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RESEARCH ARTICLE

First Report of Antimicrobial Resistance of *Mannheimia haemolytica* from Phan Rang Sheep in Vietnam

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A B S T R A C T

Mannheimia haemolytica is the principal agent associated with respiratory diseases in different animals, however, the prevalence and antimicrobial resistance of this Gram-negative bacteria in Phan Rang sheep, a local sheep of Vietnam, are still unknown. In the present study, 31 *M. haemolytica* isolates were obtained from 170 samples from clinically sick and healthy sheep. A higher prevalence of *M. haemolytica* was detected in clinically sick samples (25/85) than in healthy ones (6/85). The antimicrobial susceptibility of 31 *M. haemolytica* isolates was determined using eleven antibiotics. Among them, *M. haemolytica* strains were susceptible to ofloxacin (100%), ciprofloxacin (96.77%), enrofloxacin (93.55%), and chloramphenicol (90.32%), whereas, they were resistant to oxytetracycline (74.19%), tulathromycin (70.97%), erythromycin (67.74%), penicillin (51.61%), and ampicillin (41.93%). More than 74% of *M. haemolytica* isolates exhibited multidrug resistance, of which 54.84% of isolates resisted 3 to 4 antibiotics, and 19.35% of isolates resisted 5-6 antibiotics. A good correlation between genotype and resistance phenotype for oxytetracycline was detected as 25/31 isolates carrying at least one gene (*tetB* or/and *tetH*). On the other hand, a poor correlation between genotype and phenotype for β-lactam, and macrolide antibiotic groups were observed as none of the resistance phenotype harboring bla_{TEM}, *erm42*, *ermB*, and *mphE* gene. These findings provide the first reported evidence of the prevalence and antibiotic resistance of *M. haemolytica* that have contributed to Phan Rang sheep respiratory disease in central Vietnam.

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INTRODUCTION

Mannheimia haemolytica is the causative agent of pasteurellosis in ruminants, associated mainly with respiratory diseases. It is primarily responsible for pneumonic pasteurellosis (Marru *et al.*, 2013; Legesse *et al.*, 2018) and also causes mastitis and septicemia in sheep (Omaleki *et al.*, 2016). Furthermore, the bacterium is an important agent for causing opportunistic infection in the upper part of the respiratory tract (tonsils and nasopharynx) of healthy sheep (Klima *et al.*, 2011). Certain conditions of immunosuppression allow the bacterium to establish and multiply rapidly, it penetrates the lungs during inhalation and initiates an active infection of the alveolar epithelium (Lima *et al.*, 2016).

Pasteurellosis in livestock usually exhibits typical pneumonia symptoms and antimicrobials are a standard method of treatment (Andrés-Lasheras *et al.*, 2019), however, over time, routine use of antimicrobials induces drug resistance of *M. haemolytica*. Consequently, several studies have reported that *M. haemolytica* was isolated worldwide, exhibiting resistance to a growing number of antibiotics (Katsuda *et al.*, 2009; Klima *et al.*, 2014; Sahay *et al.*, 2020). Antimicrobials are also widely used in animal husbandry in Vietnam, most farmers use a high portion of antimicrobials, but little is known about the drivers that impact this usage (Truong *et al.*, 2019). Accordingly, livestock in Vietnam containing 247.3mg antibiotic/kg animal compared to 151.5mg in the EU, resulting in high rates of antibiotic-resistant bacteria

being detected in animal products and food sources (Carrique-Mas *et al.,* 2020). A recent study reported that more than 69% of *Haemophilus parasuis*, a member of the Pasteurellaceae family, isolated from pigs in central Vietnam exhibited resistance to trimethoprim/sulfametoxazole, colistin, chloramphenicol, penicillin, gentamycin, and erythromycin. In another study conducted on *P. multocida* isolated from pigs indicated that at least 59% of isolates were resistant to amoxicillin and tetracycline (Vu-Khac *et al.*, 2020).

Vietnam has a locally bred small ruminant, named Phan Rang sheep which has been reared in Ninh Thuan province for over a hundred years, with an estimated population of 200,000 individuals in this area. The sheep have a small size than those from Australia, France, and the USA but larger than those from South East Asea origin. Importantly, sheep can adapt and grow well in Ninh Thuan, the hottest and driest area of Vietnam.

However, to our knowledge, there is no information about the prevalence of *M. haemolytica* in sheep as well as other livestock in Vietnam. This study aimed to investigate the prevalence of *M. haemolytica* in Phan Rang sheep and provide the current state of antimicrobial resistance of the bacteria, which may help in further incorporating appropriate strategies to manage the *M. haemolytica* in sheep.

MATERIALS AND METHODS

Ethical approval: Ethical approval was not required for this study.

