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Isolation and Identification of Bovine *Pasteurella Multocida* Type A and Its Drug Sensitivity Analysis

Haqmal Mohammad Aman[1*], Mohmand Noor Ali[2], Wasim Wakil Ahmad[3], Afghan Rohullah[4], Mohammadi Aminullah[5], Tookhy Nazir Ahmad[6], Mirzaee Ali Ahmad[7]

[1,4,5]Department of Food Technology, Faculty of Veterinary Science, Herat University, Herat, Afghanistan.
[2,3,7]Department of Pre-Clinic, Faculty of Veterinary Science, Herat University, Herat, Afghanistan.
[6]Department of Para-Clinic, Faculty of Veterinary Science, Herat University, Herat, Afghanistan.

*Corresponding author: mahaqmal786@gmail.com

ABSTRACT

Pasteurella multocida is a bacteria frequently associated with pneumonia in bovine respiratory disease. *P. multocida* was initially discovered in 1878 AC in cholera-infected poultry as causitive agetnt for serious infectious diseases such as avian cholera, bovine hemorrhagic septicemia, and porcine atrophic rhinitis. This study aimed to isolate and identify *Pasteurella multocida* type A, and conducted a drug sensitivity analysis. To identify *P. multocida* type A. 30 samples were collected and sent to a lab from farms and free-range farmers in Changchun, Yushu, Siping, Nong'an, Yanji, Baicheng, Dunhua, Baishan, and other areas of Jilin Province during 2020 and 2022. The study was designed to perform bacterial isolation and identification, preservation, and drug susceptibility testing. The PCR amplification procedure used 16S rDNA primers. The desired 1450 bp target bands were attained. The target bands were isolated, attached to the pMD18-T vector, and transformed into DH5-competent cells. Positive cells were then selected by screening. The clones were sequenced, and the resulting sequences were compared with those in GenBank using the BLAST algorithm. In the result, a total of Twelve *P. multocida* strains were discovered. The MIC responses of the examined strains to the 12 antibacterial medications showed notable variations. The maximum MIC values for gentamycin and kanamycin were 64 µg/mL and 32 µg/mL, respectively, for the tested strains of tilmicosin were ≤32 µg/mL. The MIC values for florfenicol and doxycycline appeared greater than 512 µg/mL. The MIC for ciprofloxacin and enrofloxacin is up to 16 µg/mL. There are significant disparities in the levels of antibiotic resistance among the tested strains. The drug resistance rates of the tested strains to several antibacterial medications were tallied, and tilmicosin had the highest drug resistance rate (68%), followed by terramycin (63%). The other strains displayed varied degrees of drug resistance, with resistance rates below 50%. However, sulfadiazine didn't shown any resistance.

Keywords: Bovine respiratory disease, Drug sensitivity analysis, Identification, Isolation, *P. multocida*.

INTRODUCTION

Pasteurella multocida was initially discovered in 1878 in poultry affected by cholera; however, it was not isolated until 1880, at which time it was named in honor of Louis Pasteur (Harper, 2020). This bacterium, part of the Pasteurellaceae family, is the causative agent of several economically significant diseases in livestock. Pasteurellosis is a multifactorial respiratory disease, while haemorrhagic septicemia (HS) is a severe, acute septicemic illness primarily affecting cattle and buffaloes (Harper, 2020). HS, explicitly caused by *P. multocida*, remains a critical veterinary health concern, particularly in regions of Asia and Africa (Mahmood et al., 2023).

Strains of *P. multocida* are categorized based on serological classification into 16 somatic lipopolysaccharide types and five capsular types (A, B, D, E, and F) (Rasheed et al., 2021; World Organisation for Animal Health [WOAH], 2022). Molecular identification methods, such as outer membrane protein (OMP) and 16S ribosomal RNA (rRNA) typing, further elucidate its diversity. Recent molecular studies reveal significant heterogeneity in capsular forms, OMP types, and host range (Bote et al., 2021). Serotypes

B2 and E2 are particularly associated with HS, primarily affecting livestock in Africa and Asia (Mahmood et al., 2023).

