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Original article

ISOLATION AND ANTIMICROBIAL RESISTANCE OF *MANNHEIMIA HAEMOLYTICA* ON DAIRY FARMS IN SIBERIA

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Summary

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The frequency of *Mannheimia haemolytica* isolation on dairy farms in Siberia and the Republic of Kazakhstan in the period from 2006 to 2016 has been determined. Characterisation of phenotypes, biochemical and antimicrobial resistance profiles was carried out for 54 strains. Typing by the leukotoxin gene (*lktA*) was performed using the PCR. The isolates were classified into 7 *lktA*-negative and 47 *lktA*-positive strains. The highest prevalence of antimicrobial resistance was observed to benzylpenicillin (33.3%) and the lowest prevalence – to tulathromycin and sulfonamides (1.9%). Resistance of *M. haemolytica* strains isolated on the farms of the first category to the new generation antimicrobial drugs (spectinomycin and tulathromycin), suggested that these strains have been imported from abroad: a strong reason to implement a continuous system for surveillance of the circulation of resistant bacterial variants with respect to effective antimicrobial therapy.

Key words: antimicrobial resistance, cattle, *Mannheimia haemolytica*

INTRODUCTION

Respiratory diseases of cattle are widespread on dairy farms and are the primary cause of economic damage, including calf deaths, reduction in milk production, growth rates and weight gain in recovered animals, and also the increased costs of treatment and other activities (Glotov et al., 2002). Violation of feeding conditions, commingling, crowding and other factors can increase the risks of bronchopneumonia associated with the pathogens of viral and bacterial origin (Glotov et al., 2014a,b).

Mannheimia haemolytica is recognised as the most important bacterial pathogen associated with the bovine respiratory disease complex because it causes the most severe pathology and rapidly proliferates after stress or viral infection (Rice *et al.,* 2007).

Mannheimia haemolytica is a Gram-negative non-motile non-spore forming oxidase-positive facultative anaerobic bacterium, which is a commensal inhabitant of the nasopharynx and mucous

membrane of the upper respiratory tract and tonsils of cattle (Angen *et al*., 2002).

Of the 12 capsular serotypes, S1 and S6 are the principal etiologic agents of acute fibrinous bronchopneumonia in cattle accounting on average for 70 and 25% of the isolates from this animal species (Al-Ghamdi *et al.,* 2000). Serotype S2 is more common as a symbiont in the upper respiratory tract of healthy animals and in some cases is isolated from the affected lungs (Singh *et al*., 2011; Klima *et al*., 2014).

The bacterium has several factors of virulence – leukotoxin, capsule, lipopolysaccharide, iron-regulated outer membrane proteins, lipoproteins, a sialoglycoprotease, a neuraminidase and two potential immunoglobulin proteases, of these, leukotoxin (LCT) is the most well-studied. For *M. haemolytica*, the log phase of growth in the lungs is the most important virulence factor (Zecchinon *et al*., 2005). This protein is encoded by a 4-gene cluster - *lkt A-D*, of which the *lktA* gene is characterised by the greatest polymorphism and is serotype specific (Klima *et al*., 2016).

Antibiotics are widely used to treat respiratory diseases of cattle, resulting in the development of antimicrobial resistance (AMR) and isolation of multidrug-resistant (MDR) bacterial strains, as repeatedly reported by a number of authors (Katsuda *et al.*, 2013; Lubbers & Hanzlicek, 2013; Pipoz *et al*., 2016). Since 2006, the Russian Federation and the Republic of Kazakhstan massively imported livestock from Europe, Canada and Australia which could carry bacterial strains differing in pathogenic properties and AMR from local strains (Moore *et al.,* 2015).

Therefore, the aim of this study was to compare the morbidity and mortality of animals from respiratory diseases on dairy farms with different animal stocks and origin and to compare *M. haemolytica* strains isolated from animals of different origin with respect to their biochemical properties, the *lktA* gene and antimicrobial resistance profiles.