Study period, location, and sample collection: Sample collection was carried out according to the previous report (Klima *et al*., 2011). Briefly, samples were collected by rotating deep-guarded culture swabs in the nasopharynx of the sheep. A total of 170 nasal swab samples were collected from 85 healthy sheep and 85 clinically sick sheep (coughing and breathing discomfort, and nasal discharge) in different farms in Ninh Thuan province, the greatest sheep husbandry zone of Vietnam, from April to May 2021 and February to March 2022. The samples were put in 2mL of tryptone soya broth and transported and processed at the laboratory of cells, Institute of Biotechnology, Hue University.

Isolation of *Mannheimia haemolytica* : The nasal swabs were streaked on 5% sheep blood agar plates and incubated at 37 ^{o}C for 24–48 hr. Typical suspected colonies with white-grey, hemolytic, and non-mucoid were considered as *Mannheimia* and were further subcultured and analyzed with regard to motility catalase, and oxidase according to Klima *et al*. (2011). Positive colonies were further confirmed by multiplex PCR with specific primers.

***M. haemolytica* identification by PCR:** Colonies from the presumptive isolates of *Mannheimia* were collected for genomic DNA extraction using the Qiagen kit (Hilden, Germany) as per the manufacturer's instructions. A multiplex PCR assay using specific primer pairs targeted to *M. haemolytica* serotype-1 specific antigens (*PHSSA*) and methyltransferase (*Rpt2*) was applied to identify *M. haemolytica* according to the previously described method (Klima *et al.*, 2014). Briefly, the PCR was carried out in the PCR machine (MJ mini thermal cycler, Biorad, USA) using OneTaq® 2X master mix with standard Buffer (New England, Biolabs, UK) in a final volume of 25µL containing 1µL of each primer (10pM), 1µL of template DNA, and 9.5µl of nuclease-free water. The PCR program was an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 0.5 min at 94 °C for denaturation, 0.5 min at 55 °C for primer annealing, and 1 min at 68 °C for the extension. The PCR products were further verified by being subjected to 1.2% agarose gel electrophoresis and stained with SYBR™ Green I (Invitrogen, Thermo Fisher) for visualization under the MUPID® One LED Illuminator (Nippon genetics, EU). The PCR reaction using nuclease-free water and the DNA of *M. haemolytica* from the collection of Central Vietnam Veterinary Institute was carried out as negative and positive controls, respectively.

Antimicrobial susceptibility: The sensitivity profile of *M. haemolytica* isolates to antimicrobials was determined using the disc diffusion method, according to the guideline of the Clinical and Laboratory Standards Institute (CLSI, 2018). In brief, all isolates were inoculated in BHI broth with a shaking incubator at 37 ^{o}C, 150rpm/min, until cell density at 600 nm absorbance was approximately 0.5, cells were then swabbed over the surface of Mueller-Hinton plates (Himedia, India). Followed by placing 11 different antibiotic agents: ampicillin (10µg), penicillin (10µg), kanamycin (30µg), ciprofloxacin (5µg), chloramphenicol (30µg), tulathromycin (30µg), erythromycin (15µg), gentamicin (10µg), ofloxacin (5µg), enrofloxacin (5µg), and oxytetracycline (30µg) onto the plates and incubated at 37 ^{o}C for 24 hours. The antimicrobial susceptibility was determined based on the diameter of the inhibition zone of antimicrobials. *Escherichia coli* ATCC 35218 was used as the quality control strain.

Resistance genes: PCR assay with specific primers listed in Table 1, was used to identify antibiotic resistance genes of *M. haemolytica* isolates. The selected genes, bla_{TEM} and bla_{ROB1} for screening isolates resist ampicillin and/or ampicillin, and *tetB* and *tetH* for screening isolate resisted oxytetracycline. Isolates exhibiting resistance to erythromycin were screened for *erm*42, and *ermB* genes. Isolates were screened for *aacA4* and *floR* for resistance to chloramphenicol, and gentamicin, respectively. PCR was carried out in a similar thermal cycler, and the procedure was described above.

Statistical analysis: Statistical analysis was carried out by SPSS 20 (SPSS Inc., Chicago, USA), the differences in the bacterium isolation and antimicrobial susceptibility frequencies were determined by the Chi-square test, and P-value ≤ 0.05 was considered significant.

RESULTS

Isolation of *M. haemolytica:* A total of 45 suspected bacteria were isolated from 170 samples based on phenotypes and biochemical tests. Among them, 31 isolates were identified as *M. haemolytica* using a multiplex PCR assay with specific primers (Fig. 1),

Table 1: Primer pairs for identification of *M. haemolytica*, and detection of antibiotic resistance genes

