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Molecular identification and characterisation of *Mannheimia haemolytica*

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ABSTRACT

Mannheimia haemolytica is known as one of the major bacterial contributors to Bovine Respiratory Disease (BRD) syndrome. This study sought to establish a novel species-specific PCR to aid in identification of this key pathogen. As well, an existing multiplex PCR was used to determine the prevalence of serovars 1, 2 or 6 in Australia. Most of the 65 studied isolates originated from cattle with a total of 11 isolates from small ruminants. All problematic field isolates in the identification or serotyping PCRs were subjected to whole genome sequencing and bioinformatic analysis. The field isolates were also subjected to rep-PCR fingerprinting. A total of 59 out of the 65 tested isolates were conformed as *M. haemolytica* by the new species-specific PCR which is based on the *rpoB* gene. The confirmed *M. haemolytica* field isolates were assigned to serovars 1 (24 isolates), 2 (seven isolates) and 6 (26 isolates) while two of the isolates were negative in the serotyping PCR. The two non-typeable isolates were assigned to serovar 7 and 14 following whole genome sequencing and bioinformatic analysis. The rep-PCR typing resulted in five major clusters with serovars 1 and 6 often within the same cluster. The *M. haemolytica*-specific PCR developed in this work was species specific and should be a valuable support for frontline diagnostic laboratories. The serotyping results support the relative importance of serovars 1 and 6 in bovine respiratory disease.

1. Introduction

Bovine respiratory disease (BRD) is one of the major problems currently being faced by the cattle feedlots in Australia (Alhamami et al., 2021) and all around the world (Constable et al., 2017). The quality of the meat obtained from such cattle is inferior thus resulting in economic losses for the cattle meat industry (Blakebrough-Hall et al., 2020). The disease is characterised by coughs, nasal discharge and dyspnea, depression, loss of appetite, high temperature ranging from 40° to 41°C and a variable response to treatment (Constable et al., 2017).

Different environmental factors can affect the prevalence of BRD in cattle. Physical stresses such as changes in the environmental conditions like ventilation and humidity, herd density, long durations of transportation from one farm to the other, as well as, contact with the cattle from different farms can affect the overall health of the cattle making them susceptible to various viral and secondary bacterial infections. The disease morbidity has been reported to range between 10% and 50% which would depend on the feedlot age, the degree of predisposing factors such as stressors as well as the animal's immune status (Constable et al., 2017). Major bacterial causative agents of BRD include *Mannheimia haemolytica*, *Pasteurella multocida*, *Mycoplasma bovis, Histophilus somni* and *Trueperella pyogenes*. *M. haemolytica* is a commensal in the bovine upper respiratory tract which means that simple presence is not an indication of disease. The organism is an opportunistic pathogen and only causes disease when the host is immune compromised (Rice et al., 2007). A 2007 American review stated that viral agents often predispose animals to bacterial infections, and *M. haemolytica* is the most frequently isolated organism in cattle with BRD (Duff and Galyean, 2007). In a literature review, Rice et al. (2007) indicated that *M. haemolytica* was a key player in both BRD in feedlot cattle and enzootic pneumonia in all types of neonatal calves.

In Australia, *M. haemolytica* has been known as a major bacterial agent in ovine and bovine pneumonia (Blackall et al., 2002) as well as sheep mastitis (Omaleki et al., 2012). However, recent studies demonstrated a lower prevalence of *M. haemolytica* in feedlot cattle compared to *P. multocida* possibly due to a wide usage of inactivated vaccines

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(Alhamami et al., 2021).

Serotyping is very important for selection of vaccine strains of *M. haemolytica*, as antibodies against killed whole cell vaccines mostly protect against homologous serovars (Gilmour et al., 1979). There are 12 *M. haemolytica* serovars, named as serovars 1, 2, 5–9, 12 – 14, 16 and 17 (Angen et al., 1999a; Angen et al., 1999b). The genetic region for the capsule antigens has been identified and fully sequenced in the *M. haemolytica* serovar type strains with an online serotyping scheme, using draft genome assemblies, now available (Christensen et al., 2021).