MATERIALS AND METHODS

Animals

The studies were conducted in eight regions of Western and Eastern Siberia and Northern Kazakhstan in 2006–2016. A total of 54 dairy farms, divided into three categories, were included in the study. The first category comprised 11 farms rearing only imported highly productive animals and their offspring (from 800 to 2000 heads). The animals were located in restricted access complexes under conditions excluding contact with aboriginal animals. The second category comprised 21 large farms with capacity of 500–1000 cows which in terms of productivity and rearing conditions were similar to first category farms but were free from imported animals. The third category included 23 medium and small dairy farms with cow population of 100 to 500, characterised by lower productivity and seasonal grazing on pastures. In all cases the cultivation of calves and heifers from birth to slaughter or insemination took place directly on the farm.

For the study, samples were derived from the lungs of culled or forcibly killed calves with symptoms of respiratory diseases. A total of 732 calves aged between 14 days and 12 months were sampled.

Bacteriological examinations

At the first stage of research conducted in 2006–2016 the frequency of *M. haemo-*

lytica isolation in calves with respiratory diseases was determined and a set of isolates was collected. For this purpose the lung samples obtained from culled or forcibly killed calves with symptoms of respiratory diseases were examined.

Samples (2–3 cm^3) were obtained from several sites on the boundary between normal and affected tissues not later than 2 hours post mortem. The samples were placed in sterile containers, cooled and stored for not more than two days at a temperature not higher than +4 ^oC until examination in the laboratory. In the laboratory the samples were homogenised in sterile physiological solution and the suspension was applied to the surface of blood agar supplemented with 5% defibrinated sheep blood cultured in an atmosphere of 5% CO_2 at 37 °C for 18–24 hours. Colonies with morphological characteristics of *M. haemolytica* (white-gray, round, medium-sized, non-mucous, with haemolysis) from each test were tested for catalase and oxidase production. Testing for oxidase production was carried out using available commercial test strips (Biovitrum, Russia), the catalase test was carried out on glass with 3% hydrogen peroxide. Isolates with morphological characteristics of *M. haemolytica* colonies, as well as positive for catalase and oxidase, were additionally tested by the PCR using specially developed primers for the *sodA* gene to identify *M. haemolytica* (Nefedchenko *et al*., 2016) (the PCR setting procedure is described below). A total of 235 isolates were derived, some of which were stored at –80^{o}C in brain heart infusion (BHI) broth supplemented with 40% glycerol for further study.

At the second stage of research conducted in 2015–2016 biochemical properties, antimicrobial susceptibility and identification of the *lktA* gene were performed in the preserved isolates. Out of a total of 235 strains, only 54 strans were successfully preserved, restored and used in subsequent studies. For this purpose 1 mL of the stored suspension was transferred to blood agar with the addition of 5% defibrinated sheep blood and cultured in 5% CO_2 at 37 ^oC for 24–48 hours. The grown colonies were purified, typed in a reaction to catalase and oxidase and by the PCR method for the *sodA* gene.

Phenotypic identification

Phenotyping of the strains was performed as described by Garrity *et al.* (2005) using reagent sets (Biovitrum, Russia). The phenotypic tests were used to identify α-fucosidase, β-galactosidase, β-glucosidase, β-xylosidase, D-xylose, indole, L-arabinose, maltose, mannitol, ornithine decarboxylase, sorbitol, trehalose and urease.

Antimicrobial susceptibility testing

The isolates were tested by the AMR agar diffusion method in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2013). Each isolate was tested for resistance to 24 antimicrobial drugs. The studies were carried out on a Mueller-Hinton medium supplemented with 5% sheep blood. The cultures were applied with a sterile cotton swab moistened with a bacterial suspension diluted to 0.5 according to the McFarland standard. The plates were then incubated at 37 °C for 24 h. The resistance breakpoints were interpreted according to the criteria provided for by CLSI documents M31–A4 CLSI, 2013 and Pharmacotherapy Research Centre (Russia). *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were

Table 1. Primer sequences and amplicon sizes used in the PCR assay

Gene target	Primer sequence 5'–3'	Annealing temp (°C)	Amplicon size (bp)	References
sodA	GACTACTCGTGTTGGTTCAGGCT CGGATAGCCTGAAACGCCT	57	126	Nefedchenko *et al.,* 2016
lktA	TGTGGATGCGTTTGAAGAAGG ACTTGCTTTGAGGTGATCCG	55	1146	Fisher *et al.,* 1999

used as quality control strains for interpretation of the results.