P. multocida can be isolated from blood samples and the upper respiratory tracts of calves with high morbidity and mortality. It is also responsible for economically important diseases, including avian cholera, bovine haemorrhagic septicemia, and porcine atrophic rhinitis (Bote et al., 2021). The bacterium often resides harmlessly in the tonsils and nasopharynx of healthy cattle but can become an opportunistic pathogen under specific conditions. Factors such as exposure to mechanical dust, heavy rainfall, transportation stress, and concurrent infections—including Foot-and-Mouth Disease (FMD) and parasitic infestations—increase susceptibility to pasteurellosis (Bote et al., 2021). These stressors, when combined with infection by *P. multocida*, can result in significant morbidity and mortality, whereas isolated stressors rarely lead to widespread outbreaks (Mahmood et al., 2023).

The diagnosis of pasteurellosis relies on clinical symptoms, distinctive pathological changes, morbidity and mortality trends, and confirmation through pathogen isolation and molecular methods (Rasheed et al., 2021). In Ethiopia's Benshangul Gumuz Regional State, frequent movement of cattle to markets, grazing areas, and water sources poses a risk of disease transmission. Cattle from Sudan and Egypt, where *P. multocida* serotypes B2 and E2 are endemic, often intermingle with Ethiopian livestock, increasing the risk of HS outbreaks, especially among unvaccinated herds (WOAH, 2022; Mahmood et al., 2023). The cross-border movement of livestock from neighboring regions such as Oromia also contributes to the potential for disease transmission. Recent studies emphasize the importance of coordinated vaccination programs and the monitoring of livestock movement to mitigate risks associated with *P. multocida* infections (Mahmood et al., 2023; Rasheed et al., 2021).

MATERIALS AND METHODS

Sample collection

From 2020 to 2022, 30 samples were collected from farms and free-range farmers in Changchun, Yushu, Siping, Nong'an, Yanji, Baicheng, Dunhua, Baishan, and other areas of Jilin Province, China, and then sent to the basic veterinary medicine laboratory. Sterile cotton swabs were used to collect the nasal mucus from animals exhibiting low energy, poor appetite, purulent nasal discharge, and dyspnea. The nasal swabs were placed in sterilized centrifuge tubes, stored at low temperature, and then returned to the laboratory promptly.

Isolation and identification of bacteria

Nasal swabs were collected aseptically and placed into pre-prepared test tubes containing 5 mL of sterilized Brain Heart Infusion (BHI) medium, followed by overnight incubation. The resulting bacterial culture was streaked onto BHI agar plates and incubated overnight at 37°C. Tiny, transparent colonies were selected and inoculated into fresh BHI medium, which was then cultured overnight on a shaker at 37°C. A 1 mL aliquot of the overnight culture was centrifuged at 8000 rpm for 6 minutes in a 1.5 mL sterile EP tube, and the supernatant was discarded. The pellet was resuspended in 50 µL of 1× TE buffer (pH 8.0), boiled for 10 minutes, cooled in an ice bath for 5 minutes, and then centrifuged at 12,000 rpm for 2 minutes. The resulting supernatant served as the template for DNA. For 16S rDNA identification, primers and PCR reaction conditions (as detailed in Tables 1 and 2) were employed, and three µL of the PCR product were analyzed using 1% agarose gel electrophoresis. A clear and bright target band of approximately 1450 bp was observed and sent to Sangon Bioengineering (Shanghai) Co., Ltd. for sequencing. Sequencing results were assembled and analyzed using the NCBI database. Additionally, for KMT1 and capsular serotype identification, the Pm species-specific KMT1 gene primer sequence

designed by Townsend, along with capsular serotype-specific primers for A, B, and D serotypes (capA, capB, capD), were synthesized by Sangon Bioengineering and utilized for further analysis.

Table 1. Primer sequences, annealing time, and length of the target gene

Primer name	Sequence（5'-3'）	（Tm ℃）	Target gene length（bp）
16s	F: ATCCGCTATTTACCCAGTGG R: GCTGTAAACGAACTCGCCAC	55	1450
Kmt1	F: ATCCGCTATTTACCCAGTGG R: GCTGTAAACGAACTCGCCAC	55	457
HyaD-hyaC	F: TGCCAAAATCGCAGTCAG R: TTGCCATCATTGTCAGTGCA	54	1044
BcbD	F: CATTTATCCAAGCTCCACCC R: GCCCGAGAGTTTCAATCCC	54	345
FcbD	F: TCCGCAGAAAATTATTGACTC R: GCTTGCTGCTTGATTTTGTC	53	511

PCR reaction 50 μL system: 2×mix Taq enzyme, 25 μL; ddH2O, 17 μL; template, 4 μL; upstream primer, 2 μL; downstream primer, 2 μL.

