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Determination of Serotypes A1, A2, and A6 in *Mannheimia haemolytica* Isolates by Real-Time PCR and Molecular Characterization by BOX and RAPD PCR Methods

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ABSTRACT

In this study, the detection of serotypes A1, A2, and A6 in Mannheimia haemolytica isolates obtained from sheep was investigated using the Real-Time PCR method, and genotyping of the isolates was performed using RAPD and BOX PCR methods. A total of 44 Mannheimia haemolytica isolates isolated from sheep and preserved in the culture collection of the Department of Microbiology, Faculty of Veterinary Medicine, Van Yüzüncü Yıl University, were included. Using Real-Time PCR, 27 (61.4%) of the 44 isolates were successfully serotyped, while 17 (38.6%) could not be serotyped. Of the serotyped isolates, 22.2% were identified as serotype A1, 44.4% as serotype A2, and 33.4% as serotype A6. Genotyping by RAPD PCR revealed 18 different genotypes using the OPA 4 primer and three different genotypes using the OPA 9 primer, whereas BOX PCR analysis identified 15 different genotypes. No statistically significant association was found between Mannheimia haemolytica serotypes (A1, A2, A6) and genotypes. The Real-Time PCR method used in this study was found to be suitable for the molecular identification of these serotypes. For RAPD PCR genotyping, the OPA 9 primer was determined to be unsuitable, while the OPA 4 primer and BOX PCR BOXA1R primer were appropriate for epidemiological studies, with the BOXA1R primer providing clearer band profiles. In conclusion, serotype A2 was identified as the dominant serotype in the Van Province sheep population, followed by serotype A6, both of which should be prioritized in future regional vaccine formulations targeting ovine respiratory disease in eastern Turkey. Among the genotyping methods evaluated, BOX-PCR is recommended for routine epidemiological investigations. Identifying the prevalent serotypes and genotypes at regional and national levels is therefore of critical epidemiological importance for the development of effective prevention, control, and vaccination programs against Mannheimia haemolytica-associated pneumonia in small ruminants.

Keywords: Real-Time PCR, genotype, serotype

INTRODUCTION

Due to its extensive pasturelands, particularly in the Eastern Anatolia Region, Turkey is one of the leading countries for sheep farming.[1] According to the Turkish Statistical Institute (TUIK) data from June 2024, there are 44,080,554 sheep in Turkey. The provinces of Van, Konya, Şanlıurfa, Diyarbakır, Ankara, and Ağrı collectively account for approximately 30% of this total. The economic and public health significance of livestock production highlights the importance of animal health.[2]

Respiratory diseases are among the most critical challenges in sheep farming, particularly in intensive production systems, because of their adverse effects on productivity and mortality.[3,4]

Among the bacterial agents involved in respiratory infections in small ruminants, *Mannheimia haemolytica (M. haemolytica)* is widely recognized as a predominant and clinically important

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pathogen.[5] *M. haemolytica*, a Gram-negative, non-motile, ~0.5 × 1.2 µm rod or coccobacillus shaped, non-endospore forming, and usually encapsulated bacterium. *M. haemolytica* is a commensal organism found in the upper respiratory tract of various animal species. It has the potential to act as a primary or secondary bacterial pathogen in respiratory infections. However, studies focusing specifically on this pathogen in Turkey remain limited. Moreover, isolation of *M. haemolytica* from the upper respiratory tract alone is generally not considered sufficient to establish its role in clinical disease.[6]

In cases of pneumonia, identifying the causative agent is only the first step. To better understand disease dynamics, it is essential to determine the serotype distribution of *M. haemolytica* isolates. Certain serotypes may be more frequently associated with clinical cases, indicating their potential role in pathogenicity.[7-9] Furthermore, investigating virulence factors is essential for developing effective control and prevention strategies. Evaluating the genetic polymorphism of *M. haemolytica* strains through optimized molecular techniques may offer new insights into the pathogen's virulence mechanisms.[10]

This study aimed to serotype *M. haemolytica* isolates obtained from sheep using Real-Time PCR and to evaluate genetic polymorphism by comparing the effectiveness of RAPD-PCR and BOX-PCR genotyping methods. Ultimately, the goal was to identify the most suitable genotyping method for characterizing *M. haemolytica* isolates.

