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Genotyping and phylogenetic analysis of *Mannheimia haemolytica* isolates from cattle and buffaloes of West Azerbaijan, Iran

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Abstract

This study was conducted in West Azerbaijan province, Iran (37°27'18.022" N, 45°0'0" E) to investigate the genotyping and phylogenetic characterization of *Mannheimia haemolytica* in cattle and buffaloes from November 2022 to January 2024. *Mannheimia haemolytica* is a bacterium known to cause pasteurellosis pneumonia, a respiratory disease in ruminants, such as cattle and sheep. This is one of the main causes of economic losses in the feedlot industry. In addition to the deaths, treatment costs are also significant. The lung and nasal swab samples were collected from 378 cattle and buffaloes. The *M. haemolytica* was detected in 32 (8.46%) of the samples, with a notably higher isolation rate from lung tissue (56.25%; n = 18) compared to the nasal swabs (43.75%; n = 14). Interestingly, the study also revealed a seasonal pattern, with the highest isolation rates observed during January, February, and March. Multi-locus sequence typing demonstrated that all isolates belonged to sequence type 1 (ST1) within clonal complex 28. This finding is consistent with the global prevalence of ST1 in bovine isolates, indicating widespread distribution. Phylogenetic analysis revealed a strong correlation between ST1 and STs 30 and 54, highlighting the prevalence of ST1 in *M. haemolytica* among ruminants in West Azerbaijan, Iran. Further research is needed to investigate its potential for causing disease and its transmission pattern.

Introduction

Mannheimia haemolytica is a Gram-negative, opportunistic bacterial pathogen characterized by a coccobacillary morphology, weak hemolytic activity, lack of motility, and the ability to undergo facultative anaerobic metabolism.[1]

In healthy animals, *M. haemolytica* typically localizes in the upper respiratory tract at low levels, particularly in the tonsils and nasopharyngeal region.[2,3] However, under various stress factors, like transportation, harsh weather conditions, or inadequate nutrition, *M. haemolytica* can transform from a commensal bacterium into a significant pathogen.[4] This change may lead to a devastating respiratory disease in ruminants known as bovine respiratory disease (BRD) or shipping fever, causing substantial economic losses in the livestock industry due to morbidity, mortality, and decreased productivity.[5-7]

Two biotypes have been recognized for the taxon *Pasteurella haemolytica*, including biotype A, an isolate fermenting L-arabinose and biotype T, an isolate fermenting trehalose.[8] The *P. haemolytica* complex has been identified as 17 serotypes, including 13 A serotypes (A:1, A:2, A:5, A:6, A:7, A:8, A:9, A:11, A:12, A:13, A:14, A:16, and A:17) and 4 T serotypes (serotypes 3, 4, 10, and 15).[8] The *P. haemolytica* biotype A was later allocated to a new genus, *Mannheimia*, and renamed *M. haemolytica*, while the four T serotypes were named *Bibersteinia trehalosi*. Subsequently, serotype A:11 was reclassified as *M. glucosida*, forming a new taxon due to its distinct biochemical profile, thereby reducing the serotypes of *M. haemolytica* to 12.[9] Of these, serotypes A:1 and A:6 is primarily linked with BRD.[1]

Serotyping has been used for a long time to identify *M. haemolytica* isolates by finding capsular polysaccharide antigens using indirect hemagglutination assays.[10] This method offers the advantage of directly linking serotypes to potential virulence. However, limitations include the restricted availability of reference antibodies and presence of non-typeable isolates.[11]

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Multi-locus sequence typing (MLST) has emerged as a powerful tool for differentiating bacterial strains. It analyzes the nucleotide sequences of housekeeping genes (typically 5 - 7), offering a more discriminatory and broadly applicable approach compared to the techniques, like multi-locus enzyme electrophoresis.[12,13]

While *M. haemolytica* is known to cause pneumonia in ruminants, MLST has not been used in prior research to investigate the spread of this pathogen among cattle and buffaloes' populations in West Azerbaijan province, Iran. This lack of knowledge limits the development of targeted vaccines and molecular diagnostic assays. Developing effective vaccines against *M. haemolytica* could greatly decrease disease severity or even prevent pneumonia in affected animals.