Table 2. PCR reaction conditions

	Step	Temperature (℃)	Time (min)
	Pre-denatured	95	10
30 Cycles	Transsexual	95	5
	Annealing	55（54、5）	1
	Extend	72	2
	Last extension	72	10

Bacteria preservation

700 μL of the isolated and purified bacterial solution was taken and added to a 1.5 mL EP tube. Then, 300 μL of 80% glycerol was added. The mixture was mixed well, the tube was sealed, and stored at -80°C for later use.

Drug susceptibility testing

The micro-broth dilution method was used to detect the drug sensitivity of *P. multocida*. The test was mainly based on the micro-broth dilution method recommended by Clinical & Laboratory Standards Institute (VET08, 2018) to determine the minimum inhibitory concentration (MIC) of the test drug against the main pathogenic bacteria of the clinically isolated bovine respiratory tract since there is no relevant document stipulating the quality control bacteria for Pm drug susceptibility testing, Staphylococcus aureus ATCC29213 was selected as the quality control strain of the test drug in this study, and each strain was re-identified before the experiment to eliminate contamination, and each test was repeated 3 times.

After removing the freeze-dried or glycerin-frozen bacteria, dissolve the freeze-dried powder in Cation-adjusted Mueller Hinton broth (CaMHB) with 5% serum or allow the glycerin-frozen bacteria to thaw before transferring 20 μL into CaMHB broth containing 5% serum. Incubate in an oxygenated environment at 35°C in a constant-temperature incubator for 18 to 24 hours. If the clarified broth becomes turbid, it is determined that the bacterial recovery is successful. Take the resuscitated bacterial liquid and streak it on the blood plate (MHA+5% defibrated sheep blood) for 2~3 passages to restore vitality. Pick a single colony that has been propagated for 2-3 generations and inoculate it in CaMHB

broth containing 5% serum, shake, and culture on a shaker at 35°C and 220 rpm for 6-8 h to the logarithmic growth phase. It can be used for drug susceptibility tests when the turbidity reaches $1\sim2\times10^8$ CFU/mL. The prepared bacterial solution or colony suspension can be stored in a refrigerator at 2-8°C for 24 hours for later use. It is essential to ensure that the bacterial culture is in the logarithmic growth phase; otherwise, it should be discarded. A detailed record of the bacterial solution preparation must be maintained. When determining the bacterial MIC, the original bacterial solution must be diluted to 1×10^6 CFU/mL with CaMHB broth containing 5% serum as the test bacterial solution.

Prepare drug stock solutions according to CLSI (VET08, 2018) standards, as outlined in Table 3. After preparation, filter and sterilize the solutions, then store them at -20°C for future use. Macrolide antibiotics (such as tilmicosin, terramycin, gentamycin, and tetracycline) and sulfonamides are unstable in solution form and should be prepared and used immediately.

Table 3. Antibacterial drugs preparation method.

Drug	Solvent	Diluent	Stock Solution Concentration (μg/mL)
Florfenicol	95% Ethanol	Water	2560
Amoxicillin	0.1 mol/L pH6.0 PBS	0.1 mol/L pH6.0 PBS	1280
Ceftiofur	Water	Water	2560
Terramycin	0.015 mol/L Citric acid	Water	2560
Gentamycin	0.2 mol/L pH6.0 PBS	Water	2560
Tilmicosin	95% Ethanol	Water	2560
Kanamycin	Water	Water	2560
Enrofloxacin	Water (add NaOH until completely dissolved)	Water	2560
Doxycycline	Water	Water	2560
Sulfadiazine	0.5 mol/L NaOH	Water	12160
Tetracycline	Water	Water	20480
Ciprofloxacin	Water	Water	20480