MATERIALS AND METHODS

This study was reviewed by the Van Yüzüncü Yıl University Animal Researches Local Ethics Committee and, in accordance with Article 8-19k of the Regulation on the Working Procedures and Principles of Animal Experiments Ethics Committees, was deemed not to require ethics committee approval (Decision No. 2025/10-17).

In this study, a total of 44 *M. haemolytica* isolates obtained from sheep and preserved in the culture collection of the Department of Microbiology, Faculty of Veterinary Medicine, Van Yüzüncü Yıl University, were used. For the purposes of quality standardization, verification, and validation of the methods applied in the study, reference strains of *M. haemolytica* serotypes A1, A2, and A6 were obtained from Prof. Dr. László Fodor (University of Veterinary Medicine Budapest, Budapest, Hungary).

The reference strains and *M. haemolytica* isolates stored at −80 °C in the culture collection were thawed at +4 °C. After thawing, 50 µL of each sample was inoculated onto blood agar plates and incubated at 37 °C for 24 hours. Colonies that grew as pure cultures on the medium were used for genomic DNA extraction.

Genomic DNA of the *M. haemolytica* isolates was extracted using a commercial bacterial DNA extraction kit (ThermoFisher Scientific, USA) according to the manufacturer's instructions. The extracted DNA was subsequently used for PCR analyses.

Molecular Capsular Serotyping by Real-Time PCR

To identify serotypes A1, A2, and A6 in *M. haemolytica* isolates, a multiplex molecular capsular serotyping method described by Klima et al.[11] was employed with minor modifications. Real-Time PCR assays were performed using a commercial SYBR Green qPCR mastermix (Ampliqon A463403, Denmark). Each reaction mixture contained 10 µL of master mix, 3 µL of genomic DNA, 1 µL of each primer pair listed in Table 1, and PCR-grade water to a final volume of 25 µL.

PCR amplification was performed using a thermal cycler (Rotor-Gene Q 2plex, Corbett Research, Qiagen, Netherlands). The thermal cycling included an initial denaturation at 95 °C for 15 minutes, followed by 30/35 cycles of denaturation at 94 °C for 30 seconds, annealing at gene-specific temperatures (as listed in Table 1) for 45 seconds, and extension at 72 °C for 60 seconds. A final extension was carried out at 72 °C for 10 minutes. Positive amplification was confirmed by evaluating the sigmoidal curves generated by the Real-Time PCR software. To confirm initial results, the amplicons were subjected to 1% agarose gel electrophoresis and visualized using a gel documentation system. After carried out that the banding patterns corresponded to the sigmoidal curves, subsequent serotyping was performed exclusively by Real-Time PCR.

Table 1. Oligonucleotide sequences and annealing temperatures of primers used for Real-Time PCR amplification of serotype-specific gene regions (A1, A2, and A6) in M. haemolytica isolate.

Gene		Primer Sequence 5'-3'	Annealing(°C)
Serotype 1/ Hypothetical Protein	F	CATTTCCTTAGGTTCAGC	51
	R	CAAGTCATCGTAATGCCT	
Serotype 2/ Core-2-I-Branching enzyme	F	GGCATATCCTAAAGCCGT	53
	R	AGAATCCACTATTGGGCACC	
Serotype 6/ TupA-like ATPgrasp	F	TGAGAATTTCGACAGCACT	52
	R	ACCTTGGCATATCGTACC	

Genotyping

Genetic polymorphism among *M. haemolytica* isolates was investigated using conventional PCR with the single-stranded primers BOXA1R, OPA4, and OPA9, as listed in Table 2. PCR reactions were prepared in a total volume of 20 µL, containing 10 µL of 2X master mix, 3 µL of genomic DNA, and 1 µL of each primer, with the remaining volume adjusted using PCR-grade water. Amplification was

performed in the thermal cycler. The thermal protocol included an initial denaturation at 94 °C for 10 minutes, followed by primer-specific cycles (as detailed in Table 2) consisting of denaturation at 94 °C for 60 seconds, annealing for 45 seconds at primer-specific temperatures, and extension at 72 °C for durations specified in table 2. A final extension step was conducted at 72 °C for 8 minutes. PCR products were separated by electrophoresis on 1% agarose gels and visualized using a gel documentation system. Gel images were analyzed with ImageJ software[12], measuring the distances of unknown bands relative to a DNA ladder. Fragment sizes were then estimated using the online BioTools software [13] as described by Ling et al.[14] and subsequent studies.[15]