A multiplex PCR has been developed to differentiate *M. haemolytica*, *M. glucosida* and *M. ruminalis* (Alexander et al., 2008). However, this multiplex PCR could not always discriminate between these three species as the *M. haemolytica* primers targeted the leukotoxin gene (*lkt*) of *M. haemolytica,* a problem as isolates of *M. glucosida* and *M. ruminalis* are also capable of carrying the *lkt* gene (Omaleki et al., 2014). Other assays used the *sodA* gene in a multiplex PCR assay to identify *M. haemolytica* and differentiate it from other respiratory pathogens in cattle (Kishimoto et al., 2017) but the specificity of these assays has not been checked against other members of the genus *Mannheimia* that occupy the upper respiratory track of small ruminants. Hence, in this study we designed a *M. haemolytica* species-specific PCR assay that does not give false positives with the other *Mannheimia* species. As well, following confirmation of the identity of field isolates as *M. haemolytica,* we used an existing serotyping PCR (Klima et al., 2017) and whole genome sequencing followed by the on-line serotyping scheme of Christensen et al. (2021) to establish the serovars of any problematic isolates. Finally, we used repetitive element sequence-based PCR (rep-PCR) to look at the genetic variability of the *M. haemolytica* isolates within and across the host species.

2. Materials and methods

2.1. Bacterial isolates

A total of 65 isolates that were previously identified and stored in our culture collection as *M. haemolytica* were included in the current study. The isolates were mostly obtained from front-line diagnostic laboratories in the states of New South Wales (NSW) and Queensland (QLD) in Australia or New Zealand. The samples were collected from animals that were suffering from respiratory disease. Of these, 53 isolates originated from cattle, seven from sheep, four from goats and one from an unknown host species (Table 1). The isolates were cultured on 5% sheep blood

Table 1
List of field isolates, and their host species, used in the current study.[a].

Isolate	Host	Tissue	State	Year	Reaction in *M. haemolytica* PCR	Serovar	rep-PCR profile	rep-PCR cluster
PM176, PM177, PM186, PM187, PM189, PM192[b]	Cattle	NK	NSW	1994	Pos	1	27	5
BR241	Cattle	Lung	QLD	2002	Pos	2	18	NT
BR275, BR277, BR279; BR282-BR285[c]	Cattle	NK	NSW	2002	Pos	6	27	5
BR278[c]	Cattle	NK	NSW	2002	Pos	6	10	NT
BR280[c]	Cattle	NK	NSW	2002	Pos	6	9	2
BR296, BR297, BR300, BR302[c]	Cattle	NK	NSW	2003	Pos	6	9	2
BR298, BR299[c]	Cattle	NK	NSW	2003	Pos	1	9	2
BR303 – BR306[c]	Cattle	NK	NSW	2003	Pos	6	25	4
BR441	Cattle	Nasal cavity	QLD	2004	Pos	1	14	NT
PM1127	Cattle	Lung	QLD	2005	Pos	1	23	3
PM1135	Cattle	Lung	QLD	2005	Pos	6	24	NT
PM1147	Cattle	Lung	QLD	2005	Pos	1	8	NT
PM1156	Cattle	Lung	QLD	2005	Pos	1	24	NT
BR952	Cattle	Lung	QLD	2010	Pos	1	14	NT
BR953	Cattle	Lung	QLD	2010	Pos	2	21	NT
BR954	Cattle	Lung	QLD	2010	Pos	6	15	NT
BR1074	Cattle	Lung	QLD	2011	Pos	6	23	3
BR2764	Goat	NK	QLD	2015	Pos	2	3	NT
BR2765	Goat	NK	QLD	2016	Neg	NA	19	NT
BR2766	Sheep	NK	QLD	2017	Neg	NA	17	NT
BR2767	Sheep	NK	QLD	2017	Pos	2	16	NT
BR2768	Sheep	NK	QLD	2017	Neg	NA	22	NT
BR2769	Sheep	NK	QLD	2018	Pos	2	16	NT
BR2770	Sheep	NK	QLD	2018	Neg	NA	1	NT
BR2734	Goat	NK	VIC	2019	Pos	2	13	NT
BR2752, BR2753	Cattle	NK	New Zealand	2019	Pos	6	12	NT
BR2771	Goat	NK	QLD	2019	Neg	NA	2	NT
BR2772	Cattle	NK	QLD	2019	Pos	1	7	1
BR2773	Sheep	NK	QLD	2019	Pos	14	4	NT
BR2774	Cattle	NK	QLD	2019	Pos	6	7	1
BR2775 – BR2778[d]	Cattle	NK	QLD	2019	Pos	1	7	1
BR2779, BR2780	Cattle	NK	QLD	2019	Pos	6	5	NT
BR2781	Cattle	NK	QLD	2019	Pos	6	6	NT
BR2782	Cattle	NK	QLD	2019	Pos	7	11	NT
BR2783	Sheep	NK	QLD	2019	Neg	NA	26	NT
BR439	Cattle	Liver	QLD	2004	Pos	2	20	NT
BR440	Cattle	Lung	QLD	2004	Pos	1	14	NT
BR444	Cattle	NK	NK	2004	Pos	1	23	3
BR445	NK	NK	NK	2005	Pos	1	23	3
PM216	Cattle	NK	QLD	1994	Pos	1	28	NT
PM372	Cattle	Lung	QLD	1995	Pos	1	27	5
PM373	Cattle	Trachea	QLD	1995	Pos	1	9	2

