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Original Article

Investigating Key Virulence Factors in *Mannheimia haemolytica* for Enhanced Disease Control Strategies

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Abstract

Background & Objectives: Whereas our understanding of bovine pasteurellosis has greatly improved, it continues to be an important respiratory disease in feedlot cattle. *Mannheimia* (M.) *haemolytica*, is a primary cause of severe pneumonia in bovine respiratory disease (BRD). Although some research has been done in Iran and other countries to evaluate the prevalence of *M. haemolytica*, the general virulence factors of this bacteria are not clear yet. *M. haemolytica* 's virulence factors aid lung colonization, trigger inflammation, and help evade host immunity. Environmental factors influence the pathogenicity and spread of *M. haemolytica*, which carries complete leukotoxin (*LKT*) genes. In this study Virulence factors were validated through key mucosal pathogenic components: fimbriae, capsule, endotoxins, and leukotoxin.

Materials & Methods: *M. haemolytica* samples from goats and sheep swabs were cultured on Blood Agar and then transferred to BHI medium for growth. Then, the samples were analyzed for virulence factors using the polymerase chain reaction (PCR) to identify and verify the virulence factors of *M. haemolytica*.

Results: in This study 10 virulence genes in *M. haemolytica*, identifying key pathogenic markers in most samples were examined. Genes associated with toxin production, adhesion, and immune evasion were prevalent, while others showed variable distribution. The results demonstrate the significance of the virulence factors in the pathogenic and colonization potential of *M. haemolytica* in sheep and goats.

Conclusion: investigating these virulence factors helps refine strategies in vaccine development and disease control, aiming to reduce the pathogen's impact on livestock respiratory health.

Keywords: *Mannheimia haemolytica*, virulence factors, Polymerase Chain Reaction (PCR), Bovine Respiratory Disease Complex, Vaccine Development.

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Introduction

M. haemolytica is a beta-hemolytic, nonmotile coccobacillus from the family *Pasteurellaceae* (1). This family encompasses a variety of Gram-negative gamma-proteobacteria, including genera such as *Actinobacillus*, *Haemophilus*, *Mannheimia*, *Pasteurella*, and *Lonepinella* (2). These bacteria exhibit pleomorphism, presenting as either short bacilli or coccobacilli (1). Pasteurellosis predominantly impacts the respiratory systems of cattle, sheep, goats, and swine, with *M. haemolytica* and *P. multocida* being the primary causative agents, which may act independently or synergistically (3). Typically, *M. haemolytica* is implicated in infections among sheep and goats, whereas *P. multocida* is more commonly associated with cattle (4,5). While *M. haemolytica* is often a natural component of the upper respiratory tract microbiota, its pathogenic potential can result in considerable economic losses in livestock due to complications and mortality (6,7,8). This organism is linked to various syndromes affecting different hosts, with pasteurellosis being the principal syndrome characterized by upper respiratory tract disease (rhinitis) and lower respiratory tract disease (pneumonia) (6). Numerous strains of *M. haemolytica* function as opportunistic pathogens, leading to acute and chronic infections (9). Factors such as suboptimal environmental conditions, transportation stress, and concurrent bacterial and viral infections can predispose ruminants to respiratory diseases associated with *M. haemolytica* (5).

The virulence factors of *M. haemolytica* facilitate lung colonization and immune evasion, playing a critical role in the transition of the organism from a commensal state to a pathogenic one. Notable virulence factors include fimbriae, capsules, endotoxins, and leukotoxins (*LKT*) (10). *LKT*, a pore-forming cytolysin, affects leukocytes and platelets at low concentrations and induces cytolysis at elevated concentrations (11,12). Additional virulence factors, such as lipopolysaccharide (LPS) and outer membrane proteins (OMPs), also contribute to the pathogenesis of this organism (13).

The pathogenesis of *M. haemolytica* remains a subject of ongoing debate, attributed to the intricate nature of the disease and variable experimental findings (14). The bacteria need to navigate host immune responses and compete with indigenous flora to establish infection. Virulence factors enhance the microorganism's ability to adhere, colonize, and proliferate within host tissues. Understanding these molecular mechanisms is essential for developing effective vaccines and control measures (5,15,16). This research underscores the significance of identifying and targeting virulence factors of *M. haemolytica* to alleviate bovine respiratory disease's economic and health burdens.