Therefore, this study aims to characterize and identify *M. haemolytica* isolates obtained from cattle and buffaloes herds in West Azerbaijan province, Iran, utilizing MLST. This approach will provide valuable insights into the genetic diversity of this pathogen in the area, potentially paving the way for the development of improved control strategies.

Materials and Methods

Study area. This study was conducted in West Azerbaijan province, Iran (N 37°27'18.022", E 45°0'0"; Fig. 1), between November 2022 and January 2024. The province has a mild, semi-arid, mountainous climate with significant snowfall during winter months caused by cold northern winds.

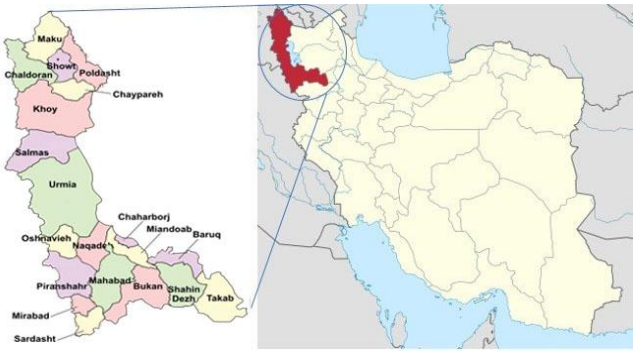

Fig. 1. A map of West Azerbaijan province in Iran where samples from cattle and buffaloes were collected.

Sample preparation. A total of 378 samples were collected randomly, comprising 192 nasal swabs and 186 lung tissue specimens. These samples were obtained from 200 cattle and 178 buffaloes. Each animal was properly restrained with the help of a handler to ensure safety and minimize stress. The external nares of the animals were disinfected with 70.00% alcohol before swab collection. Nasal swab samples were collected using sterile, un-guarded cotton swabs inserted approximately 20.00 cm into the animal's nares and then, transferred to a test tube containing 3.00 mL of tryptic soy broth (Condalab, Madrid, Spain). The inoculated tubes were transported to the laboratory in containers with ice packs.[14] Lung samples were collected from freshly slaughtered animals at abattoirs within West Azerbaijan province, Iran. These samples were immediately placed into the labeled sterile containers and maintained at a cool temperature in insulated ice boxes for transportation to the laboratory.[14] All procedures in this study were conducted in accordance with the Veterinary Ethics Committee of the Faculty of Veterinary Medicine of Urmia University, Urmia, Iran (IR-UU-AEC-3/85).

Isolation and identification of *M. haemolytica*. The isolation and identification of *M. haemolytica* followeda step-by-step protocol. Initially, the samples were streaked onto blood agar plates (Condalab, Madrid, Spain) supplemented with 7.00% sheep blood and incubated aerobically at 37.00 ˚C for 24 hr. Subsequently, the colonies developed on blood agar were subjected to Gram staining to assess cellular morphology. Gram-negative bacterial isolates were sub-cultured on both blood and MacConkey agar (Condalab) for further analyses. The colonies grown on both blood and MacConkey agar plates were meticulously examined for the presence and characteristics of hemolysis, colony morphology, and the ability to ferment lactose. Finally, the colonies underwent biochemical testing. Organisms displaying a narrow zone of hemolysis on blood agar, capable of growth on MacConkey agar, yet unable to produce indole, were identified as *M. haemolytica*.[15,16]

DNA extraction. Genomic DNA was extracted from bacterial cultures using Blood/Cultured Cells Genomic DNA Extraction Mini Kit (Favorgen, Ping Tung, Taiwan) following the manufacturer's instructions. The concentration and quality of the extracted DNA were assessed using a spectrometer (NanoDrop 2000c; Thermo Fisher Scientific, Wilmington, USA). The extracted DNA was then stored at – 20.00 ˚C until it was used in polymerase chain reaction (PCR) amplification.