[a] NA = Not applicable as isolate not *M. haemolytica;* NK = Not known; NSW = New South Wales, QLD = Queensland; VIC = Victoria.
[b] All these isolates were from the same NSW feedlot.
[c] All these isolates were from the same NSW feedlot.
[d] All these isolates were from the same QLD feedlot.
[e] Five main rep-PCR clusters were identified and numbered 1–5. NT = not assigned to one of these five main rep-PCR clusters.

agar and incubated aerobically overnight at 37 °C.

The species reference strains of the genus *Mannheimia* - *M. haemolytica* CCUG 408 ^{T}, *M. glucosida* CCUG 38457 ^{T}, *M. granulomatis* CCUG 45422 ^{T}, *M. ruminalis* CCUG 38470 ^{T} and *M. varigena* CCUG 38462 ^{T}together with an Australian field isolate of *M. pernigra* (BNO311) that has been confirmed as *M. pernigra* by Kuhnert et al. (2021) were used to test the within genus specificity of the PCR Supplementary Table 1. A further 40 strains/isolates that represented either close relatives or bacteria likely to be found in the bovine respiratory tract were also used (Supplementary Table 1).

2.2. PCR development

Genomic DNA from the *M. haemolytica* isolates was prepared by boiling a 1 µl loop of each isolate in 200 µl of DNA free H_2O for 10 min at 100 °C and residual debris was removed by centrifugation at 13,000 x g for 5 min. The supernatant was then used as template for the *M. haemolytica* species specific PCR developed in this study, as well as the multiplex serotyping PCR (Klima et al., 2017).

The novel species-specific PCR was designed based on the *rpoB* gene sequences for the *Mannheimia* species type strains as indicated above as well as *M. pernigra* CCUG 74657 ^{T} (Supplementary Table 1). The finalised primer pairs were: MropB F (5′ - AAC ACA TAA ACG CCG TAT CTC G- 3′) and MropB R (5′ -GAT ATT CGG GCC TTC AGG A- 3′), and amplify a 136 bp of the *rpoB* region.

The master mix for the PCR assay consisted of 1 x buffer containing 100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl at pH 8.3 (Roche, Mannheim, Germany), 0.2 mM of each deoxynucleotide triphosphate (dNTP), 0.5 U of *Taq* DNA polymerase (Roche, Mannheim, Germany), and UltraPure DNase/RNase-Free Distilled water (Invitrogen, Grand Island NY, USA) in a final reaction volume of 25 µl. For initial PCR optimization, 0.02 – 0.08 µM of the forward and reverse primers as well as 1–8 µl of DNA template was tested. The PCR cycles were then performed with 1 cycle at 95 °C for 2 min, 30 cycles at 95 °C for 30 s, 55–58 °C for 90 s, 72 °C for 60 s and a final cycle at 72 °C for 10 min on an Eppendorf thermocycler (Eppendorf AG 22331 Hamburg, Germany).

To test the sensitivity of the newly developed PCR assay, a dilution range of 10^7 to 10^{-1} was prepared using *M. haemolytica* strain CCUG 408 ^{T}. A McFarland No. 2 suspension (about 10^8 cfu/ml) was made and a 1 in 10 dilution series was created with the approximate endpoint of 10^{-1} cfu/ml. A 100 µl aliquot of the dilutions containing approximately 10^4, 10^3 and 10^2 cfu/ml was plated on sheep blood agar plates and incubated overnight at 37 °C. Colony counting was done using these plates to determine the number of colony-forming units in the highest dilution tube. Each tube of the dilutions series was used to extract DNA for sensitivity testing.

Matrix-assisted laser desorption-ionization time of flight analysis (MALDI-TOF) of eight isolates was performed on a Bruker MALDI Biotyper, microflex LT/SH MALDI-MS System with MBT Compass software version 4.1, MALDI Biotyper compass explorer software 4.1 and Flex Analysis software version 3.4.79.

2.3. Serotyping PCR

A previously published multiplex PCR was used to differentiate between serovars 1, 2 and 6 of *M. haemolytica* (Klima et al., 2017).