Materials and methods

To investigate the virulence factors associated with *M. haemolytica*, bacterial samples were collected from nasal and throat swabs from sheep and goats in various livestock species and subsequently transported to the laboratory under strictly controlled cold chain conditions. The samples were inoculated onto Blood Agar (BA) medium supplemented with 7% sheep blood and incubated at 37°C for 24 hours (17).

After the incubation period, several isolated colonies from the BA plates, corresponding to each animal, were transferred into 5 ml of Brain Heart Infusion (BHI) broth medium and incubated in a shaker incubator at 37°C. For

microscopy, smears were prepared from colonies suspected to *M. haemolytica*. Following heat fixation and the smears were stained using the Gram staining technique (18). The observation of Gram-negative coccobacilli under a light microscope supported the presence of *M. haemolytica*. This study aimed to evaluate the presence or absence of ten virulence genes in *M. haemolytica* through the application of polymerase chain reaction techniques. For the PCR process, DNA was initially extracted using the boiling method. Specific primers which targeting each virulence factor gene were utilized, and numerous copies of the target gene were generated using a thermocycler (Table 1). The PCR protocol comprised an initial denaturation phase, followed by a predetermined number of cycles at specified temperature (95°C) and durations (30 cycles). A final extension step was performed at a designated temperature and time for each virulence factor (19). Ultimately, the target gene was identified using an electrophoresis gel running, which facilitated the movement of the DNA on the gel and subsequent imaging.

Results

This study evaluates the presence or absence of ten virulence genes in *M. haemolytica*. The genes examined include *lkt1, lkt2, pilA, adhes, Lipopolysaccharide (lps), Irp, sodA, sodC, nanH*, and *gcp*. The results of this analysis are summarized in the Table 2.

Table 2. Number and frequency of virulence factors genes in *M. haemolytica*.

Genes	Number	Frequency (%)
lkt1	59	100
lkt2	56	95
pilA	59	100
adhes	56	95
lps	45	76.3
irp	41	69.5
sodA	57	97
sodC	17	30
nanH	54	93
gcp	50	86

The findings of this study revealed that the *lkt1* gene was present in all isolates of *M. haemolytica,* Figure 1 while the *lkt2* gene was identified in 95% of the isolates Figure2. Additionally, the *pilA* adhesin of *M. haemolytica* was detected in 100% of the isolates Figure 3, whereas the *adhes* gene was present in approximately 95% of the isolates Figure 4.

Table 1. Primer sequence and amplicon size used in the

Number	Gene	Sequence (5'→3')	Amplicon size	References
1	*lkt*	F: GCAGGAGGTGATTATTAAAGTGG R: CAGCAGTTATTGTCATACCTGAAC	206	(20)
2	*lkt2*	F: CTCTCTTTAGAAAAGCTGGAAAAC R: TTTTGCCAAGTGGTGTATTGC	170	(20)
3	*pilA*	F: TTTTAGACCGCTTGGCATTC R: CCGTAATCACGCCTTTTGTT	311	(21)
4	*Adhes*	F: CCACATTTTGAGGCGCTAAT R: AGGTCATCCGGCAACTACAC	155	(21)
5	*lps*	F: AAATTCTGCTCCCTTGTTCG R: ATTTTTCGATGCGATTACGC	385	(22)
6	*irp*	F: GGCGAAAACTTAGATATGTCGG R: CCTAAATTAAGGCGTTCAATCG	1025	(23)
7	*sodA*	F: TACCAGAATTAGGCTACGC R: TCGCACAACCGCAGGTTGCT	624	(21)
8	*sodC*	F: AAGGTGGCAAGCTCACAGCAG R: TGAGTGGTTATCGCCGCCT	230	(21)
9	*nanH*	F: GCTGAAATGGAAGCAAAAGC R: GCCACGATAGCGTAAGAAGC	660	(24)
10	*gcp*	F: GTGTTGCCATTTATGATGAAG R: AGCGAAGAAAGCCAGTGTA	912	(24)

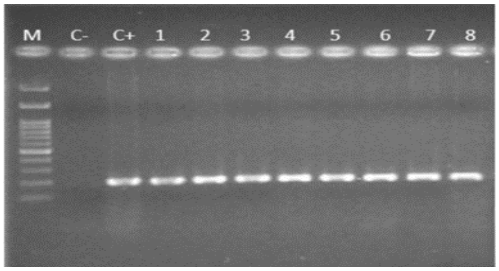

Fig 1. Presence of the *lkt1* gene in all *M. haemolytica* isolates.