Table 2. Primers and oligonucleotide sequences used in PCR for determining genetic polymorphism among M. haemolytica isolates

PCR Method /Primer name		Primer Sequence 5'-3'	Number of cycles	Annealing temperature (°C)	Extention time (min.)	Reference
BOX	BOX A1R	CTACGGCAAGGCGACGCTGACG	50	55	8	[11]
RAPD	OPA 4	AATCGGGCTG	45	32	2	[16]
	OPA 9	GGGTAACGCC				

Binary data matrices were generated based on the DNA banding patterns obtained from BOX-PCR and RAPD-PCR analyses. For each gel profile, bands of identical base pair sizes located in the same row were recorded as "1" (presence), whereas the absence of a band at that specific position was recorded as "0". The resulting binary data were organized to evaluate genotypic polymorphism among the *M. haemolytica* isolates.

Statistical Analysis

A binary matrix was constructed based on the banding patterns of the PCR products, and cluster analysis was performed using the SPSS 22.0 software (IBM SPSS Corp., Armonk, NY, USA). A dendrogram was subsequently generated to visualize the genetic relationships among the isolates.

RESULTS

Serotyping

Using the Real-Time PCR, 27 out of 44 *M. haemolytica* isolates (61.4%) were successfully serotyped. Among the serotyped isolates, 6 (22.2%) were identified as serotype A1, 12 (44.4%) as serotype A2, and 9 (33.4%) as serotype A6. The remaining 17 isolates could not be serotyped (Figures 1-4).