Antibiotic group	gene	Primer sequence (5'-3')	Annealing temperature (°C)	PCR product size (bp)	References
Ampicillin	bla_{TEM}	GAGTATTCAACATTTTCGT ACCAATGCTTAATCAGTGA	48	852	(Dayao *et al.*, 2016)
	bla_{ROB1}	CATTAACGGCTTGTTCGC CTTGCTTTGCTGCATCTTC	50	856	(Dayao *et al.*, 2016)
Tetracycline	*tetB*	CCTTATCATGCCAGTCTTGC ACTGCCGTTTTTTTCGCC	52	774	(Dayao *et al.*, 2016)
	tetH	ATACTGCTGATCACCGT TCCCAATAAGCGACGCT	49	1076	(Dayao *et al.*, 2016)
Chloramphenicol	*floR*	CACGTTGAGCCTCTATATGG ATGCAGAAGTAGAACGCGAC	51	885	(Vu-Khac *et al.*, 2020)
Gentamicin	*aacA4*	CTCGAATGCCTGGCGTGTTT TTGCGATGCTCTATGAGTGGC	56	482	(Vu-Khac *et al.*, 2020)
Erythromycin	*Erm*42	TGCACCATCTTACAAGGAGT CATGCCTGTCTTCAAGGTTT	52	173	(Dayao *et al.*, 2016)
	ermB	CATTTAACGACGAAACTGGC GGAACATCTGTGGTATGGCG	52	400	(Dayao *et al.*, 2016)
	mphE	ATGCCCAGCATATAAATCGC ATATGGACAAAGATAGCCCG	50	271	(Dayao *et al.*, 2016)
M. haemolytica detection	*RPT2*	GTTTGTAAGATATCCCATTT CGTTTTCCACTTGCGTGA	55	1022	(Klima *et al.*, 2014)
	PHSSA	TTCACATCTTCATCCTC TTTTCATCCTCTTCGTC	55	325	(Klima *et al.*, 2014)

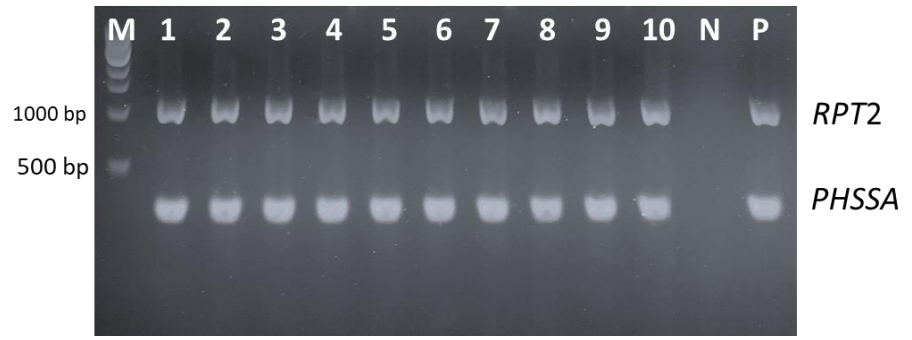

Fig. 1: Multiplex PCR for *M. haemolytica* identification using *RPT2* and *PHSSA* primers. Lane M, 1 kb DNA ladder marker (ThermoScientific, USA); lanes 1-10: detection of M. haemolytica from isolates (1-10); lane P: positive control, lane N, negative control.

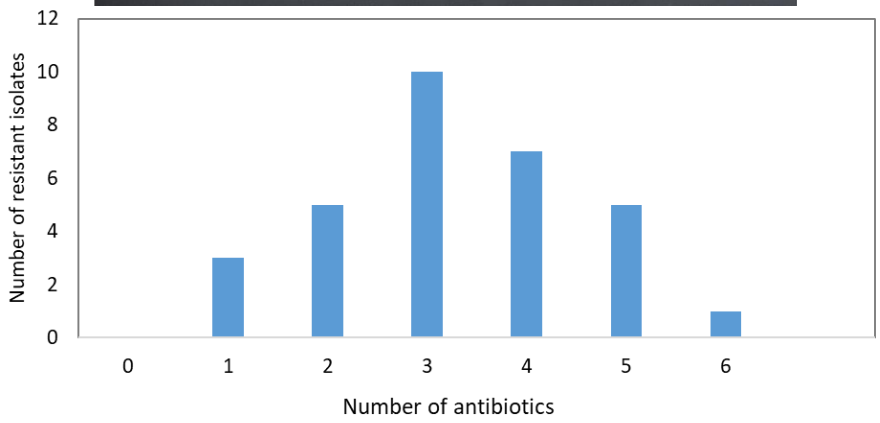

Fig. 2: Multidrug resistance profile of *M. haemolytica* isolates

of which 25 isolates (25/85) were obtained from clinically sick sheep, and 6 isolates (6/85) were isolated from healthy sheep. The results showed a significant correlation between the present of *M. haemolytica* and sheep health status ($P<0.05$) (Table S.1).

Antimicrobial susceptibility: The antimicrobial resistance profiles of *M. haemolytica* isolates are shown in Table 2. Among 31 isolates, a large percentage exhibited high resistance to oxytetracycline (74.19%), tulathromycin (70.97%), erythromycin (67.74%), penicillin (51.61%), and ampicillin (41.93%). A small number of isolates, 3 (9.68%) and 4 (12.9%) exhibited resistance to kanamycin and gentamicin, respectively. On the contrary, all *M. haemolytica* isolates were susceptible to ofloxacin (100%), and more than 90% of isolates were sensitive to ciprofloxacin (96.77%), enrofloxacin (93.55%), and chloramphenicol (90.32%).