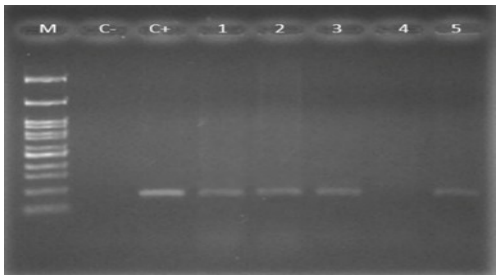

Fig 2. Presence of the *lkt2* gene in 95% of *M. haemolytica* isolates.

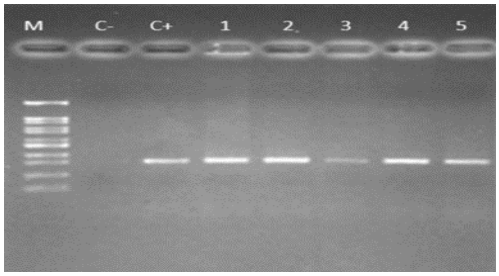

Fig 3. *Presence* of the *pilA* adhesin in 100% of *M. haemolytica* isolates.

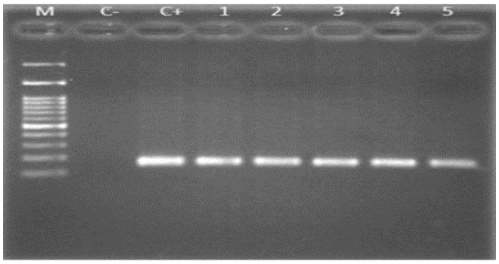

Fig 4. Presence of the *adhes* gene in approximately 95% of *M. haemolytica* isolates.

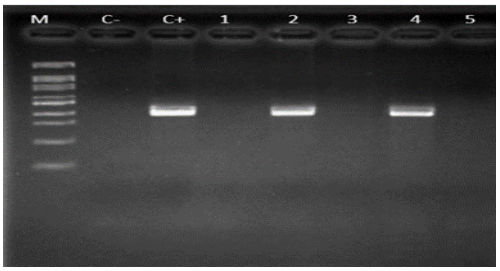

Fig 5. Presence of the lipopolysaccharide (*LPS*) gene in 76.3% of *M. haemolytica* isolates.

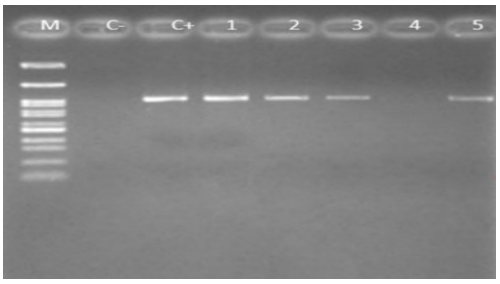

Fig 6. Presence of the *Irp* gene, essential for iron uptake, observed in 69.5% of *M. haemolytica* isolates.

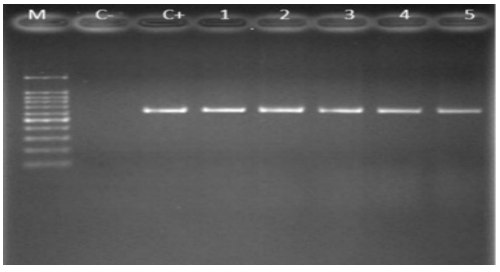

Fig 7. Presence of *SodA* superoxide dismutase in 97% of *M. haemolytica* isolates.

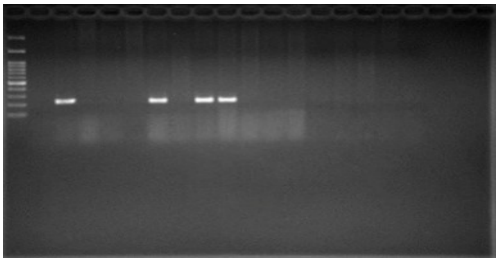

Fig 8. Presence of *SodC* superoxide dismutase in fewer than 30% of *M. haemolytica* isolates.

The frequency of this gene among the isolates in the current study was 76.3% Figure 5. The *Irp* gene, which supports the growth of *M. haemolytica* in iron-limited environments and is essential for the iron uptake system (25), was observed at a frequency of 69.5% Figure 6.

Among the various forms of superoxide dismutase, *SodA* was found in 97% of the isolates Figure 7, while *SodC* was identified in fewer than 30% of the isolates Figure 8.