Polymerase chain reaction. A PCR assay targeting the arginine-binding periplasmic protein 2 and leukotoxin acyltransferase (*artJ-lktC*) gene was used to confirm the identification of *M. haemolytica* isolates.[17] The PCR reactions were prepared in 25.00 µL volume, containing 4.00 µL of template DNA (50.00 - 100 ng μL^{-1}), 1.00 µL of each primer (0.10 nmol μL^{-1}), and 12.50 µL of master mix (Ampliqon, Odense, Denmark) contained proofreading DNA polymerase enzyme and nuclease-free water to complete the band. The oligonucleotide primer sequences specific for *M. haemolytica* genes (*artJ-lktC*) were commercially synthesized by SinaClon (CinnaGen, Tehran, Iran) and are listed in Table 1 along with the targeted amplicon sizes. A touchdown PCR program was employed in a thermal cycler (Quanta Biotech, Beverly, USA) to reduce non-specific amplification. This program starts

with a high annealing temperature decreasing gradually in subsequent cycles, promoting specific target amplification. The PCR cycling conditions employed are illustrated in Table 2. After amplification, the PCR products were run on a 2.00% agarose gel with a safe stain (Labnet, Edison, USA) and visualized using the Genius Gel Documentation System (Syngene Bio-Imaging, Cambridge, UK). The PCR products of isolates, with the amplified fragment of *artJ-lktC* gene (251 bp) for samples, were sent to Pishgam Company in Tehran, Iran, for sequencing.

Multi-locus sequence typing. All *M. haemolytica* isolates were subjected to MLST analysis to characterize their genetic diversity. This technique utilizes the nucleotide sequences of seven housekeeping genes, including *adk*, *aroE*, *deoD*, *gapDH*, *gnd*, *mdh*, and *zwf*, as shown in Table 1. The PCR amplification of the targeted genes was performed in a 25.00 µL reaction mixture, containing 1.00 µL of each forward and reverse primer (Table 2), 2.00 µL of template DNA (50.00 - 100 ng μL^{-1}), and 12.50 µL of master mix (Ampliqon) contained proofreading DNA polymerase enzyme and nuclease-free water to reach the total volume. The PCR cycling conditions employed are illustrated in Table 2. Subsequently, the PCR products were analyzed by electrophoresis on a 2.00% agarose gel, containing a safe stain (Labnet), and visualized using the Genius Gel Documentation System (Syngene Bio-Imaging). After that, the purified PCR products were delivered to Pishgam Company for sequencing and using the Sanger DNA sequencing method.[18] The resulting sequences were imported into the *M. haemolytica* MLST website (http://pubmlst.org/mhaemolytica/) for allele and sequence types designation. Phylogenetic analyses were performed using concatenated sequences of the seven MLST loci with MEGA Software (version 11.0; Biodesign Institute, Tempe, USA) to explore the genetic relationships among the *M. haemolytica* isolates.

Results

Prevalence of *M. haemolytica* in cattle and buffaloes. Out of the 378 nasal swabs and lung tissue samples collected from cattle (n = 200) and buffaloes (n = 178), *M. haemolytica* was isolated from 32 specimens, resulting in an overall prevalence of 8.46%. The *M. haemolytica* was isolated more frequently from lung tissue (n = 18; 56.25%) compared to the nasal swabs (n=14; 43.75%). The highest level of isolation was observed in January, February, and March.

Table 1. Primer sequences, amplified products, annealing temperature and primer references for the *artJ-lktC* gene and multi-locus sequence typing in *Mannheimia haemolytica* isolates.