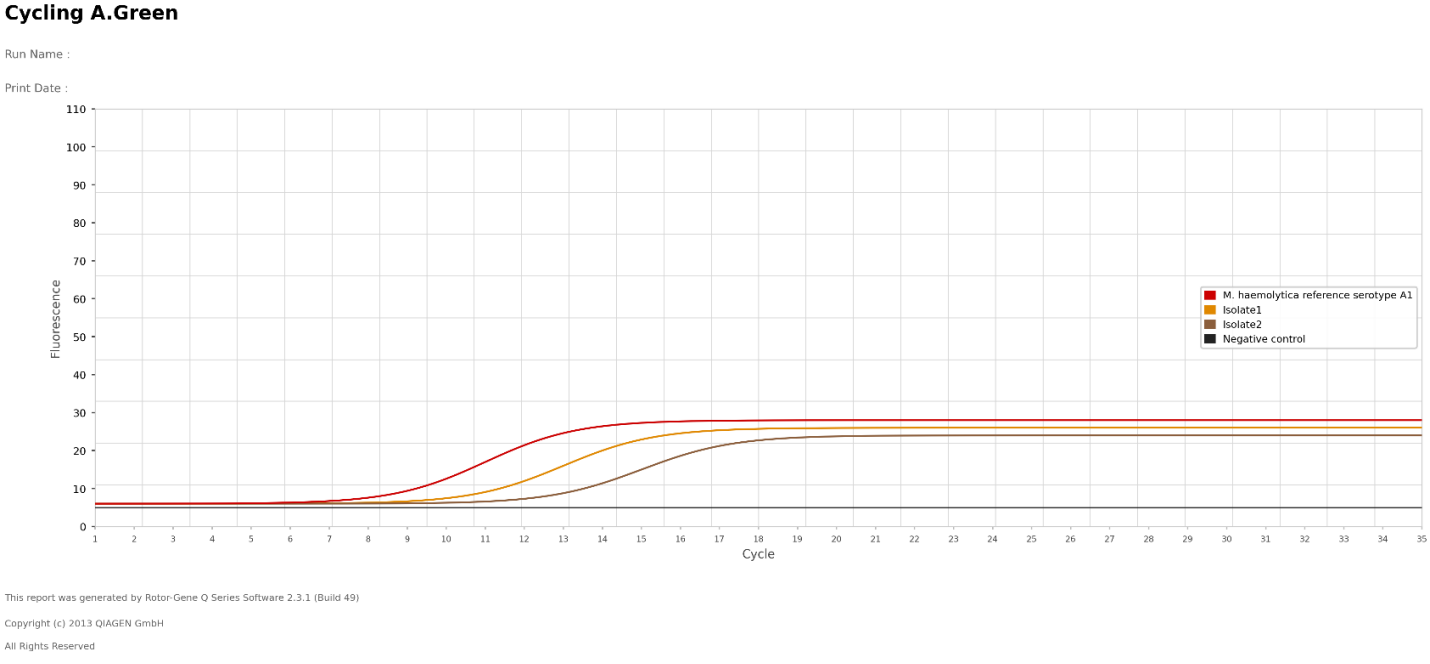

Figure 1. Sigmoidal amplification curves of *M. haemolytica* serotype A1 isolates identified as positive by Real-Time PCR.

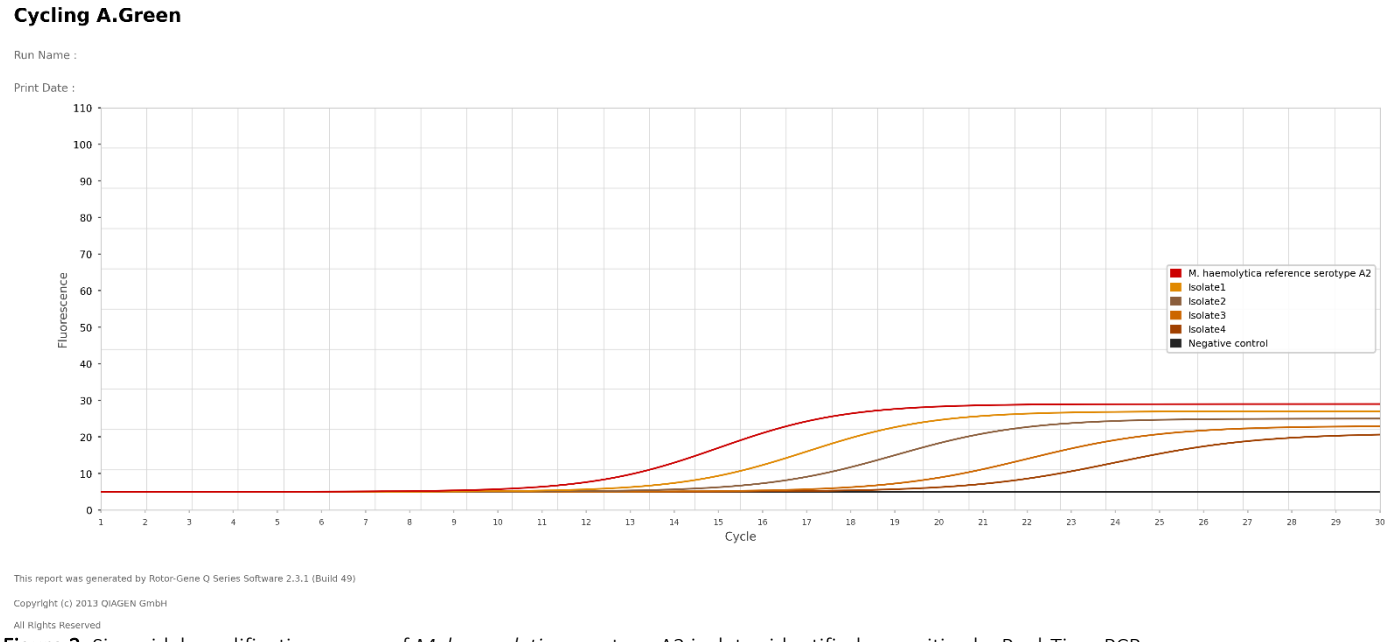

Figure 2. Sigmoidal amplification curves of *M. haemolytica* serotype A2 isolates identified as positive by Real-Time PCR.