Polymerase chain reaction

DNA from the samples of bacterial cultures was extracted using a "Ribo-Prep" reagent kit in accordance with the manufacturer's recommendations (Interlabservis, Russia). The precipitated DNA was dissolved in 100 μL of ultra pure water and stored at –20 °C until the PCR.

The base sequences and the predicted sizes of the amplified products for specific oligonucleotide primers used to identify *M. haemolytica* and to detect the *lktA* genes are presented in Table 1. The PCR mixture (50 μL) should contain 5 μL bacterial DNA, PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 3.3 mM $MgCl_2$), 200 μM of each dNTP, and 1.0 U Smart Taq DNA-polymerase (Medigen, Russia), 10 μM of each primer.

The "16" strain of *M. haemolytica* serotype S1 and the "1231" strain of P. multocida from the collection of All-Russia Research Institute of Experimental Veterinary were used as positive and negative controls, respectively.

Statistical analysis

Differences in the bacterium isolation and antimicrobial susceptibility frequencies were determined by Chi-square test, and P-value ≤0.05 was considered significant.

RESULTS

Incidence of calf morbidity and mortality from respiratory diseases

At the time of the field investigations on the fifty four farms located in eight regions of Western and Eastern Siberia and Northern Kazakhstan the situation with bovine respiratory disease in the eleven large dairy complexes belonging to the first category was the worst, especially among the calves delivered by imported pedigree heifers. A peak incidence of respiratory diseases (Fig. 1, 2) was noted, especially among the calves of the first generation at the age of up to 1 month (630 per 1000 heads), and the highest mortality (64.3%) was noted for calves aged 3–6 months. On the large dairy farms not rearing imported animals, the maximum incidence of respiratory diseases was registered among the calves 1–3 months of age: 325 per 1000 heads with mortality rate of 28.5%. On middle and small farms among the calves 1–3 months of age the incidence was 52.1% and the mortality at the age of 3–6 months was 15.8%.

Of the 738 lung samples derived from the deceased or euthanised animals *M. haemolytica* was isolated from 235 samples (38.1%). On the farms of the first and second categories the frequency of bacterial isolation did not differ significantly

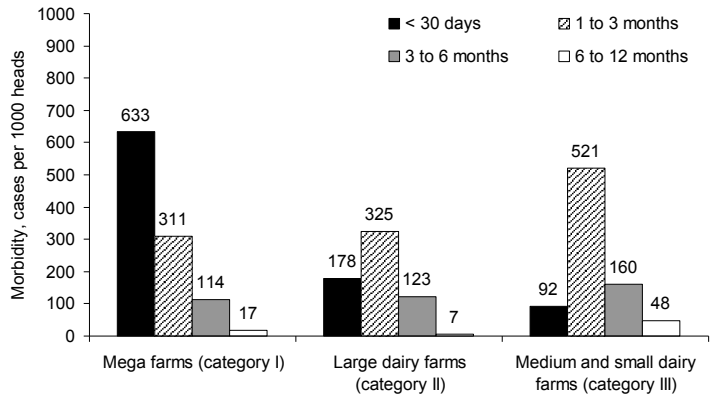

Fig. 1. Morbidity caused by respiratory diseases among the calves at farms of different categories according to the age **(**number of cases per 1000 heads).

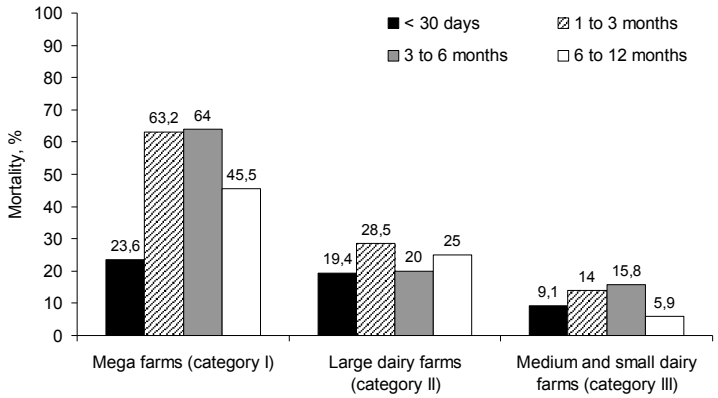

Fig. 2. Mortality from respiratory diseases among the calves at farms of different categories according to the age (% of the number of cases).

amounting to 34.2 and 35.3%, respectively. On the medium and small farms the bacterium was isolated in 26.3% of the studied samples.