2.4. Genotyping analysis of *M. haemolytica* using rep-PCR-

For rep-PCR, colonies from the overnight cultures were suspended in 200 µl of Prepman Ultra solution (Applied Biosystems, Life Technologies, Australia) and vortexed for 15 s. The suspension was heated using a heating block at 100 °C for 10 min, kept on ice for 3 min and then centrifuged (13,000 x g, 5 min) to obtain DNA. The rep-PCR assay was performed as previously described (Versalovic et al., 1991), with Bionumerics version 6.6 (Applied Maths Inc, Saint-Martens-Latem, Belgium) being used for the analysis of the obtained patterns.

2.5. Whole genome sequencing

Bacterial pellets from selected isolates were sent to the Australian Centre for Ecogenomics Sequencing Service at the University of Queensland (St Lucia QLD 4072, Australia) for DNA extraction and whole genome sequencing on an Illumina NovaSeq6000 platform (150 bp paired ends). Trimmomatic v0.39 (Bolger et al., 2014) was used for filtering the raw reads with the quality of the reads assessed before and after filtering using FastQC v0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Kraken2 was used to assess the taxonomic labels of trimmed reads (Wood et al., 2019). Shovill v1.1.0 with implemented SPAdes v3.15.3 (Bankevich et al., 2012) was used for the *de novo* assemblies with minimum length and minimum coverage set at 250 bp and 12 times, respectively. CheckM v1.0.13 (Parks et al., 2015) was used to check the completeness and contamination of *de novo* assemblies. BLASTn v2.2.31 + was used to extract the sequences for *recN* and *rpoB* gene from the draft assembles using the *recN* and *rpoB* sequences from *M. haemolytica* CCUG 408 ^{T} as query sequences. The sequence of *recN* was compared with those from the type strains of the recognised *Mannheimia* species with their genome relatedness calculated using the formula suggested by Zeigler (2003). The *rpoB* sequences were then compared with the *rpoB* gene sequences from *M. haemolytica* and *M. glucosida* type strains as well as the sequences previously published for strains associated with sheep mastitis (Omaleki et al., 2012). The draft genome assemblies were also tested against the newly developed *M. haemolytica* serotyping browser https://ivsmlst.sund.ku.dk/ (Christensen et al., 2021).

3. Results

3.1. PCR development

The optimized PCR reaction contained 0.02 µM of the forward and reverse primers, 1 µl of DNA template and was run at an annealing temperature of 58 °C for 90 s. The optimized PCR was specific for *M. haemolytica* when tested against the other type strains of species within the genus *Mannheimia* (*M. varigena* CCUG 38462 ^{T}, *M. granulomatis* CCUG 45422 ^{T}, *M. ruminalis* CCUG 38470 ^{T} and *M. glucosida* CCUG 38457 ^{T}) and the Australian reference field strain of *M. pernigra* BNO311. As well, the assay did not produce an amplicon when tested against other members of the *Pasteurellaceae* family causing respiratory disease in livestock nor a range of different Gram-positive and Gram-negative bacteria. The dilutions of the positive control for *M. haemolytica* strain CCUG 408 ^{T} showed amplifications for templates that contained approximately 7900 cfu/ml, which when using 1 µl per reaction translates to a sensitivity of 8 cfu per reaction (Supplementary Figure 1).

3.2. Identification and serotyping

A total of 59 of the 65 field isolates were identified as *M. haemolytica* when tested by our newly developed species-specific PCR with the remaining six not producing any amplicon. Four of the six negative isolates were from sheep and two from goats (Table 1).

A total of 24 and 26 bovine isolates were identified as serovars 1 and 6, respectively, while only three isolates were serovar 2 (Table 2). Except for one ovine isolate, all the ovine and caprine *M. haemolytica* isolates were identified as serovar 2 (two caprine and two ovine).

Two of the *M. haemolytica* isolates (one bovine and one ovine) together with those six isolates that did not produce any amplicon in our newly developed PCR, could not be assigned to any serovars via the multiplex PCR used.

The two non-typable *M. haemolytica* isolates together with the six isolates that could not be identified by our *M. haemolytica* species-

Table 2
Serotyping results for the *M. haemolytica* isolates[a].

	Serovar 1	Serovar 2	Serovar 6	Other serovars	Total *M. haemolytica* isolates
Bovine isolates[†]	24 (45.3%)	3 (5.6%)	26 (49.1%)	1	53
Ovine isolates[†]	0	2	0	1	3
Caprine isolates[†]	0	2	0	0	2
Unknown source	1	0	0	0	1

[a] The results are only for the 59 isolates identified as *M. haemolytica* by our newly developed PCR. Six of the previously identified *M. haemolytica* field isolates failed to produce any amplicon in the new *M. haemolytica* specific PCR.

specific PCR were subjected to MALDI-TOF analysis to confirm their identification (BR numbers 2765, 2766, 2768, 2770, 2771, 2773, 2782, 2783). The two isolates that produced positive results in the new PCR, BR2773 and BR2782, had a score of 2.16–2.36 and 2.09 – 2.53 to *M. haemolytica*, respectively. The caprine isolate BR2765 had the highest score value to *M. ruminalis* (score value 2.19) while having an score value between 1.92 and 2.12 to *M. haemolytica*.