The multi-antibiotic resistance feature of *M. haemolytica* isolates was also investigated (Fig. 2 and Table S.2). The result showed that all strains resisted at least one antibiotic tested, of which notably, 3 (9.68%) isolates had resistance to only one antibiotic, while 1 (3.22%) isolate exhibited resistance to up to 6 antibiotics. The number of strains resistant to 2, 3, 4, and 5 antibiotics was calculated as 5 (16.13%), 10 (32.26%), 7 (22.58%), and 5 (16.13%), respectively. In general, the multiple antibiotic resistance indexes (MARIs were calculated as the ratio between the number of antibiotics with resistance detected in one strain and the total number of antibiotics the strain is exposed to) ranged from 0.09 to 0.55 (Table S.2) and showed 19 different resistant patterns.

Table 2: The antimicrobial susceptibility profiles of *M. haemolytica* isolates by disk diffusion test

Antibiotic groups	Antibiotics	Number of isolates against antimicrobial agents		
		Sensitive (%)	Intermediate (%)	Resistant (%)
Penicillin	Ampicillin	16 (51.61)	2 (6.45)	13 (41.93)
	Penicillin	12 (38.71)	3 (9.68)	16 (51.61)
Fluoroquinolone	Ciprofloxacin	30 (96.77)	1 (3.22)	0
	Ofloxacin	31 (100%)	0	0
	Enrofloxacin	29 (93.55)	2 (6.45)	0
Aminoglycosides	Kanamycin	25 (80.64)	3 (9.68)	3 (9.68)
	Gentamicin	26 (83.87)	1 (3.22)	4 (12.9)
Tetracycline	Oxytetracycline	6 (19.35)	2 (6.45)	23 (74.19)
Phenicols	Chloramphenicol	28 (90.32)	2 (6.45)	1 (3.22)
Macrolide	Erythromycin	5 (16.13)	5 (16.13)	21 (67.74)
	Tulathromycin	6 (19.35)	3 (9.68)	22 (70.97)

Table S1: Prevalence of *M. haemolytica* from Phan Rang sheep samples

Sheep	Number of samples	Number of isolates (%)	X^2/p-value
healthy	85	6 (7.05)	14.242/0.001
clinical sick	85	25 (29.41)	

*Significance at P<0.05.

Table S2: The antibiotic resistance pattern of *M. haemolytica* isolates

Isolates	KAN	AM	CIP	C	ENR	GM	E	P	OFX	OTC	TUL	MARIs	Phenotype
NT1	S	R	S	S	S	S	S	R	S	R	S	0.2727	AM, P, OTC
NT2	I	S	S	S	S	S	R	I	S	R	R	0.2727	E, OTC, TUL
NT3	S	R	I	S	S	R	R	R	S	S	R	0.4545	AM, GM, E, P,TUL
NT4	S	I	S	S	S	S	R	S	S	R	R	0.27	E, OTC, TUL
NT5	S	R	S	S	S	S	I	R	S	S	R	0.27	AM, P, TUL
NT6	I	S	S	R	S	R	R	R	S	R	R	0.55	C, GM, E, P, OTC, TUL
NT7	S	S	S	S	I	S	R	S	S	R	R	0.27	E, OTC, TUL
NT8	S	R	S	S	S	R	R	S	S	R	R	0.45	AM, GM, E, OTC,TUL
NT9	R	R	S	I	S	S	R	R	S	R	I	0.45	KAN, AM, E, P, OTC
NT10	S	S	S	S	S	S	R	R	S	R	R	0.36	E, P, OTC, TUL
NT11	S	S	S	S	S	S	R	R	S	R	R	0.36	E, P, OTC, TUL
NT12	S	S	S	S	S	S	I	R	S	I	R	0.18	P, TUL
NT13	S	R	S	S	S	R	R	S	S	R	R	0.45	AM, GM, E, OTC,TUL
NT14	S	R	S	S	S	S	R	I	S	R	R	0.36	*AM, E, OTC,TUL*
NT15	S	R	S	S	S	S	R	S	S	R	R	0.36	*AM, E, OTC,TUL*
NT16	I	R	S	S	S	S	S	S	S	R	R	0.27	AM, OTC, TUL
NT17	S	I	S	S	S	S	R	R	S	R	R	0.36	E, P, OTC, TUL
NT18	R	R	S	S	S	S	R	R	S	S	R	0.45	KAN, AM, E, P, TUL
NT19	R	R	S	S	S	S	R	S	S	R	S	0.36	KAN, AM, E, OTC
NT20	S	R	S	S	S	S	R	S	S	R	R	0.36	*AM, E, OTC,TUL*
NT21	S	S	S	S	S	I	R	S	S	R	R	0.27	E, OTC,TUL
NT22	S	S	S	S	S	S	R	S	S	R	R	0.27	E, OTC,TUL
NT23	S	R	S	S	S	S	S	R	S	S	S	0.18	AM, P
NT24	S	S	S	S	S	S	R	R	S	R	S	0.27	E, P, OTC
NT25	S	S	S	S	S	S	I	S	S	I	R	0.09	TUL
NT26	S	S	S	S	S	S	R	S	S	R	R	0.27	E, OTC, TUL
NT27	S	S	S	S	S	S	S	R	S	S	R	0.18	P, TUL
NT28	S	S	S	S	S	S	I	R	S	R	S	0.18	P, OTC
NT29	S	S	S	I	S	S	I	R	S	S	I	0.09	P
NT30	S	S	S	S	S	S	S	I	S	R	S	0.09	OTC
NT31	S	S	S	S	I	S	R	S	S	R	I	0.18	E, OTC