Phenotypic characterisation of isolates, typing for lktA alleles

The phenotypic properties of all 54 isolates were characteristic of *M. haemolytica*. The isolates were Gram-negative nonmotile coccobacilli exhibiting haemolytic activity and fermenting mannitol, glucose, maltose, sorbitol and sucrose with the formation of gas and not fermenting trehalose, b-glucosidase, l-arabinose and not generating indole and urease. All 54 isolates were typed by the PCR as *M. haemolytica.*

According to the PCR results the isolates were divided into 2 groups – *lktA*-positive and *lktA*-negative. Of the 54 isolates, 47 (87.0%) carried the *lktA* gene, and 7 (13.0%) did not.

Table 2. Incidence of *M. haemolytica* strains resistant to antimicrobial drugs by farm category and leukotoxin gene typing (disc diffusion data)

Antimicrobial drugs	All strains (n=54)			Prevalence of resistant strains among different groups (%)				
				By farm category			By typing on the leukotoxin gene	
	% S	% I	% R	I (n=13)	II (n=19)	III (n=22)	positive (n=47)	negative (n=7)
Aminoglycosides								
Gentamicin	55.6	27.8	16.7	15.4	21.1	13.6	17.0	14.3
Kanamycin	68.5	7.4	24.1	23.1	36.8	13.6	25.5	14.3
Spectinomycin	92.6	1.9	5.6	23.1**	0.0**	0.0**	6.4*	0.0*
Streptomycin	77.8	5.6	16.7	23.1	15.8	13.6	17.0	14.3
Ansamycins								
Rifampicin	63.0	20.4	16.7	15.4	26.3	9.1	17.0	14.3
β-lactams								
A/CA	85.2	9.3	5.6	7.7	10.5	0.0	6.4	0.0
Amoxicillin	50.0	31.5	18.5	0.0	21.1	27.3	19.1	14.3
Ampicillin	57.4	20.4	22.2	15.4	31.6	18.2	23.4*	14.3*
Benzylpenicillin	66.7	0.0	33.3	38.5	36.8	27.3	31.9	42.9
Carbenicillin	35.2	37.0	27.8	23.1	26.3	31.8	29.8	14.3
Cephalosporins								
Cefalexin	85.2	11.1	3.7	7.7	5.3	0.0	2.1	14.3
Cefazolin	85.2	9.3	5.6	0.0	10.5	4.5	6.4	0.0
Chloramphenicols								
Levomycetin	46.3	25.9	27.8	15.4	21.1	40.9	31.9	0.0
Glycopeptides								
Vancomycin	51.9	27.8	20.4	23.1	26.3	13.6	23.4	0.0
Lincosamides								
Lincomycin	66.7	9.3	24.1	15.4	26.3	27.3	23.4	28.6
Macrolides								
Oleandomycin	68.5	9.3	22.2	15.4	31.6	18.2	25.5	0.0
Tulathromycin	92.6	5.6	1.9	7.7**	0.0**	0.0**	2.1	0.0
Tylosin	70.4	14.8	14.8	23.1	10.5	13.6	14.9	14.3
Polypeptides								
Polymixin	55.6	20.4	24.1	23.1	36.8	13.6	25.5	14.3
Quinolones								
Ciprofloxacin	83.3	7.4	9.3	7.7	10.5	9.1	8.5	14.3
Enrofloxacin	88.9	3.7	7.4	7.7	10.5	4.5	6.4	14.3
Sulfonamides								
T/S	90.7	7.4	1.9	0.0	5.3	0.0	0.0*	14.3*
Tetracyclines								
Oxytetracycline	55.6	18.5	25.9	46.2	15.8	22.7	27.7	14.3
Tetracycline	72.2	11.1	16.7	23.1	15.8	13.6	14.9	28.6

* $P \leq 0.05$; ** $P \leq 0.01$ in the chi-square test.