While the remaining five isolates all had the first best match to *M. haemolytica,* the scores were all at the lower limits of the high-confidence identification (between 2.08 and 2.3).

3.3. Whole genome sequencing

The same eight isolates examined by MALDI-TOF were subjected to WGS for further analysis. The draft genome assemblies of these isolates resulted in 27–67 contigs with genomes size between 2.22 and 2.49 Mbp, a GC content of 40.9–41.46% and a genome completeness of more than 99% (Supplementary Table 2). Comparing the extracted *recN* and *rpoB* sequences from the WGS data of the two *M. haemolytica* PCR positive but non-serotypable isolates with the sequences from *M. haemolytica* strain CCUG 408 ^{T}, confirmed the ovine isolate BR2773 and the bovine isolate BR2782 as *M. haemolytica* based on the 100% similarity (Table 3).

Prediction of the serovar from the whole genomic data of the two non-serotypable *M. haemolytica* isolates identified the ovine isolate BR2773 as serovar 14 and the bovine isolate BR2782 as serovar 7. However, BR2782 appeared to carry 2 single nucleotide changes (T669G and C1144T) compared to that of serovar 7 sequence from *M. haemolytica* strain HPA136 in the data-base.

Genome similarity indices (SI_{genome}) between these six *M. haemolytica*-specific PCR negative isolates with those of the recognised *Mannheimia* species type strains were calculated based on the similarity matrix between their *recN* genes (Supplementary Table 2). The results suggested that the caprine isolate BR2765 was more distantly related to the other five isolates, sharing between 96% and 80% similarity in the *rpoB* and *recN* gene to those from *M. haemolytica* CCUG 408 ^{T} strain, respectively. This isolate also shared only 80% similarity with the *recN* from *M. glucosida* CCUG 38457 ^{T} (SI_{genome} of 0.51). The highest similarity was shared with *recN* of *M. ruminalis* CCUG 38470 ^{T} (89.56 and SI_{genome} of 0.72). Analysis of the trimmed Illumina reads from isolate BR2765 also indicated that 39% of the reads could not be classified while 51% were classified as *Mannheimia* species using kraken2 (Supplementary Table 3).

The remaining five isolates had a SI_{genome} of 0.91–0.95 with that from *M. haemolytica* and *M. glucosida,* type strains respectively while sharing a SI_{genome} of 0.41–0.72% to the rest of the named species within the genus *Mannheimia*.

The summary of the analysis of the *rpoB* sequences of the six isolates negative in the *M. haemolytica*-specific PCR is presented in Supplementary Table 2. The *rpoB* gene from the ovine isolate BR2766 was 100% identical to *rpoB* from isolate A5 previously obtained from sheep mastitis and identified as *M. glucosida* by Omaleki et al. (2010). The ovine isolate BR2770 and caprine isolate BR2771 were 100% identical in their partial *rpoB* to each other and to that from isolate H1 previously obtained from sheep mastitis (Omaleki et al., 2010). These two isolates had 7 nucleotide differences in their 1101 bp alignment of *recN* gene (99.36% identical).

Alignment of the extracted *rpoB* gene of these six non-*M. haemolytica* isolates with our newly designed primers, demonstrated differences in the binding regions of the primers (Fig. 2). BR2768 and BR2783, both obtained from ovine hosts, sharing 100% similarity in their *rpoB* gene to that from *M. glucosida* CCUG 38457 ^{T}*,* had two nucleotide difference in the forward primer target sequence and three nucleotide differences in the reverse primer target sequence. BR2766, also obtained from an ovine host, had one nucleotide difference in the forward and reverse target sequences. The rest of the non-*M. haemolytica* isolates had one nucleotide difference in the forward primer and two nucleotide difference in the reverse primer target sequences (Fig. 1).

Overall, based on the above results, the six isolates that were negative in the *M. haemolytica* PCR could not be confidently assigned to any currently recognised species within the genus *Mannheimia.*

3.4. rep-PCR

The ovine and caprine isolates had very different rep-PCR patterns to the bovine isolates. The majority of the bovine isolates formed five distinct main clusters (.

