pathogen combinations	No. +ve farms (%)
Actinobacillus pleuropneumoniae serovar 15 + *P. multocida* PCR-LPS type 3	1 (1.85%)
A. pleuropneumoniae serovar 15 + *P. multocida* PCR-LPS type 4	2 (3.70%)
A. pleuropneumoniae serovar 15 + *P. multocida* PCR-LPS type 6	1 (1.85%)
A. pleuropneumoniae serovar 15 + App 7	3 (5.56%)
A. pleuropneumoniae serovar 7 + *P. multocida* PCR-LPS type 3 + *P. multocida* PCR-LPS type 6	1 (1.85%)
P. multocida PCR-LPS type 1+ *P. multocida* PCR-LPS type 6	1 (1.85%)
G. australis + *P. multocida* PCR-LPS type 6	1 (1.85%)

et al., 2010) would require a study directly comparing the assays as different methodologies such as DNA extraction may explain the apparent differences in the performance of the assays (Freschi et al., 2005). The detection limit of the Multiplex Ver 2 PCR was 40 CFU per reaction for *G. australis*. Since the *G. australis* was a new species, there was no reference to compare the sensitivity. However, the limit of detection for *G. australis* was about 10-fold greater than that for *A. pleuropneumoniae* and *P. multocida* in the Multiplex Ver 2 PCR. However, when a high concentration of *G. australis* (10^7 CFU/ml) was mixed with the lowest detectable concentration of *A. pleuropneumoniae* or *P. multocida* (10^5 CFU/ml), only *G. australis* could be amplified. This may be due to the differences in the sensitivity of the multiplex PCR between *G. australis* and the other two species. This problem with multiplex PCR having different efficiency on different targets has been previously described and suggested to be due to the more complex nature of multiplex assays (Hanapi et al., 2015). An optimization for one target might not result in an optimal assay for another target. Therefore, multiplex assays can be a compromise. In the current study, our focus was to optimize the assay for *G. australis*, as *P. multocida* and *A. pleuropneumoniae* are found easily in tissue, as both of these pathogens are normally present in large numbers if lesions are found at the abattoir (Turni et al., 2021).

As noted above, the pure culture work indicated that the Multiplex Ver 2 PCR was not as sensitive as some existing assays (none of which can detect *G. australis*). However, the assay gave significantly more positive results than conventional culture for the detection of both *A. pleuropneumoniae* and *P. multocida* at the sample level and significantly more than culture for the detection of *P. multocida* at the farm level. There are a number of explanations for the difference in the PCR and culture methods. The pathogenic bacteria may have lost viability during the transportation to the central laboratory, a problem which would not have impacted the PCR. It is also possible that the two swabs, one for culture and one for PCR, may have been slightly different, despite all efforts to sample the same region of the lesion. Another possibility is that the pathogenic bacteria present and detected by PCR may have been overgrown by contaminants.

In a prior study on a member of the family *Pasteurellaceae*, *Avibacterium paragallinarum,* our laboratory reported a 100% agreement between PCR and culture when swabs were collected from birds and processed within a few hours (Chen et al., 1996). However, when the culture and the PCR for *Av. paragallinarum* were compared in a central laboratory in China using clinical samples (chicken heads) transported to the laboratory, the PCR outperformed traditional culture (Chen et al., 1998). It is likely that for most veterinary laboratories, a molecular assay is a better option than traditional culture, particularly for samples transported to the laboratory from the field and particularly for fragile bacteria, such as members of the family *Pasteurellaceae.*

The Kappa analysis suggest that differential survival may not explain the difference in the results of culture and the multiplex assay. The Kappa analysis indicated that there was a moderate agreement between the culture and PCR for the two bacteria that are most affected by suboptimal conditions in regards to survival, *A. pleuropneumoniae* and *G. australis*. In contrast, the Kappa analysis showed only a minimal agreement between the assays for *P. multocida*. This minimal agreement occurs despite the fact that *P. multocida* is a robust bacterium with retrieval of live bacteria even possible from relatively old carcases (CABI, 2019) and therefore, independence observed in the Kappa statistics of these two tests for *P. multocida* suggests that the PCR might be indeed more sensitive than culture for *P. multocida*. Hence, it seems unlikely that a loss of viability explains the minimal agreement between culture and PCR for *P. multocida.* It should be noted that the number of tissues positive (in either assay) for *A. pleuropneumoniae* and *G. australis* was much lower than the positive numbers for *P. multocida,* a factor that may have influenced the outcome of the Kappa analysis.

This study did not involve random sampling but used targeted sampling to enhance the chances of finding *G. australis* if present. Lesions similar to *A. pleuropneumoniae* lesions were targeted and all sampling, except for the sampling in Queensland, was done by a single person who was experienced in the lesions associated with *G. australis* infection. The prevalence rate of the current study for *G. australis* was 5.56%, which was lower than expected, as in the initial study that described this organism there were 17 *G. australis* isolates from 14 farms (Turni et al., 2017). Only 2 farms in the current yielded *G. australis* by culture while PCR analysis detected *G. australis* from one more farm, findings which would suggest that the new species may not be very prevalent.