Antimicrobial susceptibility of strains

Out of the strains isolated on the farms of the first and second categories, only three did not demonstrate antimicrobial drug (AMD) resistance (Table 2). At the same time, the number of isolates resistant to common AMD, which have been in use for a long time (penicillin, tetracycline, chloramphenicol, gentamicin and kanamycin), ranged from 16.7% for gentamicin to 33.3% for benzylpenicillin. At the same time, the isolates showed relatively higher frequency of resistance (> 20%) to medications infrequently used on the farms: lincomycin and polymyxin. In each group of AMDs, resistance to common drugs was significantly higher than to the new ones. The susceptibility was significantly ($P<0.01$) higher to spectinomycin and kanamycin of the aminoglycoside group, to the combination of amoxicillin/clavulanic acid (A/CA) and cephalosporin of the β-lactams group, and to the macrolide tulathromycin ($P<0.01$).

The bacterial strains isolated on the farms of different categories had different antimicrobials resistance profiles. On average, each of them was resistant to 4 AMD. The isolates of different categories were ranked for their resistance to AMD (Table 3). On the farms of the second and third categories the largest group was made up of the isolates resistant to 1–3 AMDs as compared to 4–6 AMDs on the first category farms. On the farms of the first and second categories several strains were sensitive to all AMDs studied, and on the farms of the second category one strain was resistant to 10 AMDs.

Strains resistant to spectinomycin and tulathromycin were isolated only on the farms of the first category, and no strains resistant to amoxicillin, cefazolin and sulfonamides were isolated, although for the latter the differences were unreliable.

No significant differences were detected in the incidence of *lktA*-positive and *lktA*-negative strains on the farms of different categories. When comparing antimicrobial resistance profiles (Table 2), significant differences ($P\leq0.05$) were found between *lktA*-positive and *lktA*-negative strains with respect to their AMR to spectinomycin, ampicillin and sulfonamides.

Table 3. Ranking *M. haemolytica* strains in groups according to their antimicrobial resistance profile

	Prevalence of resistant strains among different groups, %				
	By farm category			By leukotoxin gene typing	
	I (n=13)	II (n=19)	II (n=22)	positive (n=47)	negative (n=7)
median	4	5	3.5	5	4
range*					
0	7.7	10.5	0	4.3	14.3
1–3	38.5	36.8	50.0	42.6	42.6
4–6	46.2	26.3	27.3	31.9	28.6
7–10	7.7	21.1	22.7	19.1	14.3
≥10	0	5.3	0	2.1	0

The results are given in %, except median; * number of AMD to which strains are resistant.

DISCUSSION

According to many researchers (Noyes *et al.,* 2015; Murray *et al*., 2017), *M. haemolytica* is the most important etiologic agent in the outbreaks of "shipping fever" among weaned feedlot calves, which is primarily associated with prolonged shipping, stress and prior viral infections (Hotchkiss *et al*., 2010).

Our previous studies indicated that bronchopneumonia of this form also occurred in imported heifers on the dairy farms of Siberia (Glotov *et al*., 2014a). In large dairy complexes in the first two to three years upon arrival the respiratory diseases of imported animals are caused by associations of several bacterial and viral agents (bovine viral diarrhoea virus, bovine respiratory syncytial virus, bovine herpes virus 1, bovine parainfluenza virus 3, *Pasteurella multocida* A and D, *Histophilus somni*). The prevailing agents are bovine viral diarrhoea virus and *M. haemolytica* (Glotov *et al*., 2014b).

Due to intrauterine infection with viral diarrhoea viruses and infectious bovine rhinotracheitis or transmission of highly pathogenic *M. haemolytica* isolates from mothers (Klima *et al*., 2014) the calves born from imported animals are more likely to develop respiratory diseases, especially in the first month of life, which was supported by the isolation of *M. haemolytica* from the lungs.

On the large farms not rearing imported animals as well as on the medium- and small-sized farms the situation with respiratory diseases was different. On such farms the animals are not normally purchased from other farms, but are reared from birth to insemination, which precludes exogenous infections. Respiratory diseases are more common in animals aged 1–3 months following commingling. The mortality in calves due to respiratory diseases is the highest at this age. The lack of scheduled laboratory examinations and erratic use of AMDs results in the accumulation of antimicrobial resistant strains. Such strains may circulate together with multidrug-resistant bacteria clones providing for high prevalence and mortality rates in young animals.