Table 4. Drug dilution and quality control scope

Drug	Dilution range	Quality control range（μg/mL） Staphylococcus aureus ATCC29213
Florfenicol	0.25-128	2-8
Amoxicillin	0.06-32	-
Ceftiofur	0.06-32	0.25-1.0
Terramycin	0.25-128	2-8
Gentamycin	0.25-128	0.5-4
Tilmicosin	0.25-128	1-4
Kanamycin	0.25-128	1-4
Enrofloxacin	0.02-8	0.03-0.12
Doxycycline	0.06-32	0.12-0.5
Sulfadiazine	2.28-1216	≤9.5
Tetracycline	0.06-512	1-4
Ciprofloxacin	0.02-512	0.03-0.06

The MIC was determined using the micro-broth dilution method, following the guidelines outlined in the CLSI VET08 document (2018). This method involves serially diluting the antimicrobial agent in a liquid growth medium to assess the lowest concentration that effectively inhibits bacterial growth.

The drug stock solution was diluted with CaMHB broth to prepare a solution with a concentration of 256 μg/mL, which was then serially two-fold diluted using the same broth. A total of 100 μL of each diluted drug solution was added to the 1st to 10th column wells of a microwell plate in decreasing concentrations, resulting in final concentrations in the wells of 256 μg/mL, 128 μg/mL, 64 μg/mL, 32 μg/mL,

16 µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL, and 0.5 µg/mL. Subsequently, 100 µL of a test bacterial solution (10^6 CFU/mL) was added to the same wells, resulting in final drug concentrations ranging from 128 µg/mL to 0.25 µg/mL. For controls, 200 µL of the diluted bacterial solution was added to the 11th column as a positive control (bacteria without drugs), and 200 µL of CaMHB broth was added to the 12th column as a negative control (sterile without drugs). The bacteria-drug mixture was thoroughly mixed, and the plate was sealed with adhesive tape. It was then placed in an enamel square plate with wet gauze and incubated at 35°C for 18–24 hours. After incubation, the plate was observed against a black bottom under light to assess bacterial growth, indicated by diffuse turbidity or precipitate at the U-shaped bottom of the wells. The lowest drug concentration that inhibited visible bacterial growth was recorded as the MIC. Each batch of tests included quality control using *Staphylococcus aureus* ATCC29213 and Streptococcus species, ensuring that the MIC values for these controls fell within the expected range. MIC determinations for each test strain were performed in duplicate, with a third test conducted in cases of inconsistent results.

RESULTS

Isolation and identification

PCR amplification was performed using 16S rDNA primers, and the results are presented in Figure 1. Target bands of approximately 1450 bp were obtained, purified, ligated into the pMD18-T vector, and transformed into DH5α competent cells, followed by screening for positive clones. The clones were sequenced, and the sequence results were further compared and analyzed with sequences registered in GenBank using BLAST search software. This analysis yielded 12 strains of *P. multocida.*

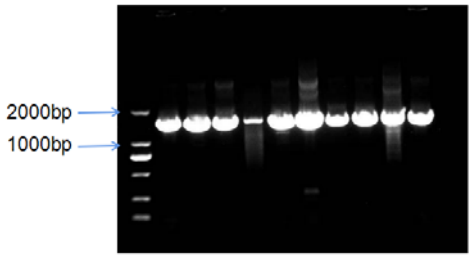

Fig. 1 The PCR results of the 16S rDNA of clinical isolation

The P. multocida drug susceptibility test results:

The isolated strains were tested for susceptibility to florfenicol, ceftiofur, tetracycline, gentamicin, ciprofloxacin, enrofloxacin, doxycycline, tilmicosin, sulfadiazine, terramycin, amoxicillin, and kanamycin using the micro-broth dilution method. Their test results against 12 antibacterial drugs are presented in Table 3.

There are significant differences in the MIC results of the tested strains to the 12 Among them, the MIC values of tested strains for tilmicosin were ≤32 µg/mL, and the maximum MIC values for gentamycin and kanamycin were 64 µg/mL; the MIC values for florfenicol and doxycycline appeared to be greater than 512 µg/mL; the MIC for ciprofloxacin and enrofloxacin is up to 16 µg/mL; the MIC for ceftiofur is up to 4 µg/mL, and the MIC for terramycin is up to 16 µg/mL, but it is generally concentrated around 8 µg/mL. It can be observed that the tested strains exhibit significant differences in their resistance to various antibiotics.