Genes	Primer sequence (5' to 3')	Amplicon size (bp)	Annealing temperature	References
artJ-lktC	F: TATAAGGATTACCACTTTAACGCA R: ATAATCAGAAGAGAAAAAGGAGTGT	251	60.00 ˚C	17
adk	F: GCAAAGGTACGCAAGCTCAG R:AAAATTTTCGCTAACTCAGCAC	604	55.00 ˚C	18
aroE	F: GCTTCTGGAGCAAAAGGTTG R: CCTGTCCAACCAACATTCCT	580	55.00 ˚C	18
deoD	F: TCCACACATTAACGCACCTG R: GCTCCATACTCTGCCGCTAC	576	55.00 ˚C	18
gapDH	F: CCGTATCGGTCGTATCGTTT R: TTTTGCGTTGCAGTAGTTGC	519	55.00 ˚C	18
gnd	F: GTGATTGGACTCGCCGTAAT R: TTCGATACCGTTGTGAACCA	551	55.00 ˚C	18
mdh	F: AGTAACCGGTTTTGCAGGTG R: GCTTTTGCCTCAACCACTTC	503	55.00 ˚C	18
zwf	F: TGATGAAGTCGCAAAAGTGC R: ACGGTTTTCGCCATACTTTG	671	55.00 ˚C	18

Table 2. Cycling conditions of the primers during polymerase chain reaction (PCR).

Genes	PCR cycling conditions
artJ-lktC	Initial denaturation: 95.00 ˚C for 4 min, 35 cycles of touchdown: 60.00-66.00 (5) for 40 sec; denaturation: 95.00 ˚C for 40 sec; annealing: 60.00 ˚C for 40 sec; extension: 72.00 ˚C for 1 min; final extension: 72.00 ˚C for 7 min
adk ***aroE*** ***deoD*** ***gapDH*** ***gnd*** ***mdh*** ***zwf***	Initial denaturation: 96.00 ˚C for 5 min, 30 cycles of denaturation: 96.00 ˚C for 30 sec; annealing: 55.00 ˚C for 30 sec; extension: 72.00 ˚C for 1 min; final extension: 72.00 ˚C for 10 min.

Cultural and biochemical characteristics. Bacterial identification was performed following the established protocols outlined by Quinn *et al*.,[16] including colonial morphology, Gram staining, and a series of biochemical tests. Among the isolates, 32 displayed mucoid, grey colonies with slight β-hemolysis, a characteristic colonial morphology associated with *M. haemolytica* species. All isolates exhibiting this morphology were identified as Gram-negative, oxidase-positive, and indole-negative rods, being unable to ferment trehalose, aligning with the criteria for *M. haemolytica*. Additionally, these isolates grew on MacConkey agar, forming pinpoint red colonies.

Molecular confirmation of *M. haemolytica*. After initially identifying the isolates based on colony morphology, Gram staining, and biochemical tests, a PCR assay targeting the *artj-lktc* gene was employed to confirm them as *M. haemolytica.*[17]

The PCR reactions and visualization of amplicons were conducted as described previously. Briefly, amplification produced the expected bands at the targeted sizes for all 32 isolates, as shown in Figure 2. Followed by Sanger DNA sequencing of the PCR product, all the 32 isolates tested for the presence of *M. haemolytica* were identified as positive. The sequences were deposited in the GenBank database under the accession number of PP532883. Nucleotide sequence analysis of the *artJ-lktC* gene in *M. haemolytica* demonstrated substantial homology between Iranian strains and *M. haemolytica* strains from diverse clinical lesions, hosts, and geographical locations worldwide, as deposited in GenBank. Sequence identities between the isolated Iranian strains and different *M. haemolytica* strains uploaded in GeneBank revealed 98.00 - 100% homology.

Multi-locus sequence typing and phylogenetic analysis. As previously described by Petersen *et al*.,[18] PCR amplification of the seven housekeeping genes *(adk*, *aroE*, *deoD*, *gapDH*, *gnd*, *mdh*, and *zwf)* was performed for all 32 *M. haemolytica* isolates. Electrophoresis of the PCR products on a 2.00% agarose gel verified the presence of amplicons at the expected sizes (Fig. 2). After confirmation, the PCR products were purified and undergone Sanger DNA sequencing. The obtained sequences for each of the seven housekeeping genes were analyzed using BLAST against the GenBank database to verify their identity. Subsequently, these sequences were imported into the *M. haemolytica* MLST website (http://pubmlst. org/mhaemolytica/) to assign allele and sequence types to the isolates.