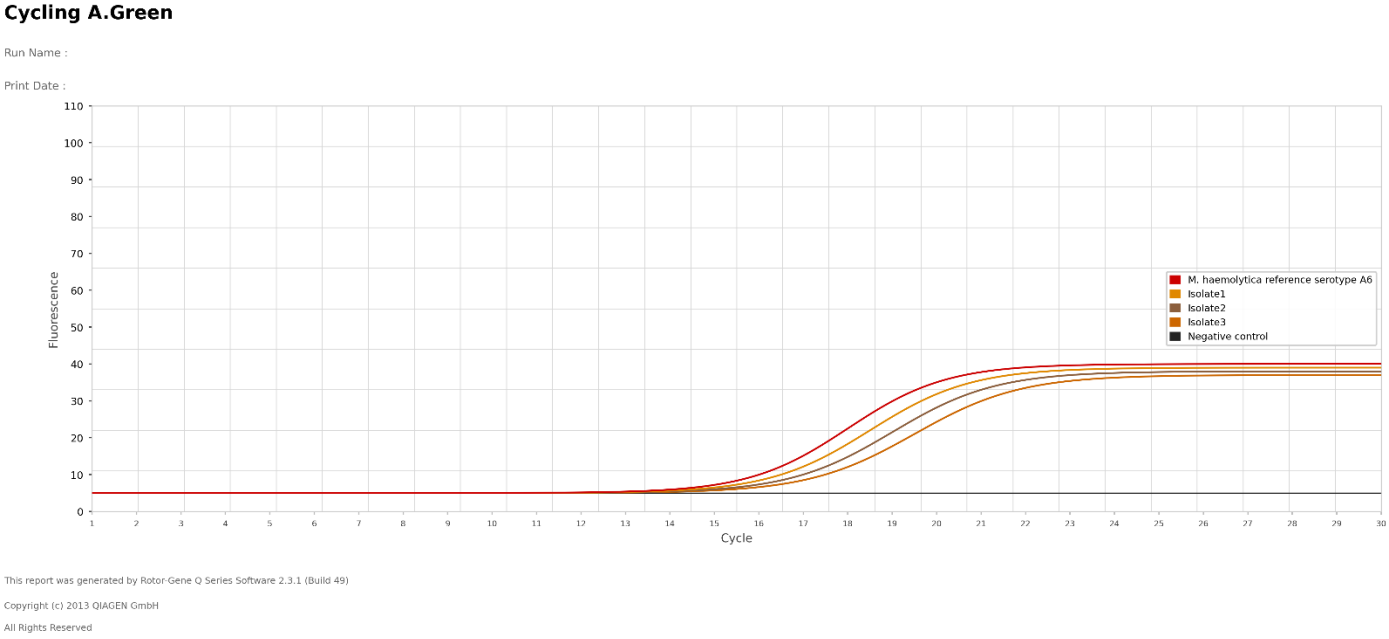

Figure 3. Sigmoidal amplification curves of *M. haemolytica* serotype A6 isolates obtained by Real-Time PCR.

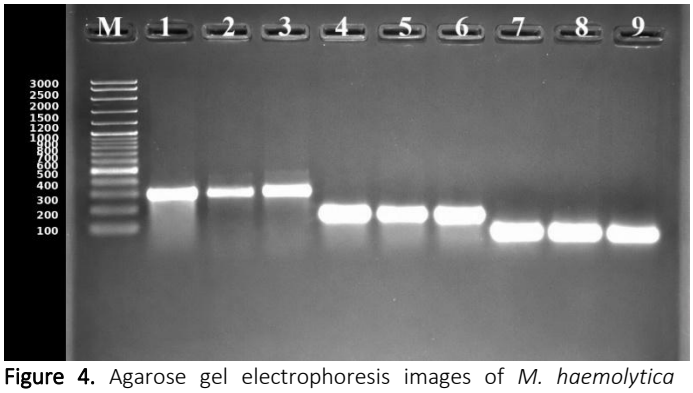

Figure 4. Agarose gel electrophoresis images of *M. haemolytica* serotypes identified by Real-Time PCR (M: Marker; 1: *M. haemolytica* reference serotype A1; 2–3: *M. haemolytica* serotype A1 positive isolates; 4: *M. haemolytica* reference serotype A2; 5–6: *M. haemolytica* serotype A2 positive isolates; 7: *M. haemolytica* reference serotype A6; 8–9: *M. haemolytica* serotype A6 positive isolates).