Finally, among the various enzymes of *M. haemolytica*, the presence of genes related to *nanH* and *Gcp*, which belong to the families

of neuraminidases and sialoglycoproteases, respectively, has been examined. The frequencies of *nanH* and *Gcp* were found to be 93% Figure 9 and 86% Figure 10, respectively.

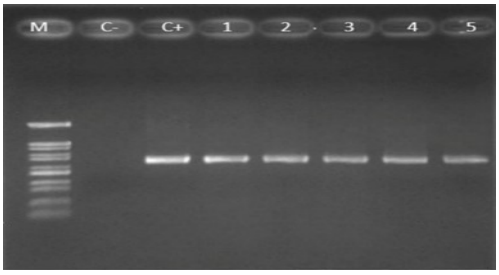

Fig 9. Presence of the *nanH* gene, a neuraminidase enzyme, in 93% of *M. haemolytica* isolates.

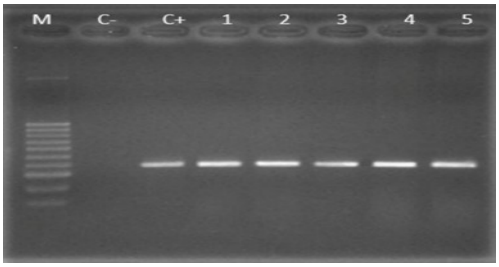

Fig 10. Presence of the *Gcp* gene, a sialoglycoprotease enzyme, in 86% of *M. haemolytica* isolates.

Discussion

The results of the present study are compared with prior research findings. The *lkt1* and *lkt2* genes, which encode leukotoxins, are recognized as significant virulence factors in *M. haemolytica*. These leukotoxins specifically target and lyse leukocytes, enhancing the bacterium's ability to circumvent the host immune response and induce respiratory disease in cattle. The prevalence of the *lkt1* gene was noted in all isolates, while the *lkt2* gene exhibited a frequency of 95%, aligning with the observations of Vougidou et al. (2013), who reported a 100% prevalence of these genes (26).

Furthermore, the *Adhes* gene plays a pivotal role in the adhesion of *M. haemolytica* to the respiratory mucosa, a critical step in its pathogenicity. The *PilA* gene encodes a pilin protein that contributes to the formation of pili on the bacterial surface, facilitating attachment to host cells, which is essential for colonization and infection. The *nanH* gene encodes a neuraminidase enzyme that cleaves sialic acids from host glycoproteins, a vital process for bacterial colonization and invasion. The frequencies of the *Adhes* (95%), *PilA* (100%), and *nanH* (93%) genes in this study are in close alignment with the findings of García-Alvarez et al. (2018), who reported frequencies of 97.5%, 100%, and 99%, respectively (27).

Lipopolysaccharides (*LPS*) are integral components of the outer membrane in Gram-negative bacteria such as *M. haemolytica*, supporting the structural integrity of the bacteria and triggering strong immune responses in the host as well. The *Irp* gene is essential for bacterial survival in iron-limited environments, as iron is a crucial nutrient for various bacterial processes. The *SodA* gene encodes a manganese-dependent enzyme, while the *SodC* gene encodes a copper and zinc-dependent enzyme, both confer protection against oxidative stress. The frequencies of *LPS* (76.3%), *Irp* (69.5%), *SodA* (97%), and *SodC* (less than 30%) are consistent with the findings of Gharibi et al. (2021), who reported frequencies of 93%, 70%, 92%, and 21%, respectively (28).

Lastly, the *Gcp* gene is implicated in the biosynthesis of glycoproteins, which are important for maintaining both bacterial cell wall integrity and function; and playing roles in adhesion, immune evasion, and other virulence-related processes. The frequency of the *Gcp* gene was recorded at 86%, which is comparable to the findings reported by Klima et al. (2014), who documented a frequency of 96% (29).

Conclusion

The examination of multiple virulence factors in *M. haemolytica*, including *lkt1*, *lkt2*, *PilA*, *Adhes*, *LPS*, *Irp*, *sodA*, *sodC*, *nanH*, and *Gcp* genes, highlights their significant role in the pathogenicity and colonization of this bacterium in sheep and goats. The findings derived from this study may contribute to developing more effective vaccines and management strategies for respiratory diseases in animals.

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Conflicts of interest

The authors declare that there are no competing interests.

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