Kanamycin (KAN); Ampicillin (AM); Ciprofloxacin (CIP); Chloramphenicol (C); Enrofloxacin (ENR); Gentamicin (GM); Erythromycin (E); Penicillin (P); Ofloxacin (OFX); Oxytetracycline (OTC); Tulathromycin (TUL).

The highest frequency of multiresistant phenotypes was observed for erythromycin, tulathromycin and oxytetracycline (E, OTC, TUL) with 19.35% (6/31), followed by the phenotype (P, E, OTC, TUL) and the phenotype (AM, E, OTC, TUL) with 9.68% (3/31) each.

Antimicrobial resistance genes of *M. haemolytica* isolates: Antimicrobial resistance genes are important factors, associated with antibiotic resistance of bacteria and the genes are widely investigated as potential markers for phenotype prediction. As per results shown in Table 3 and Fig. 3, 80.64% of isolates harbored either *tetB* or *tetH*, in which 22.58% of isolates carrying *tetB*, 4 isolates were resistant to oxytetracycline. On the other hand, 70.97% of isolates (22/31) harbored *tetH*, and 18 of them were detected in oxytetracycline-resistant strains.

Interestingly, 5/31 isolates were positive for bla_{TEM}, and 3 isolates harbored the *floR* gene, but none of them was resistant to ampicillin/ penicillin or chloramphenicol. Conversely, 19/31 isolates were positive for bla_{ROB1}, and of which 13 strains exhibited resistance phenotype to ampicillin or penicillin and 3 strains resisted both antibiotics. Moreover, among 6 isolates that harbored the *aac*A4 gene (Table 3), only one isolate exhibited gentamicin resistance. Surprisingly, none of the isolates harbored *erm*42, *ermB* and *mphE* was detected, although 67.74 and 70.97% of isolates were resistant to erythromycin or tulathromycin, respectively.

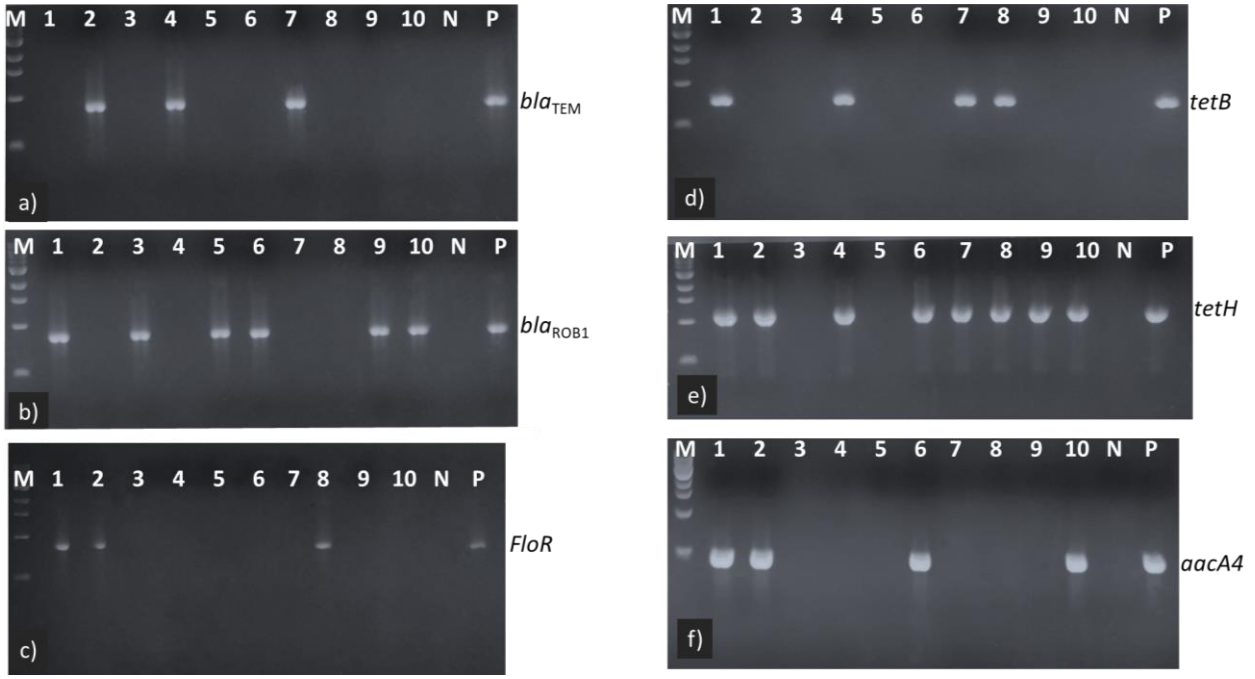

Fig. 3: PCR detection of the distribution of bla_{TEM} (a), bla_{ROB1} (b), *floR* (c), *tetB* (d), *tetH* (e) and a*acA4* (f) genes in *M. haemolytica isolates*. M: 1kb DNA ladder; N: Negative control; P: Positive control; 1-10: Samples.