Molecular Characterization

RAPD-PCR Findings

The analysis using the OPA4 primer yielded amplicons ranging from 268 bp to 2547 bp. Based on the resulting banding patterns, dendrogram analysis identified 18 distinct genotypes (Figures 5 and 7). In contrast, the OPA9 primer produced amplicons between 206 bp and 1579 bp, with corresponding dendrogram analysis revealing only 3 distinct genotypes (Figures 6 and 8).

BOX-PCR Findings

In this study, BOX-PCR analysis was conducted using the BOXA1R primer to assess genetic diversity among *M. haemolytica* isolates. The analysis produced amplicons ranging from 150 bp to 3202 bp. Based on these banding patterns, dendrogram analysis identified 15 distinct genotypes (Figures 9 and 10).

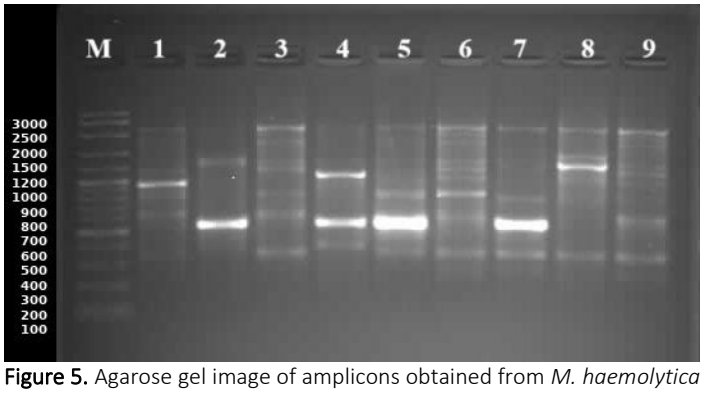

Figure 5. Agarose gel image of amplicons obtained from *M. haemolytica* isolates using the OPA4 primer in RAPD-PCR (M: Marker; 1–9: *M. haemolytica* isolates).

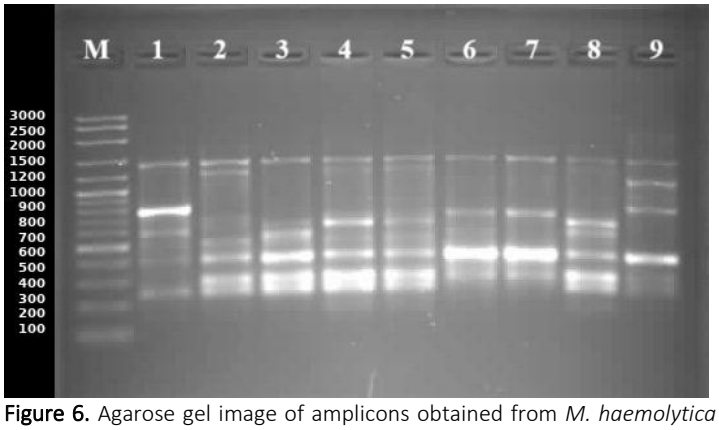

Figure 6. Agarose gel image of amplicons obtained from *M. haemolytica* isolates using the OPA9 primer in RAPD-PCR (M: Marker; 1–9: *M. haemolytica* isolates).

DISCUSSION

In intensively raised sheep, respiratory infections frequently occur when the immune system is suppressed. Factors such as inadequate and imbalanced nutrition, harsh climatic conditions, transportation stress, and poor ventilation contribute to this suppression. These infections result in significant economic losses both in Turkey and worldwide.[16] Respiratory tract infections in sheep involve various bacterial, viral, and parasitic agents. Among these, *M. haemolytica* is commonly found in the upper respiratory tract as a commensal organism. However, numerous studies have shown that it often acts as a primary or secondary pathogen in pneumonia cases.[11,17-20]

Previous studies have identified 12 capsular serotypes of *M. haemolytica*, with A1, A2, and A6 being most commonly associated with pneumonia in sheep and cattle. Identifying regionally dominant serotypes is essential for developing effective vaccination strategies within local control programs.[11,21] Various researchers have used indirect and passive hemagglutination assays to identify *M. haemolytica* capsular serotypes.[22-28] Due to the time-consuming protocols, high costs, and cross-reaction risks of traditional serotyping methods, there is increasing interest in faster, more reliable, and cost-effective molecular techniques for detecting the most common *M. haemolytica* serotypes (A1, A2, and A6).[11,21]

Several studies have examined the capsular serotypes of *M. haemolytica* in ovine pneumonia cases, although their number remains limited. Traditional methods such as indirect hemagglutination and coagulation tests have been commonly employed.