Table 1. The MIC detection of *P. multocida* strains to antibacterial drugs

Strain number	Tilmicosin	Gentamycin	Sulfadiazine	Enrofloxacin	Ciprofloxacin	Florfenicol	Tetracycline	Doxycycline	Terramycin	Kanamycin	Amoxicillin	Ceftiofur
Pm-1	2	<	<	<	<	1	2	<	16	16	0.5	<
Pm-2	8	1	1	<	<	2	1	<	<	2	<	<
Pm-3	4	<	<	1	1	1	4	<	<	2	<	<
Pm-4	8	1	8	<	<	>	8	>	8	2	4	<
Pm-5	16	8	1	16	16	32	4	1	16	4	<	4
Pm-6	32	1	4	<	<	2	4	<	16	1	<	<
Pm-7	4	1	1	<	<	1	4	<	<	4	1	<
Pm-8	4	<	<	<	<	1	8	<	<	4	0.5	<
Pm-9	32	64	<	<	<	2	8	0.5	8	16	<	<
Pm-10	8	1	1	2	2	1	2	<	8	8	<	<
Pm-11	8	1	1	8	8	2	2	<	4	4	<	<
Pm-12	8	<	1	<	<	2	2	<	8	64	<	<

Resistance of isolated strains to 12 antibacterial drugs

The drug resistance rates of the tested strains to different antibacterial drugs were determined, and among them, the highest drug resistance rate was observed for tilmicosin (68%), followed by terramycin (63%). Sulfadiazine did not detect any drug-resistant strains, and the remaining strains exhibited varying degrees of drug resistance, with drug resistance rates below 50%, as shown in Figure 2.

Results of multidrug resistance of isolated strains

Following the (CLSI) guidelines for drug susceptibility testing, the resistance profiles of different bacterial strains against 12 antibacterial drugs were analyzed, as presented in Table 3.4. Among these strains, one exhibited resistance to seven antibacterial drugs, another to six, two strains showed resistance to five drugs, two strains to three drugs, and two strains to two drugs. No resistance was observed against a single antibacterial drug. There were four antimicrobial strains; 6 strains were resistant to 3 or more antibacterial drugs, accounting for 50% of the total, and six were resistant to 3 or fewer antibacterial drugs. The results are shown in Figure 2 and 3.

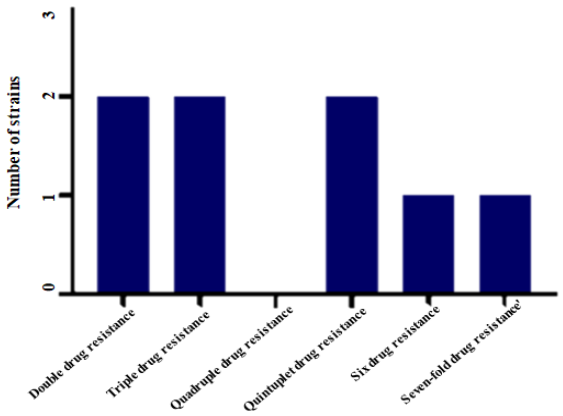

Fig. 2 Resistance rate of isolated strains to different antibiotics

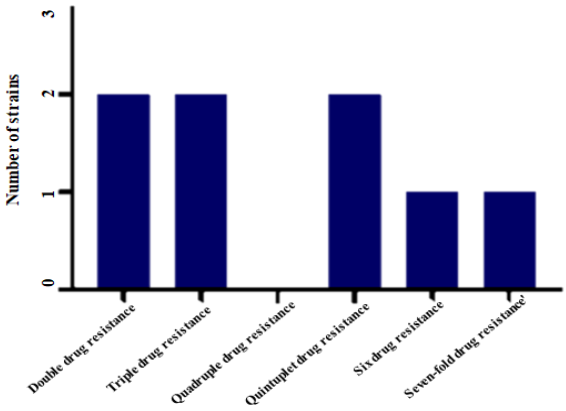

Fig. 3 Multi-drug resistance of isolated strains to 12 kinds of antibacterial drugs