Table 3: Correlation between the presence of antibiotic resistance genes and their phenotype in *M. haemolytica* Phan Rang sheep isolates

Antimicrobial agent	Resistance genes	Positive isolates		Resistance phenotype		Resistant antibiotic
		Number (percentage)	X^2/p-value	Number (percentage)	X^2/p-value**	
Ampicillin (n=31)	bla_{TEM}	5 (16.13)	13.325/0.0003	0	16.449/<0.0001*	0
	bla_{ROB1}	19 (61.29)		13		Penicillin[$]
Chloramphenicol (n=31)	*floR*	3 (9.68)	-	0	-	
Gentamicin (n=31)	*aacA4*	6 (19.35)	-	1	-	Gentamicin
Oxytetracycline (n=31)	*tetB*	7 (22.58)	24.641/<0.00001*	4	29.509/<0.00001*	Oxytetracycline
	tetH	22 (70.97)		18		Oxytetracycline
	tetB or tetH	25 (80.64)		25		
Erythromycin (n=31)	*erm42*	0		0		
	ermB	0	-	0	-	
	mphE	0		0		

[$] Penicillin, ampicillin: * significance at P<0.05: ** compare to resistance genes.

DISCUSSION

Pasteurellosis is a common disease in livestock, caused by two main agents, *Pasteurella multocida* and *M. haemolytica*, which account for 30% of sheep deaths every year worldwide (Sahay *et al.*, 2020). Several studies on Pasteurellosis have been carried out in Vietnam, which focused on *P. multocida* causing respiratory disease in pigs (Townsend *et al.*, 1998; Vu-Khac *et al.*, 2020).

The present study is the first report on the prevalence and antibiotic resistance profile of *M. haemolytica* in animals in Vietnam. We revealed that *M. haemolytica* was more frequently isolated from Phan Rang sheep with respiratory problems (29.41%) than from healthy ones (7.05%). These incidences are close to that of Timsit *et al*. (2017) who reported that 30.5% and 13.1% of *M. haemolytica* were isolated from the bovine with the respiratory disease and healthy ones, respectively. Higher incidences were reported in the study of Sahay *et al*. (2020), in which *M. haemolytica* was detected in 66.7% of diseased samples and 35.6% of nasal samples from healthy sheep. Likewise, the ratio of *M. haemolytica* isolated from sheep with pneumonia symptoms ranged from 32.62 to 34.21% (Legesse *et al.*, 2018; Akane *et al.*, 2022). The difference in the rate of isolation of *M. haemolytica* may be from sampling techniques, sample size, different isolation methods, misidentification, and environmental conditions. In general, our findings are in agreement with those of previous studies *(*Timsit *et al*., 2017; Bello *et al.*, 2019; Sahay *et al.*, 2020), which reported that *M. haemolytica* was mainly isolated from sheep with respiratory infections. This indicates that *M. haemolytica* plays an important role in respiratory diseases.

Antibiotics are still an effective treatment for respiratory diseases caused by *M. haemolytica* (Klima *et al.*, 2011). However, due to indiscriminate use of incorrect dosage, more and more strains of bacteria, including *M. haemolytica*, become drug-resistant (Bahr *et al.*, 2021). Especially, Phan Rang sheep are usually kept with other livestock, which rearing is on a small scale, and farmers are not properly trained to use antibiotics and to write a diary of animal health. At present, no report on the antimicrobial resistance profiles of *M. haemolytica* in Vietnam livestock. Our study reveals that ofloxacin, ciprofloxacin, and enrofloxacin were the most effective (>90%) drugs against *M. haemolytica* isolates. These results are similar to the previous studies, of which 89-100% of *M. haemolytica* was susceptible to enrofloxacin (Katsuda *et al.*, 2009; Timsit *et*

al., 2017), while 83.3-88.9% of isolates were inhibited by ciprofloxacin and enrofloxacin, respectively (Nefedchenko *et al.*, 2019).