The allelic profiles assigned to seven loci were 1, 2, 1, 2, 1, 2, and 1 for *adk*, *aroE*, *deoD*, *gapDH*, *gnd*, *mdh*, and *zwf* genes, respectively. This allelic profile corresponds with sequence type 1.

Interestingly, all 32 *M. haemolytica* isolates belonged to a single sequence type, sequence type 1. This represents the first reported identification of ST1 in *M. haemolytica* isolates from the West Azerbaijan province of Iran.

To further explore, the identified sequence type was aligned with representative sequence types from each sequence type complex downloaded from the *M. haemolytica* MLST website (http://pubmlst.org/mhaemolytica/). The ST1 detected in this study was compared with those available in the *M. haemolytica* MLST database (https://pubmlst.org/mhaemolytica/) using e-BURST analysis and grouped into clonal complex 28 (Fig. 3).

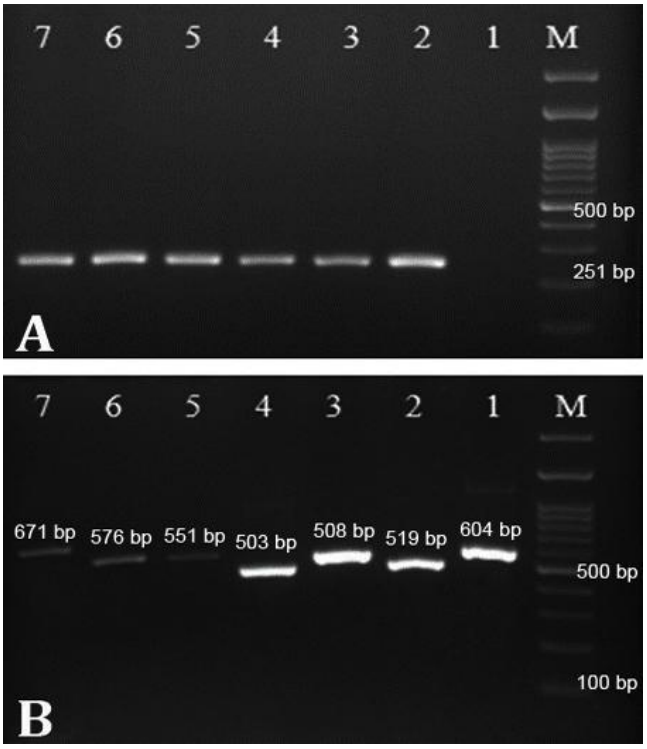

Fig. 2. Molecular confirmation of *M. haemolytica*. **A)** Agarose gel image of amplified fragment of *artJ-lktC* gene (251 bp). Lane M: 100 bp DNA marker; Lane 1: Negative control; Lane 2: Positive control; Lanes 3-7: Positive samples for *M. haemolytica.* **B)** An amplified segment of seven housekeeping genes from *M. haemolytica* on an agarose gel. Lane M: 100 bp DNA marker; Lane 1: *adk* gene (604 bp); Lane 2: *gapDH* gene (519 bp); Lane 3: *aroE* (580 BP); Lane 4: *mdh* gene (503 bp); Lane 5: *gnd* gene (551 bp); Lane 6: *deoD* gene (576 bp); Lane 7: *zwf* gene (671 bp).

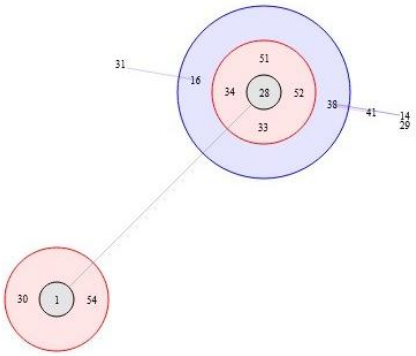

Fig. 3. The e-BURST analysis of sequences types in *M. haemolytica* multi-locus sequence typing database. The ST1 was detected in this study. Each number represents a unique sequence type.

