10.22059/IJVM.2022.333838.1005209

Iranian Journal of Veterinary Medicine
Volume 16- Issue 4

Original Article
Online ISSN: 2252-0554

Prevalence and Antibiotic Resistance Pattern of *Mannheima haemolytica* and *Pasteurella multocida* Isolated from Cattle Lung Samples from an Industrial Abattoir: A Study from Northeastern Iran

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Abstract

BACKGROUND: Bovine respiratory disease (BRD) is still one of the most important infectious diseases in dairy farms. *Pasteurella multocida* and *Mannheimia hemolytica* are the main bacterial pathogens of BRD.

OBJECTIVES: This study aimed to determine the prevalence and pattern of antibiotic resistance of *M. hemolytica* and *P. multocida* isolated from lung samples of cattle slaughtered in an industrial abattoir in Golestan province, northeastern Iran.

METHODS: Samples were taken from diseased and normal-appearing lungs of cattle slaughtered in an industrial abattoir. The samples were processed bacteriologically for the isolation of both *Pasteurellaceae* species. The isolates were identified by cultural and biochemical tests, and polymerase chain reaction (PCR) technique. Antimicrobial susceptibility of the isolates was determined using the Kirby–Bauer disc diffusion method.

RESULTS: A total of 120 samples were collected. The bacteriological examination of the samples resulted in the isolation of 36 (30%) *M. hemolytica* and *P. multocida* strains. Of the isolates, 14 (11.6%) and 22 (18.3%) were positive for *P. multocida* and *M. hemolytica,* respectively. Clear difference was observed between the populations of *M. haemolytica* recovered from apparently healthy lung tissues versus pneumonia samples. All the isolates were susceptible to tulathromycin, and the highest resistance was observed to penicillin and erythromycin. Multiple drug resistance was observed in both *Pasteurellaceae* species.

CONCLUSIONS: The results highlight the importance of continued surveillance of BRD pathogens in monitoring their antibiotic resistance patterns to update treatment protocols. Surveillance studies are also necessary to develop policies for limiting the spread of resistance.

KEYWORDS: Antimicrobial resistance, Cattle, Prevalence, *Pasteurella multocida, Mannheimia haemolytica*

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Received: 2022-01-10

Accepted: 2022-04-13

How to Cite This Article
Ashrafi, F ., Ahani Azari, A., Fozouni, L. (2022). Prevalence and Antibiotic Resistance Pattern of *Mannheima haemolytica* and *Pasteurella multocida* Isolated from Cattle Lung Samples from an Industrial Abattoir: A Study from Northeastern Iran. *Iranian Journal of Veterinary Medicine, 16*(4), 414-422.

Introduction

Bovine respiratory disease (BRD) is still one of the most important infectious diseases in dairy farms. It is the main cause of economic loss due to animal death, low milk yield, reduction in weight gain and growth rates, as well as increase in therapeutic costs and other economic measures (Nefedchenko *et al*., 2019). The economic burden of the disease is estimated to be around $1 billion in the United States alone (Lubbers & Hanzlicek 2013). Environmental stressors such as climate change, change in feed or housing, or transportation make animals more susceptible to BRD caused by viral and bacterial agents (Hodgins *et al*., 2002). *Pasteurella multocida*, *Mycoplasma bovis*, *Histophilus Somni*, *Arcanobacterium pyogenes*, and *Mannheimia hemolytica* are considered as BRD bacterial pathogens. These commensal bacteria colonize bovine nasopharynx and cause infection when cattle are under stress (Confer AW, 2009).

M. haemolytica is an opportunistic pathogen that is commonly isolated from the respiratory tract of healthy cattle. This organism, formerly known as *Pasteurella haemolytica*, is the main bacterial pathogen associated with BRD (Klima *et al*., 2014). *P. multocida* is another bacterial agent that causes BRD. This Gram-negative zoonotic bacterium can cause infection in domestic animals which can lead to significant economic losses.

In treatment of BRD in large commercial farms, the focus is on treating sick animals using metaphylactic antimicrobial therapy. The sick animals that do not respond to initial treatments are usually treated with another antimicrobial drug. Recently, treatment strategies for BRD have been investigated due to the observed antimicrobial resistance, especially multiple drug resistance (MDR) in isolates from cattle (Noyes *et al*., 2015).