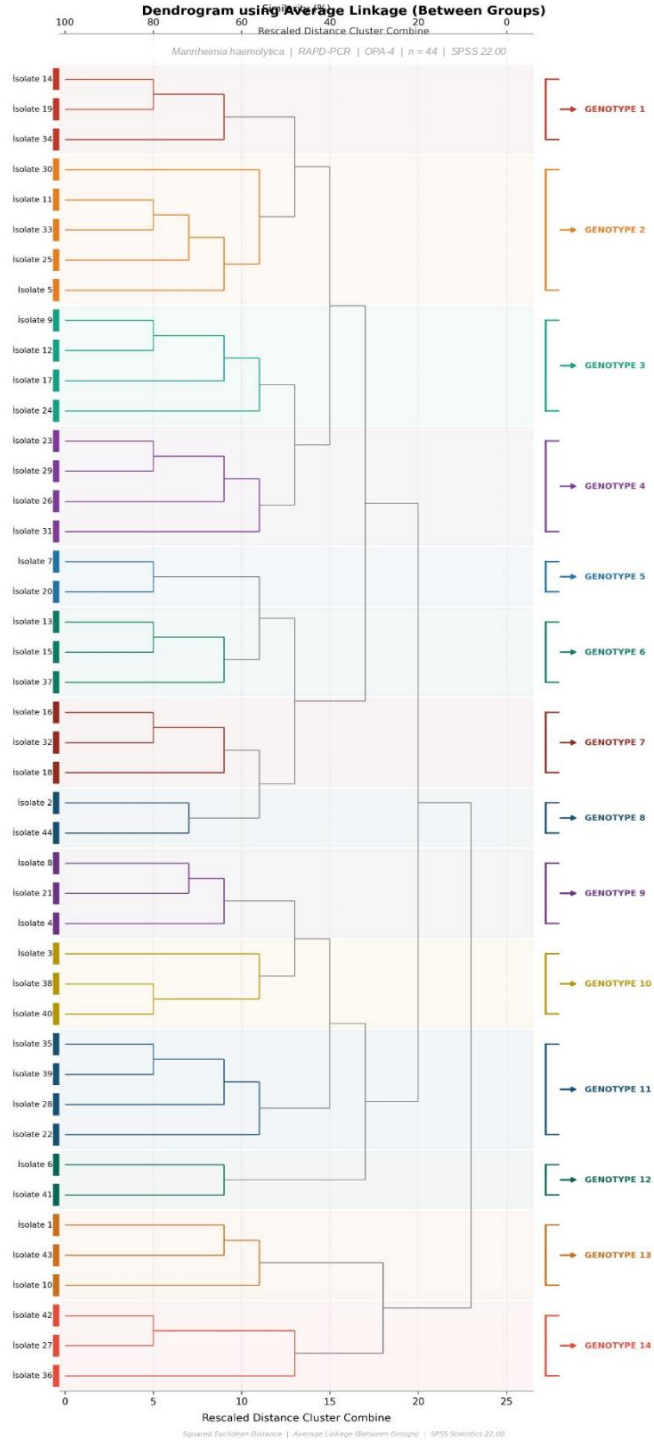

Figure 7. Dendrogram analysis of data obtained from *M. haemolytica* isolates using the OPA4 primer in RAPD-PCR.