On the other hand, oxytetracycline, tulathromycin, and erythromycin showed less effectiveness against *M. haemolytica* from Phan Rang sheep since approximately 70% or more isolates exhibited resistance to these antibiotics. This finding reflects those of Timsit *et al.* (2017) who also found that 71.8, and 74.4 % of *M. haemolytica* isolates were highly resistant to tulathromycin, and oxytetracycline, respectively. However, the resistance levels observed in this investigation were much higher compared to those of other studies (Andrés-Lasheras *et al.*, 2019; Katsuda *et al.*, 2009) which reported that about 20% of *M. haemolytica* strains were resistant to these drugs. In contrast, high sensitivity toward tulathromycin (>92%) and intermediate resistance to oxytetracycline (<50%) of *M. haemolytica* strains were reported (Nefedchenko *et al*., 2019; Schönecker *et al.*, 2020). Similarly, Omaleki *et al.* (2016) reported that all *M. haemolytica* is susceptible to erythromycin.

It is known that chloramphenicol is a highly effective drug against a variety of both Gram-negative and Gram-positive bacteria including pasteurellosis bacteria (Sahay *et al.*, 2020). In the present study, only one isolate (3.22%) exhibited resistance to this antibiotic. This result supports evidence from previous observations (Katsuda *et al.*, 2009; Marru *et al.*, 2013; Sahay *et al.*, 2020). Similarly, high sensitivity (95.2%) to chloramphenicol was also found in *P. multocida* isolated from a pig in Vietnam (Vu-Khac *et al.*, 2020). Moderate efficacy (>80%) of kanamycin and gentamicin against *M. haemolytica* isolates was also observed. This finding supports evidence from previous observations (Katsuda *et al.*, 2009; Singh *et al*., 2019; Sahay *et al.*, 2020), which showed that more than 80% of *M. haemolytica* strains were susceptible to these antibiotics. This outcome is contrary to that of Marru *et al*. (2013) who found that 100% *M. haemolytica* strains isolated from sheep in Ethiopia exhibited resistance against gentamicin.

Due to the wide use and incorrect dose, the resistance rate to the β-antibiotics has been increasing in several countries such as Spain (Cuevas *et al.*, 2020), Taiwan (Yeh *et al.*, 2017), and Vietnam (Vu-Khac *et al.*, 2020). Consistent with the literature, this study found that penicillin and ampicillin showed low efficacy (38.71 and 51.61% susceptibility, Table 2) against *M. haemolytica* isolates. This also accords with earlier observations, which showed varying resistance levels of *M. haemolytica* toward penicillin, and ampicillin (Marru *et al*., 2013; Schönecker *et al*., 2020). Higher resistance levels were observed in the study of Bahr *et al.* (2021) who found that 100% of *M. haemolytica* strains isolated from different hosts in Egypt resist ampicillin and penicillin, and 83.3% of isolates exhibited resistance to tetracycline. In contrast, ampicillin and tetracycline were the most effective drugs for the treatment of 96.4, and 88.9% of *M. haemolytica* strains isolated from sheep in India (Sahay *et al.*, 2020). Similarly, all *M. haemolytica* strains were isolated from mastitis in sheep, susceptible to ampicillin, and penicillin (Omaleki *et al.*, 2016). The difference in antibiotic resistance of *M. haemolytica* strains may be due to geographical origin and antibiotics used previously in the given population (Kehrenberg *et al.*, 2001).

Increasing multidrug resistance of *P. multocida* isolates was observed in the study of Lizarazo *et al.* (2006) who showed that isolates exhibiting resistance to at least four antibiotics increased from 7.93% to 61.9% in 14 years in Spain. A similar study conducted in China by Tang *et al.* (2009) indicated that 47% of isolates were resistant to at least five antibiotics in 2003, which increased to 97.1% in 2007. On the other hand, Klima *et al.* (2020) observed the prevalence of multidrug resistance of *M. haemolytica* in Canada decreased, in particular isolates resistant to more than 7 antibiotics fell from 50% in 2011 to 26% in 2016. Multidrug resistance was also found to be higher in *M. haemolytica* compared to in *P. multocida* isolates (Andrés-Lasheras *et al.*, 2019; Singh *et al*., 2019). The present study found a high prevalence, 74.19% (23/31) *M. haemolytica* strains from Vietnam exhibited multidrug resistance, of which 54.84% of isolates resisted 3-4 antibiotics, 19.35% of isolates resisted 5-6 antibiotics (Fig.2 and Table S2). These prevalence were very close to the study of Anholt *et al.* (2017), in which 53.22% of *M. haemolytica* isolates exhibited resistance to more than 3 antibiotics. But the rate is much higher than that of Sahay *et al*. (2020) who found 25.9% of *M. haemolytica* isolates have multidrug resistance.

The antibiotic resistance of *M. haemolytica* may depend on many factors including the accessibility of the isolates to reservoirs of resistance genes (Kehrenberg *et al.*, 2001). In the current study, 26/31 isolates harbored at least one resistance gene demonstrating their important role in antibiotic resistance of *M. haemolytica* sheep isolates from Vietnam. A good correlation between phenotype and genotype (presence of resistance gene) (p-value < 0.05, Table 3) for oxytetracycline resistance was detected since *tetB* and *tetH* were detected in 25 isolates, of which 18/20 isolates exhibited resistance to oxytetracycline, carrying at least one of these resistance genes, and of which *tetH* was higher prevalent than *tetB*. Similar results were also observed in previous studies, in which *tetH* was detected in 16/18 strains exhibiting resistance to oxytetracycline (Klima *et al.*, 2011) or in all 312 *M. haemolytica* 2b strains isolated from cattle (Clawson *et al.*, 2016). Comparison of the findings with those of other studies conducted on *P. multocida* strains confirms the strong agreement between genotype and phenotype for oxytetracycline resistance (Dayao *et al.*, 2016; Petrocchi-Rilo *et al.*, 2019; Vu-Khac *et al.*, 2020). However, in *P. multocida* strains, *tetB* was reported to be more prevalent than *tetH*.