Antimicrobial resistance, especially in isolates with MDR, has been reported by many researchers (Nefedchenko *et al*., 2019). Given the importance of BRD for animal husbandry economy and animal health, it is critical to gather more data on the prevalence and antimicrobial resistance of BRD-causative agents (Noyes *et al*., 2015).

Due to the limited data, this study aimed to determine the prevalence and pattern of antibiotic resistance of *M. hemolytica* and *P. multocida* isolated from lung samples of cattle slaughtered in an industrial abattoir in Golestan province, northeastern Iran.

Materials and Methods

Sample Collection

For this cross-sectional descriptive study, the lung samples were collected randomly from the cattle slaughtered in an industrial abattoir in Golestan province. Each sample was separately placed in a sterile ziplock plastic bag and transferred to the laboratory of Microbiology, Islamic Azad University, Gorgan Branch, not later than 2 hours after the animal`s death and stored cool not more than two days at 4°C until examination. From diseased samples, samples of 2-3 cm^3 were taken from cranioventral lung lobes showing hyperemia, rough surface, and consolidation symptoms.

Bacterial Isolation and Identification

After homogenizing the samples in a sterile physiological solution, the suspension was cultured on blood agar supplemented with 5% defibrinated sheep blood and incubated at 37°C for 24 h. The colonies showing the morphology of *M. haemolytica* (white-grayish, medium-sized round, non-mucoid, and β-hemolytic) and *P. multocida* (non-hemolytic, greyish, medium-sized round, and non-mucoid) were selected. The suspicious colonies were streaked on blood agar and McConkey agar and incubated at 37°C for 24 h. Afterwards, the samples were tested for catalase and oxidase production and Gram staining reaction. In addition, TSI, SIM, urea agar, MRVP, nitrate, gelatin, simmons citrate agar, and culture media containing sugars such as glucose, arabinose, mannose, trehalose, ONPG, and NPG were used to identify the microorganisms. The cultures were incubated at 37°C for 24 h (Khalili *et al*., 2016; Nefedchenko *et al*., 2019). A narrow zone of hemolysis and growth on McConkey agar plate but unable to produce indole, was considered *M. hemolytica* (Al-Haj Ali and Al-Balla, 2019). While Gram-negative coccobacilli, catalase, oxidase and indole positive and citrate utilization, methyl red and VP tests, gelatin hydrolysis negative that did not grow on McConkey agar with no

hemolysis on blood agar were considered *P. multocida* (Khamesipour *et al.*, 2014).

Species Confirmation by PCR

Preliminary identification of the isolates was carried out by standard cultural and biochemical tests. PCR assay was used to confirm the isolates. The bacterial isolate was inoculated into the brain-heart infusion (BHI) broth and incubated at 37°C for 18-24 h. Genomic DNA was extracted from the isolated overnight culture using Geno Plus TM Genomic DNA Extraction Miniprep System (Viogene, China). The quality and quantity of the extracted DNA were determined by agarose gel electrophoresis (1%). The primers used to detect the species are displayed in Table 1 (Rawat *et al*., 2019).

Table 1. Primers used for detection of *P. multocida* and *M. hemolytica*

Microorganism	Gene	Primer Sequence	Amplicon size (bp)
M. hemolytica	*PHSSA*	F5`TTCACATCTTCATCCTC3` R 5` TTTTCATCCTCTTCGTC3	327
P. multocida	*KMT1*	F5`ATCCGCTATTTACCCAGTGG3` R5`GCTGTAAAGAACTCGCCAC3`	457

Amplification was carried out in a 25-µL total reaction volume containing 0.5-µLTaq DNA polymerase, 5 µL of 5× reaction buffer (Bioline, UK), 1 µL of each primer, 1 µL of template DNA, and 16.5 µL of DEPC water. The PCR amplification for *PHSSA* gene was done with 35 cycles of denaturation at 95°C for 1 min, annealing at 48°C for 1 min, and elongation at 72°C for 30 s. The initial denaturation and final extension were 95°C for 3 min and 72°C for 5 min, respectively. For amplification of the *KMT1* gene, PCR was performed with 35 cycles of denaturation at 95°C for 45 s, annealing at 56°C for 45 s, and elongation at 72°C for 1 min. The initial denaturation and final extension were 95°C for 3 min and 72°C for 5 min, respectively (Rawat *et al*., 2019). The PCR products were detected by electrophoresis on a 1.5% agarose gel stained with Gel Red.