A phylogenetic tree was constructed using concatenated sequences of the seven MLST loci and MEGA Software to investigate the evolutionary relationships among the isolates. The analysis indicated a close genetic association between ST1, ST30, and ST54, suggesting a recent common ancestor for these strains (Fig. 4).

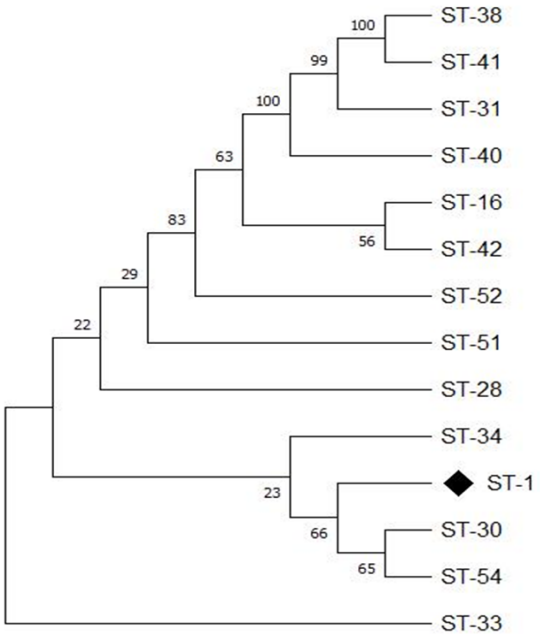

Fig. 4. The phylogenetic tree for ST1 isolate is displayed with a diamond shape indicating the isolate from this study. The phylogenetic tree is based on the concatenated sequences of sequences types included in the *Mannheimia haemolytica* multi-locus sequence typing database (https://pubmlst.org/mhaemolytica/). This phylogenetic tree was constructed using the Neighbor- Joining method. The bootstrap consensus tree inferred from 1,000 replicates represents the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50.00% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions *per* site. This analysis involved 14 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+noncoding. All ambiguous positions were removed for each sequence pair using the pairwise deletion option. There were 3,186 positions in the final dataset. Evolutionary analyses were conducted in MEGA Software (version 11.0; Biodesign Institute, Tempe, USA).

Discussion

Pasteurellosis pneumonia presents a significant challenge in animal husbandry due to its high morbidity and mortality rates, resulting in significant financial losses throughout the production chain.[5] Stressors, such as overcrowding, inadequate nutrition, cold temperatures, and poor hygiene, can compromise the innate defenses of ruminants, increasing cortisol levels and reducing serum immunoglobulin G concentrations. Also, these stressors can disrupt the mucociliary clearance mechanism, facilitating bacterial proliferation within the respiratory system.[19] Specifically, *M. haemolytica* as a commensal organism can become pathogenic, infiltrating deep into the respiratory system and causing disease, establishing itself as a primary causative agent of pasteurellosis pneumonia in cattle.[20] Given the susceptibility of cattle and buffaloes of all ages and sexes to this disease, effective control measures are crucial for its prevention, particularly in regions like Iran where it leads to severe pneumonia in both cattle and buffaloes. Therefore, this study aimed to investigate the pathogenicity of *M. haemolytica* in cattle and buffaloes to assist in disease prevention and the development of multivalent vaccines. Previous research has not explored the genetic properties of *M. haemolytica* isolates in West Azerbaijan, Iran.

In our study, *M. haemolytica* isolates were identified using the PCR test targeting the *artJ-lktC* gene. This gene is known for its high conservation among strains of this bacterium. Additionally, some studies have used this gene as a target for establishing detection methods for these bacteria.[17] Based on the molecular findings of the study, 8.46% of the investigated buffaloes and cattle were found to be carriers of *M. haemolytica*. The frequency of *M. haemolytica* isolates varies significantly and can sometimes be high, depending on the source of isolation.