In Russia, the serological typing of *M. haemolytica* is unavailable, so we could not determine the serotypes of the derived isolates. Leukotoxin plays an important role in the pathogenesis of *M. haemolytica*- associated bovine respiratory disease by mediating cell lysis and apoptosis, thereby inducing fibrinous bronchopneumonia. Four genes present in all bacterial isolates are required to synthesise leukotoxin (Klima *et al*., 2011). Leukotoxin is synthesized *in vivo* by bacteria of all serotypes, but its production and biological characteristics differ between serotypes (Burrows *et al.,* 1993), which is mostly due to the polymorphism of the *lktA* gene. For instance, the bacterial strains of the first and sixth serotypes isolated from the cattle belong to lktA allele type 1.1, and the second serotype to *lktA* allele types 2.1 and 2.2. (Davies *et al*., 2001).

Initially, we intended to type the isolates by the *lktA* gene to determine their haemolytic activity and, accordingly, the pathogenicity as described in Fisher *et al.* (1999). However, *lktA*-negative strains also exhibited haemolytic activity and had a biochemical profile similar to that of *lktA*-positive strains. Comparison of the fragments of this gene in bacteria of different serotypes published in the GenBank database and the PCR simulation with the use of Vector NTI software and our proprietary DNA fragments were used to provisionally refer *lktA*-negative strains to non-pathogenic (commensal) serotype S2, and *lktA*-positive strains – to serotypes S1

and S6 that are highly pathogenic to cattle. Indirectly our classification is supported by the number of *lktA*-negative strains (13.0%), which agrees with the data of Singh *et al.* (2011) on the frequency of serotype S2 detected in cattle.

A number of authors (Katsuda *et al.,* 2013; Klima *et al.,* 2014) reported some differences in AMR isolates of different serotypes. According to our data, among the *lktA*-positive strains there were significantly ($P \leq 0.05$) more strains resistant to spectinomycin, ampicillin and susceptible to sulfonamides.

On average each *lktA*-positive strain was resistant to 5 AMDs whereas each *lktA*-negative strain was resistant only to 4 AMDs. Both groups included the strains sensitive to all AMDs but the first group had more multidrug-resistant strains to four or more AMDs.

Thus, typing bacteraemia strains and isolates by the leukotoxin gene can be a useful tool in the study of AMR pathogenic and non-pathogenic variants of the bacterium, which will allow searching for the most effective AMDs.

Antimicrobial drugs remain to date one of the most effective tools to control *M. haemolytica*-associated infections. According to many authors, antimicrobial drug resistance is a growing problem as the number of antimicrobial-resistant strain isolates increases while the efficacy of the drugs that have been used for a long time declines (Portis *et al*., 2012; Benedict *et al*., 2013; DeDonder & Apley, 2015).

As demonstrated in our studies, out of 54 strains, the majority showed multiple antimicrobial resistance – on the farms of the first category each strain was resistant on average to 4 AMDs, in the second category – to 5, and in the third – to 3.5 AMDs. A large number of multidrug-resistant strains on the farms of the first and second categories may be associated with the use of AMDs to prevent diseases in newborn calves. On the small and medium-sized farms this technique is generally not used, but AMDs are used only for the treatment of sick animals.

Analysis of the data obtained suggests that import of animals may lead to the migration of new highly pathogenic *M. haemolytica* strains previously not encountered in the country. This is supported by resistance to new previously unused antiobiotics (spectinomycin and tulathromycin) in bacterial strains isolated in the large complexes rearing animals imported from abroad. Long-term circulation of such strains is not excluded among livestock at large breeding complexes, the period of which is not defined. In addition, the erratic use of antimicrobial drugs can also lead to the emergence of multidrug resistant clones of bacteria on dairy farms not rearing imported animals. The cocirculation of such clones with viral pathogens can lead to high morbidity and mortality of calves in the first months of life.

CONCLUSION

M. haemolytica is an important respiratory pathogen of calves on the dairy farms of Siberia and the Republic of Kazakhstan. Different resistance of bacterial isolates derived from the large and small farms and resistance to old and new antimicrobial drugs require a system for surveillance of the circulating resistant *M. haemolytica* variants and the efficacy of antimicrobial therapy.

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