Antimicrobial Susceptibility Testing

Using the Kirby–Bauer disk diffusion method, the antimicrobial susceptibility testing of the isolates was performed according to the Clinical and Laboratory Standards Institute (CLSI, 2018) guidelines. The plates were incubated at 37°C for 18 h, and the results were interpreted after measuring the inhibition zone against each of the isolates. The antimicrobial agents (purchased from Padtanteb Company) including ceftiofur (30 µg), enrofloxacin (5 µg), florfenicol (30 µg), oxytetracycline (30 µg), spectinomycin (10 µg), tilmicosin (30 µg), tulathromycin (30 µg), erythromycin (30 µg), cotrimoxazole (1.25 µg), streptomycin (30 µg), ampicillin (30 µg), and penicillin (10 µg) were used in this study. The results were interpreted as resistant (R), intermediate (I), and susceptible (S) according to the criteria provided by the CLSI. *Escherichia coli* ATCC 25922 was used as the quality control strain for the interpretation of results (provided from Tehran University, Faculty of Veterinary Medicine).

Statistical Analysis

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

Results

A total of 120 lung samples, including apparently healthy (n=60) and those containing hepatization and consolidation in cranioventral lobes (n=60), were collected randomly from an industrial slaughterhouse in Golestan province during April to September 2020. The bacteriological examination of the samples resulted in the isolation of 36 (30%) *M. hemolytica* and *P. multocida* strains that were further confirmed by PCR technique. Of the isolates, 14 (11.6%) and 22 (18.3%) were positive for *P. multocida* and *M. hemolytica,* respectively. Eleven apparently healthy lungs were positive for both bacteria (Table 2). Significant difference was observed between the populations of *M. haemolytica* recovered from apparently healthy lung tissues versus diseased samples ($P \leq 0.05$), while no significant differences were found between the populations of *P. multocida*.

Table 2. Incidence of *M. hemolytica* and *P. multocida* in respiratory tract of apparently healthy and diseased lungs

Samples	Number of the samples	Number (percentage) of positive isolates for	
		P. multocida	*M. hemolytica*
Apparently healthy	60	6 (10)	5 (8.3)
Diseased	60	8 (13.3)	17 (28.3)
Total	120	14 (11.6)	22 (18.3)

In addition, other bacterial species such as *Staphylococcus aureus*, *Staphylococcus epidermidis, Streptococcus pneumonia*, and *E. coli* were isolated and identified according to the phenotypic characteristics and biochemical tests.

Antimicrobial resistance profiles of the isolates are displayed in Tables 3 and 4. Based on the results, all the isolates were susceptible to tulathromycin. Resistance to erythromycin (55.5% of the isolates) was remarkable due to their importance in animal production and human health. Among the *M. hemolytica* isolates, the highest resistance was observed to penicillin, ampicillin, and erythromycin (54.5%). Of these isolates, 28.5% were MDR. In addition to tulathromycin, all the *P. multocida* isolates were susceptible to florfenicol and tilmicosin. In these isolates, the highest level of resistance was found to penicillin and erythromycin (57.1%), and 22.7% of the isolates were MDR.

M. haemolytica and *P. multocida* isolates were further identified by the specific amplification of the *PHSSA* (327 bp) and *KMT1* (457 bp) genes, respectively (Figures 1 and 2).

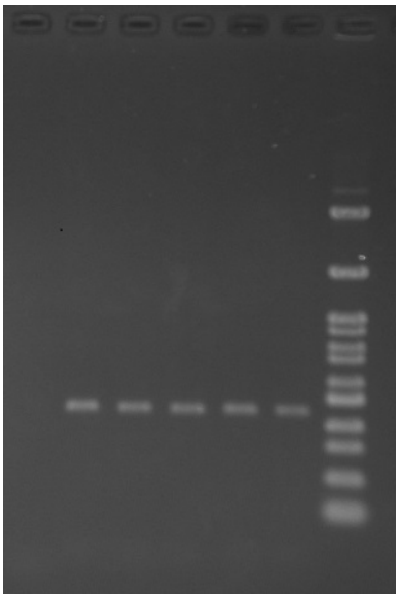

Figure 1. Amplification of *KMT1* gene (457 bp) specific to *Pasteurella multocida*