Studies investigating the prevalence of *M. haemolytica* in cattle have produced a range of findings. El-Seedy *et al*.,[21] reported a prevalence of 8.40%, being similar to the observations in the current study. Zaki *et al*.,[22] found a higher prevalence of *Pasteurella multocida* (19.90%) compared to the *M. haemolytica* (8.80%) in pneumonic calves. Similarly, Ahmad *et al*.,[23] observed a higher prevalence of *Pasteurella* species in the upper respiratory tract of cattle, with 30.00% for *P. multocida* and 20.00% for *M. haemolytica*. Other studies investigating *M. haemolytica* presence in lung and lymph node samples from cattle with respiratory disease have shown varied results. Ashrafi *et al*.,[24] identified *M. haemolytica* in 18.30% of samples using a combination of culture, biochemical, and PCR methods. Nefedchenko *et al*.,[25] reported isolation rates of 14.30% from lung samples and 11.10% from lymph node samples. In contrast, studies focusing on healthy cattle lungs have generally reported lower prevalence. Cengiz *et al*.,[26] detected *M. haemolytica* in only 4.80% of lung samples using PCR, and Abera *et al*.,[27] found a 3.95% positivity rate in Ethiopia using combined PCR and culture methods. Similarly, Haji Hajikolaei *et al*.,[28] isolated *M. haemolytica* from just 1.60% of cows using nasopharyngeal swab and blood samples. Interestingly, Khalili *et al*.,[29] did not detect any *M. haemolytica* in a study of sheep lung samples, suggesting potential species-specific variations in prevalence.

The variation among these results may stem from differences in health facilities, predisposing factors in the animals, sampling methods, diagnostic techniques, and the use of related vaccinations.[30] For instance, conventional culture methods may yield negative results in detecting pathogenic agents in healthy animals due to the low bacterial count in the sample. Additionally, vaccinations can reduce the occurrence of lesions and carriage of bacteria.

Traditionally, *M. haemolytica* typing relied on methods, like ribotyping, restriction enzyme analysis, random amplified polymorphic DNA analysis, and pulsed-field gel electrophoresis (PFGE).[31-33] For a significant period, PFGE served as a gold standard. However, Villard *et al*.,[34] demonstrated phenotypic and PFGE pattern changes in isolates after 50 sub-culture rounds in laboratory settings.

Several virulence-associated genes (VAGs) have been identified in *M. haemolytica*, including *lktA*, *adhes*, *fhaC*, and etc.[1,35] Virulence-associated gene typing (VAGT) has emerged as a valuable tool for the molecular characterization of bacterial pathogens. This technique allows researchers to identify specific VAG profiles in *M. haemolytica* isolates, potentially aiding in epidemiological investigations and understanding strain variations.[36]

Recent studies have provided valuable insights into the prevalence of VAGs in *M. haemolytica*. For example, Abed *et al*.,[6] demonstrated that 80.00% of the examined isolates harbored both the *gcp* and *lktC* genes, while only 60.00% possessed the *ssa* gene. These findings corroborate earlier research by Singh *et al*.,[2] identifying the *lkt* gene as a species-specific marker for *M. haemolytica* in ruminant hosts. Furthermore, Klima *et al*.,[37] consistently detected the *lktC* and *gcp* genes across all tested *M. haemolytica* isolates. Notably, the *ssa* gene, encoding a serotype-specific outer membrane protein serine protease, is considered a critical virulence factor in *M. haemolytica* isolates. Complementing these observations, Gharib Mombeni *et al*.,[38] reported that all *M. haemolytica* isolates in their study possessed the *lkt*, *pomA*, and *nanH* genes, being implicated in the pathogenesis and virulence of this bacterial pathogen, as highlighted by Klima *et al*.[39]

While VAGT offers valuable insights, recent studies by García-Alvarez *et al*.,[13] and Klima *et al*.,[37] have highlighted limitations in solely relying on VAG profiles to predict virulence. These studies observed similar VAG profiles in *M. haemolytica* isolates from both healthy and diseased animals. This suggests that the presence of individual VAG may not necessarily translate to virulence and the additional factors, potentially involving complex gene regulation and host interactions, may be critical for pathogenicity.