The results of the prevalence study revealed that the infection with *A. pleuropneumoniae* serovars 15 and 7 had a prevalence of 37.03% and 11.11%, respectively. Both serovars are moderately pathogenic (Frey, 1995; Tumamao et al., 2004) and might cause a sub-clinal disease after the outbreak on farms and hence it is not surprising that these serovars were found in healthy pigs at slaughter as has been reported previously (Gottschalk, 2015). While *A. pleuropneumoniae* serovar 15 has been the predominant serovar detected in Australian pigs (Blackall et al., 2002; Turni et al., 2014), serovar 7 has been also a commonly encountered serovar for many years (Blackall and Pahoff, 1995; Blackall et al., 1999; Turni et al., 2013).

The most prevalent of the three target bacterial pathogens was *P. multocida,* being detected in 55.6% of the samples by a combination of Multiplex Ver 2 PCR and culture. *P. multocida* was isolated from 31% of pneumonic lungs in the 21 reports published from 1922 to 1990 (Straw et al., 1996). In the samples from an Australian porcine pleurisy study, 52% of *P. multocida* positive farms also yielded other primary pathogen like *A. pleuropneumoniae*, *Mycoplasma hyopneumoniae* and Porcine Circovirus 2 (Turni et al., 2021). In the current study, among the three pathogens looked for, *P. multocida* was also found in combination with other pathogens; 24% *P. multocida* positive farms were co-infected with *A. pleuropneumoniae* or *G. australis* detected by a combination of Multiplex Ver 2 PCR and culture. The presence of *P. multocida* in lungs examined at the abattoirs also increases the probability of other pathogens being present and is associated with a high frequency of recovering lesions (Fablet et al., 2012). Therefore, finding pure *P. multocida* isolates in large numbers in the current study is not surprising. On 5 farms, only *P. multocida* (7 samples) was yielded with high plate score, which indicted that these strains may be the main contributor of these lung lesions.

5. Conclusion

The results suggested that the modified multiplex PCR was specific, sensitive and could be used in place of traditional culture when examining lung swabs. The prevalence of *G. australis* was not high, but it was the dominant pathogen in the two infected pig herds detected by culture.

Funding source

This work was supported by the Australian Pork Limited [project number 2018/0028].

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2021.106360.

Declaration of Competing Interest

None.

Acknowledgement

We would like to thank Edina Lobo and Nusrat Nahar for helping with the development of the PCR.

References

Blackall, P.J., Pahoff, J., 1995. Characterisation of porcine haemophili isolated from Australian pigs, 1988-1992. Aust. Vet. J. 72, 18–21.

Blackall, P., Bowles, R., Pahoff, J., Smith, B., 1999. Serological characterisation of *Actinobacillus pleuropneumoniae* isolated from pigs in 1993 to 1996. Aust. Vet. J. 77, 39–43.

Blackall, P.J., Klaasen, H.L., van den Bosch, H., Kuhnert, P., Frey, J., 2002. Proposal of a new serovar of *Actinobacillus pleuropneumoniae*: serovar 15. Vet. Microbiol. 84, 47–52.

CABI, 2019. *Pasteurella multocida* infections. In: In: Invasive Species Compendium. CAB International, Wallingford, UK. www.cabi.org/isc/datasheet/70916.

Chen, X., Miflin, J.K., Zhang, P., Blackall, P.J., 1996. Development and application of DNA probes and PCR tests for *Haemophilus paragallinarum*. Avian Dis. 40, 398–407.

Chen, X., Chen, Q., Zhang, P., Feng, W., Blackall, P.J., 1998. Evaluation of a PCR test for the detection of *Haemophilus paragallinarum* in China. Avian Path 27, 296–300.

Fablet, C., Marois, C., Dorenlor, V., Eono, F., Eveno, E., Jolly, J.P., Le Devendec, L., Kobisch, M., Madec, F., Rose, N., 2012. Bacterial pathogens associated with lung lesions in slaughter pigs from 125 herds. Res. Vet. Sci. 93, 627–630.

Freschi, C.R., Carvalho, L.F.O.S., Oliveira, C.J.B., 2005. Comparison of DNA-extraction methods and selective enrichment broths on the detection of *Salmonella typhimurium* in swine feces by polymerase chain reaction (PCR). Braz. J. Microbiol. 36, 363–367.

Frey, J., 1995. Virulence in *Actinobacillus pleuropneumoniae* and RTX toxins. Trends Microbiol. 3, 257–261.

Gottschalk, M., 2015. The challenge of detecting herds sub-clinically infected with *Actinobacillus pleuropneumoniae*. Vet. J. 206, 30–38.