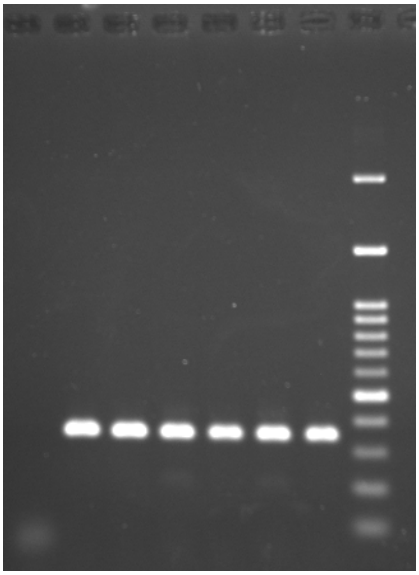

Figure 2. Amplification of *PHSSA* gene (327 bp) of *Mannheimia haemolytica*

Table 3. Antimicrobial susceptibility testing of the *M. hemolytica* isolates against 12 antimicrobial agents

Antimicrobial Agents	Number (percentage) of isolates		
	Susceptible	Intermediate	Resistant
Ceftiofur	19 (86.3)	0 (0)	3 (13.6)
Enrofloxacin	18 (81.8)	0 (0)	4 (18.1)
Florfenicol	20 (90.9)	0 (0)	2 (9)
Oxytetracycline	16 (72.7)	1 (4.5)	5 (22.7)
Spectinomycin	19 (86.3)	0 (0)	3 (13.6)
Tilmicosin	20 (90.9)	0 (0)	2 (9)
Tulathromycin	22 (100)	0 (0)	0 (0)
Erythromycin	7 (31.8)	3 (13.6)	12 (54.5)
Co-trimoxazole	17 (77.2)	0 (0)	5 (22.7)
Streptomycin	18 (81.8)	0 (0)	4 (18.1)
Ampicillin	7 (31.8)	3 (13.6)	12 (54.5)
Penicillin	7 (31.8)	3 (13.6)	12 (54.5)

Table 4. Antimicrobial susceptibility testing of the *P. multocida* isolates against 12 antimicrobial agents

Antimicrobial Agents	Number (percentage) of isolates		
	Susceptible	Intermediate	Resistant
Ceftiofur	10 (71.4)	1 (7.1)	3 (21.4)
Enrofloxacin	12 (85.7)	0 (0)	2 (14.2)
Florfenicol	14 (100)	0 (0)	0 (0)
Oxytetracycline	11 (78.5)	0 (0)	3 (21.4)
Spectinomycin	7 (50)	2 (14.2)	5 (35.7)
Tilmicosin	14 (100)	0 (0)	0 (0)
Tulathromycin	14 (100)	0 (0)	0 (0)
Erythromycin	4 (28.5)	2 (14.2)	8 (57.1)
Co-trimoxazole	7 (50)	0 (0)	7 (50)
Streptomycin	11 (78.5)	1 (7.1)	2 (14.2)
Ampicillin	8 (57.1)	0 (0)	6 (42.8)
Penicillin	6 (42.8)	0 (0)	8 (57.1)

Discussion

Considering the role of *M. hemolytica* and *P. multocida* bacterial species in causing respiratory diseases in cattle, knowledge of their frequency and pattern of antibiotic resistance is of importance. In the present study, we aimed to isolate, identify, and monitor the antibiotic resistance of *M. hemolytica* and *P. multocida* isolated from lung tissue samples collected from an industrial slaughterhouse in Golestan province. Based on the results, out of 120 bovine lung samples, 30% were positive for *P. multocida*