Regarding genes involved in β-lactam antibiotic resistance, a low prevalence of bla_{TEM} (16.13%), but a high distribution of bla_{ROB1} (61.29%) gene in *M. haemolytica* strains was detected. This finding is consistent with a recent study indicating that a higher appearance of bla_{ROB1} than bla_{TEM} was also observed (Dayao *et al.*, 2016). Klima *et al.* (2011) reported that bla_{ROB1} was present in any resistance phenotype of either ampicillin or amoxicillin/clavulanic acid, this gene was also detected in more than 68% of isolates that show resistance to ampicillin or penicillin in our study. On the other hand, some strains were susceptible to ampicillin or penicillin, and have bla_{TEM} but did not express it. A similar pattern has been reported in the study of Petrocchi-Rilo *et al.* (2019).

Surprisingly, the *aacA4* gene, which mediates gentamicin resistance, was detected in 19.35% of isolates but only 3.22% showed phenotypic resistance to gentamicin. A low frequency of *aacA4* gene was also identified in *P. multocida* strains isolated from pigs in Vietnam (Vu-Khac *et al.*, 2020). On the other hand, a higher prevalence of the *aacA4* gene was found in China (Wang *et al.*, 2017). It is interesting to note that all *M. haemolytica* isolates harboring the *floR* gene were susceptible to chloramphenicol. This finding is contrary to the results of Sahay *et al.* (2020) in which neither resistance gene of chloramphenicol was detected. A similar pattern was also observed in the macrolide resistance genes, our results revealed that none of 21 or 22 erythromycin/tulathromycin-resistant isolates carried the *mphE*, *erm42*, or *ermB* gene. This finding supports evidence from earlier observations (Dayao *et al.*, 2016; Olsen *et al.*, 2015) which showed that in the absence of these genes *M. haemolytica* strains were resistant to all macrolide antibiotics. Conversely, a high frequency of *ermB* (50%), and *erm42* (45.2%) was identified in *P. multocida* strains isolated in Taiwan (Yeh *et al.*, 2017). The uncorrelation between the genotype and phenotype of antibiotic resistance in *M. haemolytica* was not focused on in this study, however, several mechanisms such as malfunctioning mutations in resistant genes or outer membrane, alter efflux pumps… could be involved (Hassani *et al*., 2022).

In general, the current study indicated that *M. haemolytica* from Phan Rang harbors different antimicrobial resistance genes that may be transferred to other pathogenic bacteria by horizontal gene transfer (Sahay *et al.*, 2020). More importantly, our finding is a warning for managing *M. haemolytica* in this area since most isolates were resistant and multidrug resistance, particularly to some common antimicrobials (oxytetracycline, tulathromycin, erythromycin, penicillin and ampicillin) that could have a significant impact on animal health and may be associated with resistant infections in humans (Ma *et al.*, 2021).

Conclusions: This present study has provided data about the prevalence and antimicrobial resistance of *M. haemolytica* from Phan Rang sheep in Vietnam. The results indicated that *M. haemolytica* was a strong correlation with the health status of the sheep (p-value < 0.05). The most obvious finding to emerge from this study is that *M. haemolytica* exhibited high multidrug resistance against antibiotics belonging to the beta-lactam, tetracyclin, and macrolide groups. Conversely, antibiotics of fluoroquinolone, aminoglycosides, and phenicol groups were highly effective in vitro against *M. haemolytica*. Several resistance genes associated with antibiotic resistance phenotypes were also identified, *tetB* and *tetH* were highly detected in oxytetracycline-resistant isolates, and the bla_{ROB1} gene was identified in isolates resistant to ampicillin or/and penicillin. No antibiotic resistance genes were detected in erythromycin- and tulathromycin-resistant isolates, these findings suggest further study is required to determine the genes, mechanism regulation involved in resistance, and resistance phenotype.

The study provides a first glimpse of the prevalence and antimicrobial resistance of *M. haemolytica* in Vietnam, which is valuable for the clinical control of Pasteurellosis, and for the development of technical assistance policies and strategies to reduce antimicrobial resistance in sheep rearing in Vietnam.

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Authors contribution: Conceptualization and writing original draft, P.V.N and K.C.T.N; investigation and data analysis, X.T.T.H. T.L.C and P.H.T; methodology and validation; critically revising and editing, B.V.L, P.V.N and K.C.T.N. All authors have read and agreed to the published version of the manuscript.

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