Gottschalk, M., Broes, A., 2019. Actinobacillosis. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G., Zhang, J.Q. (Eds.), Diseases of Swine, 11th ed. John Wiley & Sons, Inc, Hoboken, NJ, USA, pp. 749–766.

Gram, T., Ahrens, P., 1998. Improved diagnostic PCR assay for *Actinobacillus pleuropneumoniae* based on the nucleotide sequence of an outer membrane lipoprotein. J. Clin. Microbiol. 36, 443–448.

Hanapi, U.K., Desa, M.N.M., Ismail, A., Mustafa, S., 2015. A higher sensitivity and efficiency of common primer multiplex PCR assay in identification of meat origin using NADH dehydrogenese subunit 4 gene. J. Feed. Sci. Technol. 52, 4166–4175.

Harper, M., John, M., Turni, C., Edmunds, M., St. Michael, F., Adler, B., Blackall, P.J., Cox, A.D., Boyce, J.D., 2015. Development of a rapid multiplex PCR assay to genotype *Pasteurella multocida* strains by use of the lipopolysaccharide outer core biosynthesis locus. J. Clin. Microbiol. 53, 477–485.

Hricinova, M., Holoda, E., Mudronova, D., Ondrasovicova, S., 2010. Multiplex PCR assay for detection of *Actinobacillus pleuropneumoniae, Pasteurella multocida* and *Haemophilus parasuis* in lungs of pigs from a slaughterhouse. Folia Microbiol. (Praha) 55, 635–640.

Liu, D., Lawrence, M.L., Austin, F.W., 2004. Specific PCR identification of *Pasteurella multocida* based on putative transcriptional regulator genes. J. Microbiol. Methods 58, 263–267.

McHugh, M.L., 2012. Interrater reliability: the kappa statistic. Biochem. Med. (Zagreb). 22, 276–282.

Register, K.B., Brockmeier, S.L., Zimmerman, J.K.L., Ramirez, A., Schwartz, K.J., Stevenson, G., Zhang, J.Q., 2019. Pasteurellosis. In: Diseases of Swine, 11th ed. John Wiley & Sons, Inc, Hoboken, NJ, USA, pp. 884–897.

Straw, B., Dewey, C., Erickson, E., 1996. Interpreting culture reports from swine lungs. Swine Hlth. Prod. 4, 200–201.

Tell, L.A., Foley, J., Needham, M.L., Walker, R.L., 2003. Comparison of four rapid DNA extraction techniques for conventional polymerase chain reaction testing of three *Mycobacterium* spp. that affect birds. Avian Dis. 47, 1486–1490.

Townsend, K.M., Frost, A.J., Lee, C.W., Papadimitriou, J.M., Dawkins, H.J., 1998. Development of PCR assays for species- and type-specific identification of *Pasteurella multocida* isolates. J. Clin. Microbiol. 36, 1096–1100.

Tumamao, J.Q., Bowles, R.E., van den Bosch, H., Klaasen, H.L., Fenwick, B.W., Storie, G. J., Blackall, P.J., 2004. Comparison of the efficacy of a subunit and a live streptomycin-dependent porcine pleuropneumonia vaccine. Aust. Vet. J. 82, 370–374.

Turni, C., Singh, R., Omaleki, L., Blackall, P.J., 2013. *Actinobacillus pleuropneumoniae*—a diagnostic update. In: Manipulating Pig Production, XIV, p. 215.

Turni, C., Singh, R., Schembri, M.A., Blackall, P.J., 2014. Evaluation of a multiplex PCR to identify and serotype *Actinobacillus pleuropneumoniae* serovars 1, 5, 7, 12 and 15. Lett. Appl. Microbiol. 59, 362–369.

Turni, C., Giang, N., Wu, Y., Omaleki, L., Christensen, H., Blackall, P.J., 2017. *Actinobacillus* taxon C - a new species not to be ignored. In: 2017 Australasian Pig Science Association: Manipulating Pig Production XVI, Melbourne, Victoria, 19–22 November 2017.

Turni, C., Wu, Y., Omaleki, L., Giang, N., Blackall, P.J., Christensen, H., 2020. *Glaesserella australis* sp. nov., isolated from the lungs of pigs. Int. J. Syst. Evol. Microbiol. 70, 3686–3692.

Turni, C., Meers, J., Parke, C., Singh, R., Yee, S., Templeton, J., Mone, K., McKenzie, P., Blackall, P.J., Barnes, T.S., 2021. Pathogens associated with pleuritic pig lungs at an abattoir in Queensland Australia. Aust. Vet. J. 99, 163–171.

Yee, S., Nahar, N., Lobo, E.T., Jenkin, S., Turni, C., 2019. A multiplex molecular assay for *Glaesserella australis*. Proceedings of the Seventeenth Biennial Conference of the Australasian Pig Science Association (APSA). (2019). Adv. Anim. Biosci. 10 (S1) https://doi.org/10.1017/S2040470019000050. S23,50.