(11.6%) and *M. hemolytica* (18.3%), respectively. Different isolation rates have been reported by other researchers in and outside the country. In one study, the prevalence of *M. haemolytica* isolated from feedlot cattle over a 3-year period was reported 16.9% (Alexander *et al*., 2013). Moreover, in a study conducted in Syria, the highest rate of *M. haemolytica* isolation was 21.8% from pneumonic lung tissues (Al-Haj Ali and Al-Balla, 2019). In contrast with the present findings, Haji Hajikolaei *et al*. (2010) reported that 1.6% of the sampled cattle were positive for *M. haemolytica* in an abattoir in Ahvaz (Haji Hajikolaei *et al*., 2010). Based on a study by Khamesipour *et al*. (2014), 30 *P. multocida* strains were isolated from 333 pneumonic and apparently healthy slaughtered cattle (Khamesipour *et al*., 2014). Additionally, another study showed that all 12 studied pneumonic sheep lungs were positive for *M. haemolytica* (Kumar *et al*., 2015). In a study by Noyes *et al.* (2015), *M. haemolytica* was recovered from 29% of deep nasopharyngeal swabs (Noyes *et al*., 2015). In a study from India, 5 *M. haemolytica* and 3 *P. multocida* strains were isolated from 8 clinical cases. Moreover, both *M. haemolytica* and *P. multocida* were co-isolated from two lung tissue samples (Rawat *et al*., 2019). In line with the present work, the isolation rate of *P. multocida* from 175 lung, liver, and spleen samples was reported 10.3% by Sugun *et al*. (2016). In research by Valadan *et al.* (2014), out of 1454 samples, only 54 samples (3.71%) were positive for *P. multocida* (Valadan *et al*., 2014). Sahay *et al*. (2020) recovered 27 (7.2%) *M. haemolytica* and 28 (7.4%) *P. multocida* from 374 healthy and infected samples (Sahay *et al*., 2020).

According to the antimicrobial results, all the isolates were susceptible to tulathromycin, and the highest resistance was observed to penicillin and erythromycin. The sensitivity rates for *M. haemolytica* were between 31.8% (to penicillin, ampicillin, and erythromycin) and 100% (to tulathromycin), whereas sensitivity of the *P. multocida* isolates ranged from 28.5% (to erythromycin) to 100% (to tulathromycin, florfenicol, and tilmicosin). In a study conducted by Sebbar *et al.* (2019), the sensitivity rates of *M. haemolytica* to erythromycin and streptomycin were in line with our findings (Sebbar *et al*., 2019). In the present work, all the isolates were susceptible to tulathromycin while in a study by Timsit *et al*. (2017), high levels of resistance (>70%) to tulathromycin were reported in *M. haemolytica* and *P. multocida* isolates (Timsit *et al*., 2017). In our study, high resistance rate was observed to erythromycin (55.5% of the isolates) that was in agreement with the result of Anholt *et al.* (2017). In the current study, the ampicillin resistance rate was 54.5% for *M. haemolytica*, whereas it was reported 6% by Cassidy *et al.* (2014). In a similar study, the sensitivity rates of *P. multocida* to enrofloxacin and erythromycin were in line with those of our study (Khamesipour *et al*., 2014). Moreover, in a study carried out in Russia, the prevalence of antimicrobial resistance of *M. haemolytica* to ampicillin, streptomycin, and enrofloxacin was the same as our findings (Nefedchenko *et al*., 2019). In India, the resistance rates to penicillin, oxytetracycline, and streptomycin for both pathogens were similar to our findings (Sahay *et al*., 2020). Similarly, in a study from Mashhad, more than 90% of the *M. haemolytica* tested were susceptible to florfenicol (Mohammadi *et al*., 2006). In contrast to the present study, all *P. multocida* strains isolated from cattle and buffalo from Ahwaz were sensitive to cotrimoxazole (Gharibi *et al*., 2017).

In most studies including ours, high resistance rates of *M. haemolytica* to ampicillin and penicillin have been reported, whereas in a study from Mexico City, all the *M. haemolytica* strains were susceptible to these antimicrobials (Samaniego *et al*., 2011).

As seen here, the prevalence and antibiotic resistance pattern of *M. haemolytica* and *P. multocida* significantly varied in different studies. These differences may be due to the changes in climate, season, breeding, commercial practices, treatments, and management strategies of infected cattle (Ghadrdan Mashhadi *et al*., 2009).