DISCUSSION

Bovine respiratory disease (BRD) remains one of the major threats to the global cattle industry-impacting productivity, welfare, and economics across both beef and dairy systems (White, 2020). While older estimates suggested large annual losses (in the billions of USD), more recent reviews confirm that the disease's burden continues to be substantial, driven by complex interactions of host, pathogen, environmental, and management factors (Smith & Step, 2020; Calderón Bernal et al., 2021). *P. multocida* is a conditional pathogen that can exist in the upper respiratory tract and digestive tract of healthy cattle. When cattle are exposed to adverse factors, such as stress, which causes a decline in immunity, *P. multocida* can multiply in large numbers within the body, leading to pneumonia, hemorrhagic sepsis, and other acute diseases (He et al., 2011). According to reports in 2008, Mawengo isolated *P. multocida* type A from cattle farms in Heilongjiang Province and Tianjin City for the first time (Ma & Yu, 2008). To date, there have been reports of *P. multocida* infection in various locations. In recent years, our laboratory has also isolated capsular *P. multocida* type A from different sources in the three northeastern provinces and tested the drug resistance of the isolates (Kong et al., 2014).

A recent epidemiological studies indicate that mixed infections play a major role in Bovine Respiratory Disease (BRD); for example, in one mortality study of cattle in Italy, 66.7 % of animals with co‑infection harboured one additional pathogen and 22.2 % had two additional pathogens, with *Pasteurella multocida* and *Mycoplasma bovis* among the most common bacterial partners (Liu et al, 2022). However, Gagea M I et al. conducted an epidemiological survey on cattle farms in Ontario, Canada, and found that BRD was the cause of 76% of the dead cattle. They further detected pathogens in 54 cattle that died of BRD, and the results showed that 11 cattle were mixed-infected by *M. bovis* and *P. multocida* (Li et al., 2012). However, Hotchkiss et al. detected BRD on cattle farms in Scotland, and 17% of the cases were caused by infection with *P. multocida* (Lai et al., 2015). Pardon et al. isolated the pathogen from sick cattle with BRD in some Belgian cattle farms, and the isolation rate of *P. multocida* was 26%. Currently, the treatment of bovine A-type *P. multocida* infections mainly relies on antimicrobial drugs. However, with the prolongation of use time, different degrees of drug-resistant bacteria of bovine A-type *P. multocida* have appeared, which have hindered the prevention and control of the disease and aggravated the resistance, causing economic losses due to drug problems. Hendriksen et al. tested the drug resistance of bovine *P. multocida* isolated from various parts of Europe between 2002 and 2004. Nicotine, quinolones, and other commonly used clinical drugs are sensitive (Ma & Yu, 2008); this study differs from our result because the antibacterial drugs used in different regions were different. Geovana et al. isolated a strain of multidrug-resistant bovine *P. multocida* in Germany. The drug sensitivity test results showed that it was resistant to tetracycline, spectinomycin, streptomycin, enrofloxacin, tilmicosin, and kanamycin, among others (Kehrenberg & Schwarz, 2007). These findings were far more serious than our test results. K. Katsuda et al. isolated 378 bovine *P. multocida* strains in Japan and selected nine

commonly used drugs for drug sensitivity testing, among which 102 *P. multocida* strains were resistant to at least one drug, including kanamycin and ampicillin (Hotchkiss et al., 2010). Hossein Jamali et al. isolated and identified 141 strains of *P. multocida* from beef cattle with respiratory diseases in Iran, including 126 strains of capsular A-type *P. multocida*, which accounted for 89% of the P. multocida strains. It was found in drug sensitivity testing that the isolated strains were resistant to penicillin and streptomycin. The drug resistance rate was high, and three strains of *P. multocida* showed multidrug resistance (Hu et al., 2013). Relevant researchers in China have isolated strains resistant to commonly used clinical drugs, such as cephradine, kanamycin, ampicillin, ceftiofur sodium, and amikacin, in certain areas.

CONCLUSION

In conclusion, the resistance rate of the tested strains to various antibacterial drugs was analyzed, with tilmicosin exhibiting the highest resistance rate at 68%, followed by terramycin at 63%. No drug-resistant strains were detected for sulfadiazine; however, the remaining antibacterial agents demonstrated varying levels of resistance, all of which were below 50%.

CONFLICT OF INTEREST: The authors of this manuscript declares that there is no conflict of interest.

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