Table 1 and Fig. 2). Cluster 1 consisted of isolates obtained in 2019 in QLD. This cluster contained isolates of serovar 1 and 6 with BR2774 (assigned to serovar 6) having the same rep-PCR profile as the five

Table 3
Comparison of the *recN* and *rpoB* genes of the six isolates that were negative in the novel *M. haemolyticia* PCR and the two isolates that were positive in the novel *M. haemolytica* PCR but were not serovars 1, 2 or 6. The comparisons were made to *M. glucosida* CCUG 38457 ^{T} and *M. haemolytica* CCUG 408 ^{T}.

Isolate	Host	% similarity to *recN*		% similarity to *rpoB*		Nucleotide difference in primer binding region of the novel *M. haemolytica* PCR		Reaction in *M. haemolytica* PCR
		M. glucosida	*M. haemolytica*	*M.glucosida*	*M. haemolytica*	Forward primer	Reverse primer	
BR2765	Goat	80	80	97	96	1	2	Neg
BR2766[†]	Sheep	98	98	95	97	1	1	Neg
BR2768	Sheep	99	99	100	97	1	3	Neg
BR2770[‡]	Sheep	99	98	96	97	1	2	Neg
BR2771[‡]	Goat	99	99	96	97	1	2	Neg
BR2773	Sheep	99	100	97	100	-	-	Pos
BR2782	Cattle	99	100	92	100	-	-	Pos
BR2783	Sheep	98	98	100	97	1	3	Neg

[†] 100% identical to *rpoB* from isolate A5 previously obtained from sheep mastitis (Omaleki et al., 2010)
[‡] 100% identical to *rpoB* from isolate H1 previously obtained from sheep mastitis (Omaleki et al., 2010)

Figure legend

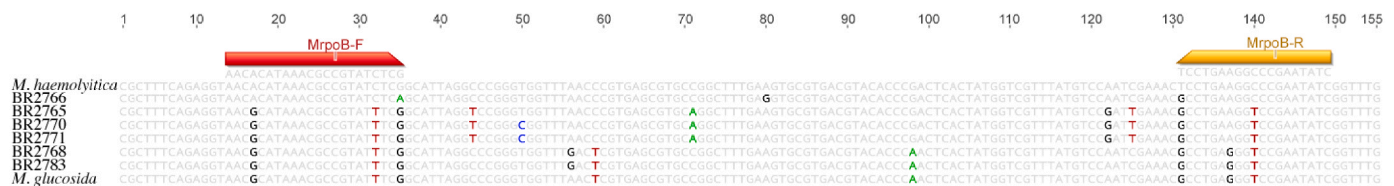

Fig. 1. Alignment of *rpoB* sequences of the sheep and goat isolates that gave negative results in the species-specific PCR with forward and reverse primers.

serovar 1 isolates. Cluster 2 contained feedlot cattle isolates of serovar 1 and 6 obtained in 2002 or 2003 with the exception of one isolate from QLD that was isolated in 1995. Cluster 3 also has serovar 1 and 6 isolates over several years, mainly from QLD. Cluster 4 is made up of serovar 6 isolates all from the same year from the same NSW feedlot. Cluster 5 consists of serovars 1 and 6 from NSW with the exception of one QLD isolate which was isolated in 1995 with the same rep-PCR pattern also shared between some of the serovar 1 and 6 isolates. The DNA fingerprint pattern of the serovar 2 isolates was different from that of serovars 1 and 6, a finding also reflected in the generated dendrogram with no serovar 2 isolates being closely clustered with either a serovar 1 or serovar 6 isolate (Fig. 2). The isolates that were found not to be *M. haemolytica* (circled in Fig. 2) were quite distinct from the confirmed *M. haemolytica* isolates.

4. Discussion

Bovine respiratory disease is one of the major problems in feedlot cattle in Australia (Alhamami et al., 2021) and other countries (Constable et al., 2017) with one of the main pathogens involved in this disease complex being *M. haemolytica*. This pathogen is also a major contributor to disease in the sheep and goat industry (Gilmour et al., 1979).

We elected to target the *rpoB* gene for a species-specific assay for *M. haemolytica* as this gene has been identified to be more reliable for discrimination between genera and species within the family *Pasteurellaceae* than 16S rRNA gene. A 10–12% difference between *rpoB* gene sequences from different *Pasteurellaceae* genera, compared to 6–7% between 16S rRNA gene sequences has been reported (Korczak et al., 2004). Other studies have shown that amplification and sequencing of *rpoB* gene could be used successfully to differentiate between *M. haemolytica* and *M. glucosida* (Lau et al., 2015; Omaleki et al., 2010).