Multi-locus sequence typing has emerged as a more robust alternative to traditional methods. The MLST can identify strains undergoing phenotypic alterations and exhibit altered PFGE patterns, while still assigning the same sequence type as a parent strain.[13] Additionally, MLST offers advantages in accuracy and portability, facilitating global result comparison. This method excels at discriminating between closely related bacterial strains, making it valuable for tracking potentially epidemic strains during outbreaks. Thus, to further characterize the isolated *M. haemolytica* strains in this study, MLST was employed to investigate their genetic diversity.

In addition to strain differentiation, MLST has been used to assess host or niche relatedness in numerous pathogens. For instance, it has been utilized to investigate the pathogenic potential of pathogens shared between humans and animals, as well as identify host- and niche-specific clones. Such approaches help in understanding host-pathogen interactions.

In this study, MLST analysis based on seven loci (*adk*, *aroE*, *deoD*, *gapDH*, *gnd*, *mdh*, and *zwf*) revealed that all isolated *M. haemolytica* strains were genetically identical. This finding suggests that these strains may originate from a single source population of *M. haemolytica*. Furthermore, it implies that the ST1 sequence type of *M. haemolytica* is prevalent in the West Azarbaijan province of Iran.

The ST1 has been previously detected in cattle from various countries. Petersen *et al*.,[18] identified ST1 in over one-third of *M. haemolytica* isolates collected from cattle sources across 10 countries. Similarly, studies in Australia and Idaho, United States, reported a high prevalence of ST1 among bovine isolates.[18]

Analysis of the *M. haemolytica* MLST database (https:// pubmlst.org/mhaemolytica/) indicated that ST1 accounts for 75.00% of bovine isolates, suggesting a global distribution for this sequence type. This finding highlights the need for continued epidemiological surveillance of ST1, particularly given its potential impact on large ruminants.

Interestingly, ST1 has also been isolated from sheep, chamois, and buffaloes, suggesting the ability of this sequence type to adapt to a wide range of animal hosts.

Omaleki *et al*., reported an outbreak of clinical mastitis in sheep. They identified five different sequence types among the 16 *M. haemolytica* isolates, with ST1 being the most common, representing 10 out of 16 isolates (62.50%).[40] Reportedly, in a study by García-Alvarez involving 121 lung samples isolated from sheep, two samples were found to be related to ST1.[13]

In Iran, where cattle and buffaloes often graze together, the presence of ST1 in both species suggests the potential for past inter-species transfer of *M. haemolytica.* Further studies are warranted to investigate this possibility.

After conducting e-BURST analysis, the ST1 isolate was classified into clonal complex 28, a finding aligning with the clonal population structure of *M. haemolytica* as seen in other typing methods.[18]

The phylogenetic analysis revealed a close relationship between ST1, identified in this study, and ST30, ST54, and

ST28. These sequence types were isolated from cattle in various countries, including the United Kingdom, United States, France, Germany, Australia, Belgium, Netherlands, Norway, and China. This close relationship suggests a common ancestor for these sequence types and potentially indicates a worldwide distribution. The findings from this study's molecular typing analysis provide valuable new insights into the genetic characteristics of *M. haemolytica* isolates obtained from both cattle and buffalo samples. This information enhances our understanding of the epidemiology of this pathogen in these large ruminants.

In conclusion, this study found *M. haemolytica* in 8.46% of cattle and buffalo samples from West Azarbaijan, Iran. All isolates belonged to the same sequence type (ST1). Identifying this dominant ST1 genotype is a crucial step towards developing an effective *M. haemolytica* vaccine to mitigate economic losses and animal welfare issues caused by BRD. Further research building on these findings is needed for vaccine development targeting the predominant ST1 strain.

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Conflict of interest

No conflicts of interest exist in the submission of this manuscript.

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