Conclusion

In summary, the prevalence of *M. haemolytica* was more than that of *P. multocida* in the lung samples obtained from the apparently healthy and diseased cattle. In addition, clear difference was observed between the populations of *M. haemolytica* recovered from apparently healthy samples versus diseased samples. All the isolates were susceptible to

tulathromycin, and the highest resistance was observed to penicillin and erythromycin. MDR was observed in both *Pasteurellaceae* species.

For the veterinarians, the results highlight the importance of constant monitoring of antibiotic resistance patterns of BRD pathogens to update treatment protocols. Surveillance studies are also necessary to develop strategies for limiting the spread of resistance.

Acknowledgments

This study was extracted from a dissertation performed by Fatemeh Ashrafi for a Master of Science degree in Microbiology. The Department of Microbiology, Islamic Azad University, Gorgan Branch is acknowledged for providing necessary laboratory facilities.

Conflict of Interest

The authors declared no conflicts of interest exist.

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10.22059/IJVM.2022.333838.1005209

Iranian Journal of Veterinary Medicine

Abstracts in Persian Language

Online ISSN 2252-0554

مجله طب دامی ایران، ۱۴۰۱، دوره ۱۶، شماره ۴، ۴۲۲-۴۱۴

فراوانی و الگوی مقاومت آنتی بیوتیکی *Mannheima haemolytica* و *Pasteurella multocida* جداشده از نمونه‌های ریوی گاو در یک کشتارگاه صنعتی: مطالعه‌ای از شمال شرق ایران

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(دریافت مقاله: ۲۰ دی ماه ۱۴۰۰، پذیرش نهایی: ۲۴ فروردین ۱۴۰۱)

زمینه مطالعه: بیماری تنفسی گاو (BRD) همچنان یکی از بیماری‌های مهم در گاوداری‌ها است. *Pasteurella multocida* و *Mannheimia haemolytica* پاتوژن‌های باکتریایی اصلی BRD هستند.

هدف: هدف از این مطالعه تعیین شیوع و الگوی مقاومت آنتی‌بیوتیکی *M. hemolytica* و *P. multocida* جداشده از نمونه‌های ریه گاوهای یک کشتارگاه صنعتی واقع در استان گلستان، شمال شرق ایران، بود.

روش کار: نمونه‌های ریه (شامل ریه‌های درگیر پنومونی و ظاهراً سالم) به‌طور تصادفی از گاوهای کشتار شده در یک کشتارگاه صنعتی جمع‌آوری شد. نمونه‌ها برای جداسازی هر دو گونه *Pasteurellaceae* از نظر باکتریولوژیکی پردازش شدند. جدایه‌ها با آزمایش‌های کشت و بیوشیمیایی و تکنیک واکنش زنجیره‌ای پلیمراز (PCR) شناسایی شدند. حساسیت ضد میکروبی جدایه‌ها با استفاده از روش انتشار دیسک کربی‌بائر تعیین شد.

نتایج: در مجموع ۱۲۰ نمونه جمع‌آوری شد. بررسی باکتریولوژیک روی نمونه‌ها منجر به جداسازی ۳۶ (۳۰٪) سویه *M. hemolytica* و *P. multocida* شد. از بین جدایه‌ها، ۱۴ (۱۱/۶٪) و ۲۲ (۱۸/۳٪) *P. multocida* و *M. hemolytica* مثبت بودند. تفاوت واضحی بین جمعیت *M. haemolytica* که از بافت‌های ظاهرا سالم ریه در مقابل نمونه‌های درگیر پنومونی به‌دست آمده بود مشاهده شد. تمامی جدایه‌ها به تولاترومایسین حساس بودند و بیشترین مقاومت به پنی‌سیلین و اریترومایسین مشاهده شد. مقاومت چند دارویی در هر دو گونه *Pasteurellaceae* مشاهده شد.

نتیجه‌گیری نهایی: نتایج بر اهمیت نظارت مستمر پاتوژن‌های BRD برای نظارت بر الگوهای مقاومت آنتی‌بیوتیکی آنها برای به‌روزرسانی پروتکل‌های درمانی تأکید می‌کند. مطالعات نظارتی نیز برای به‌کارگیری اقداماتی برای محدود کردن گسترش مقاومت ضروری خواهد بود.

واژه‌های کلیدی: فراوانی، گاو، مقاومت آنتی بیوتیکی ، *M. haemolytica*، *P. multocida*

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