MALDI-TOF MS is now routinely used in veterinary diagnostic laboratories for bacterial identification and can successfully differentiate many of the genera in the *Pasteurellaceae* family (Frey and Kuhnert, 2015). However, the differentiation provided by MALDI-TOF MS is mainly based on the fingerprints of the ribosomal proteins and has a limitation in identification of closely related species within the *Pasteurellaceae* family (Frey and Kuhnert, 2015). Hence we believe the MALDI-TOF MS identification of the six PCR negative results as *M. haemolytica* is in-correct. We have previously reported a similar issue of the mis- identification of a *M. glucosida* isolate in association with human wound infection and the limited capacity of MALDI-TOF MS analysis for correct identification (Lau et al., 2015).

Among the primers we designed for the *rpoB* gene, one pair was found to have a unique sequence for *M. haemolytica*. Using the PCR based on these primers, it was possible to differentiate *M. haemolytica* from closely related species such as *M. glucosida*, *M. granulomatis, M. pernigra, M. ruminalis* and *M. varigena*. Our newly developed PCR failed to produce any amplicon when tested against just six of the sixty-five previously tentatively identified *M. haemolytica* field isolates. Analysis of the housekeeping gene sequences extracted from the WGS of these six isolates only gave a 100% similarity of two isolates with *M. glucosida* with two other of these isolates closely related to *M. glucosida*-like isolates previously obtained from sheep mastitis cases (Omaleki et al., 2010). *M. glucosida* is known as a heterogenous species that includes different biovars together with a considerable diverse 16S rRNA gene sequences (Angen et al., 1999a). Hence, as the data-base of sequences for *M. glucosida* is limited, identification of this species is a challenge.

One of the confirmed field isolates of *M. haemolytica* (BR439) was obtained from the liver, indicating a septicaemic condition. While not a common occurrence, there is a report of *M. haemolytica* being associated with pleuropneumonia and septicaemia in a calf (Mahu et al., 2015) as well as association of *M. haemolytica* with polyserositis in veal calves (Biesheuvel et al., 2021). In addition, there are reports of human septicaemic infections, reviewed by Punpanich and Srijuntongsiri (2012) associated with either *[P]. haemolytica* or *M. haemolytica*. Hence, veterinary diagnostic laboratories need to be aware of the possibility of *M. haemolytica* occurring in body sites outside the respiratory tract.

Twelve serovars have been identified in *M. haemolytica*. However, many *M. haemolytica* like species from ruminants seem to share common capsular antigens with isolates of *M. haemolytica* (Angen et al., 1999b). *M. haemolytica* serovars 1, 2 and 6 have been identified as the most prevalent serovars worldwide (Christensen et al., 2021; Klima et al., 2017) with serovar 2 being the most prevalent in sheep (Fodor et al., 1984). Our results also identified serovars 1, 2 and 6 in the field isolates we investigated with serovars 1 and 6 being the dominant serovars. As well, we have identified two of the rarely reported *M. haemolytica* serovars (serovars 7 and 14) in association with ruminant respiratory disease in Australia.

Pulsed field gel electrophoresis and rep-PCR have been used to investigate the molecular epidemiology of *M. haemolytica* (Katsuda et al., 2003; Taylor et al., 2014) and *M. glucosida* (Omaleki et al., 2015) in ruminants. Taylor et al. (2014) reported that rep-PCR was the most informative of the two genetic typing methods used in their study of Australian and US field isolates of *M. haemolytica* and showed that the US and Australian isolates were quite distinct and did not cluster together. In our hands, the rep-PCR typing gave a somewhat similar picture – with some clusters being typically from a single State (e.g. Clusters 1 and 4, Fig. 2). Some Clusters did show a geographical diversity e.g. Clusters 2 and 5 (where a sole isolate in each Cluster was from a different State) and Cluster 3 (which was a Cluster with few isolates of possible different geographical origin and collected across a large time range).

In the current study, rep-PCR analysis clustered isolates of serovars 1 and 6 close to each other, suggesting that this method is not ideal to differentiate between serovars. The inability of PCR-based fingerprinting methods like rep-PCR and the similar enterobacterial repetitive intergenic consensus-based PCR (ERIC-PCR) to differentiate serovars has been shown already for other members of the family *Pasteurellaceae* – specifically *Glaesserella parasuis* (Oliveira et al., 2003) and *Pasteurella multocida* (Omaleki et al., 2020).

In summary, this work has demonstrated the specificity and sensitivity of a novel species-specific assay for the identification of a major pathogen – *M. haemolytica.* As well, the ability of WGS and bioinformatic analysis to serotype confirmed *M. haemolytica* isolates has been confirmed. However, the limitation of in-use data-bases for MALDI-TOF MS to human specific pathogens means that identification of some animal pathogens, particularly within the family *Pasteurellaceae,* to species level remains challenging. We believe that these tools will be important support tools for diagnostic laboratories investigating respiratory disease in cattle.

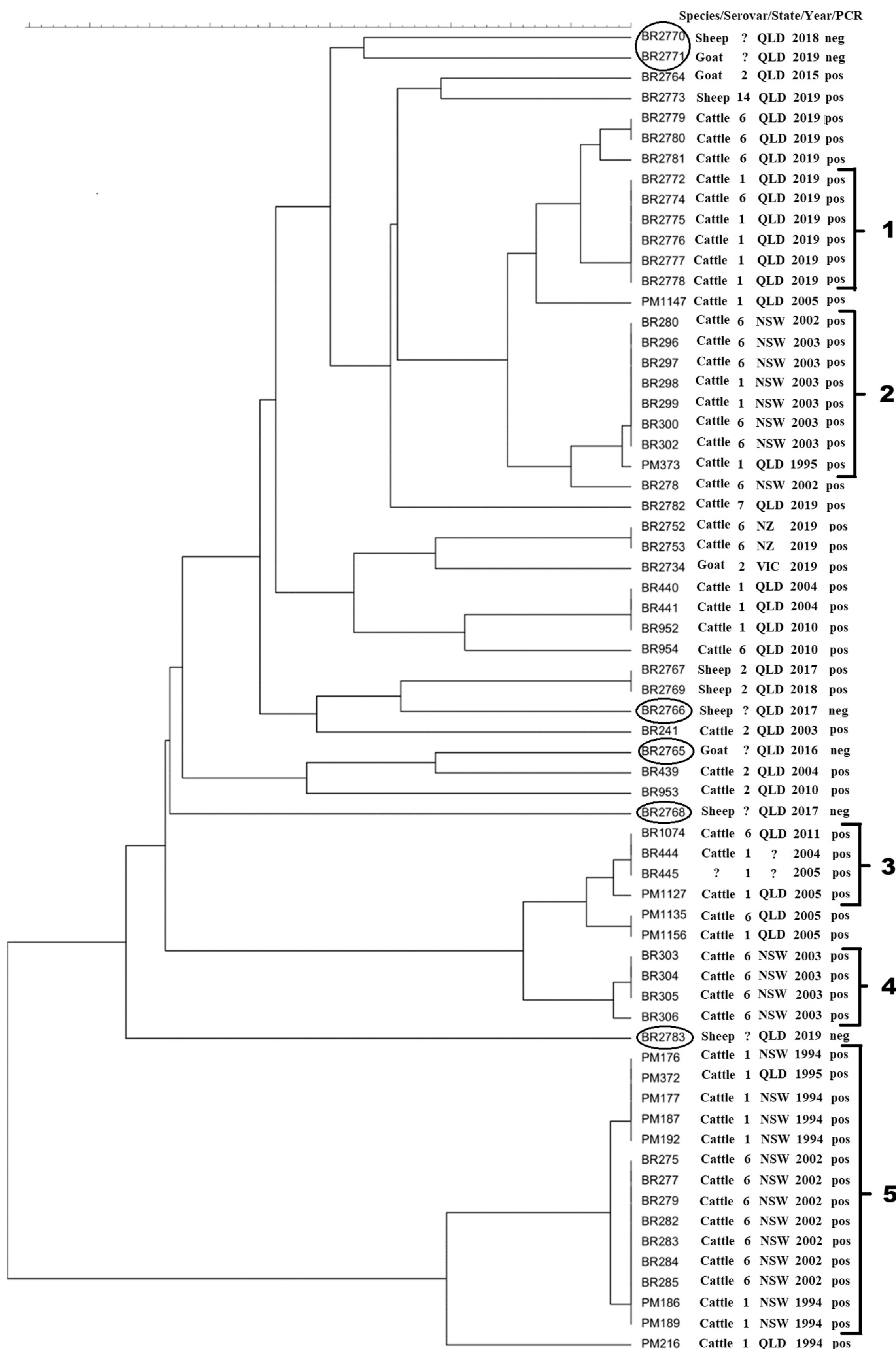

Fig. 2. rep-PCR profile of the isolates from cattle, sheep and goats. Neg = not *M. haemolytica,* pos = *M. haemolytica*. Circles indicated isolates that are not *M. haemolytica*. Five clusters (1−5) are defined that are talked about in the results. There are 28 patterns of rep-PCR, which are listed in Table 1. The question mark (?) indicates that there is no knowledge of the origin either of the state or host species or both. It also indicates that serovar was not identified as the isolate was not *M. haemolytica*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2023.109930.

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