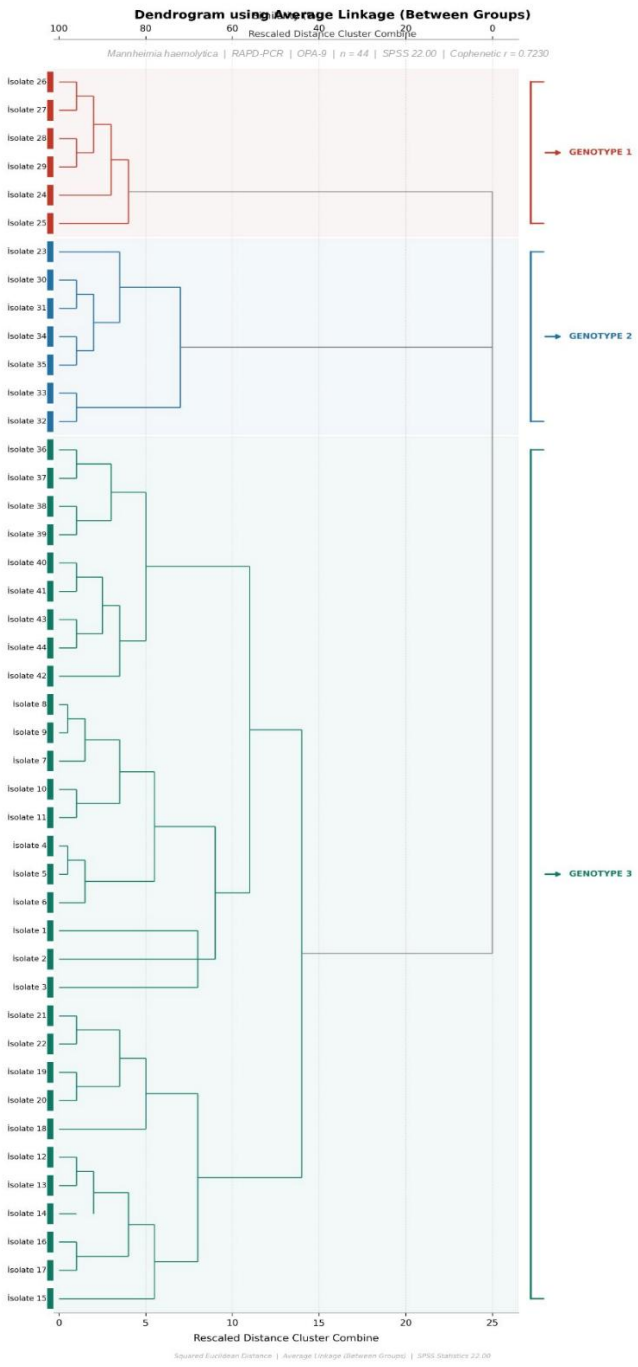

Figure 8. Dendrogram analysis of data obtained from *M. haemolytica* isolates using the OPA9 primer in RAPD-PCR.

For instance, in a comparative study, Fodor et al.[22] reported serotypes A1 (23.1%), A2 (24.6%), and A6 (7.7%) using indirect hemagglutination, with similar results from the coagulation test. However, a portion of the isolates remained untypable, highlighting the limitations of these conventional approaches. Al-Ghamdi et al.[29] found that 59.2% of the isolates were serotype A1, 25.8% A6, and 7.5% A2 using passive hemagglutination[30], emphasizing the challenges of cross-reactivity and sensitivity. In Türkiye, several regional studies provide valuable data. In Aydın Province, *M. haemolytica* isolates from sheep showed 12.5% A1, 25% A2, and 20.8% A6 serotypes.[24] İlhan and Keleş[25] reported rates of 16.6% A1, 21.2% A2, and 15.1% A6 in Van Province. Another study using passive hemagglutination identified 51.1% A2, 8.8% A6, and 7.7% A1.[26] In lambs, Gonzalez et al.[27] detected A2 (29%), A6 (12%), and A12 (18%) as the most common serotypes. In a similar study from Ethiopia, it was reported that 33.3% of the isolates from sheep were serotype A1, 11.9% as A2, and 14.3% as A6.[23] Similarly, Berhe et al.[28] found high rates of A1 (75%) and A2 (68.8%). These results collectively suggest that serotype distribution is influenced by factors such as animal species, geography, and husbandry practices, and they highlight the presence of untypable isolates across multiple studies.

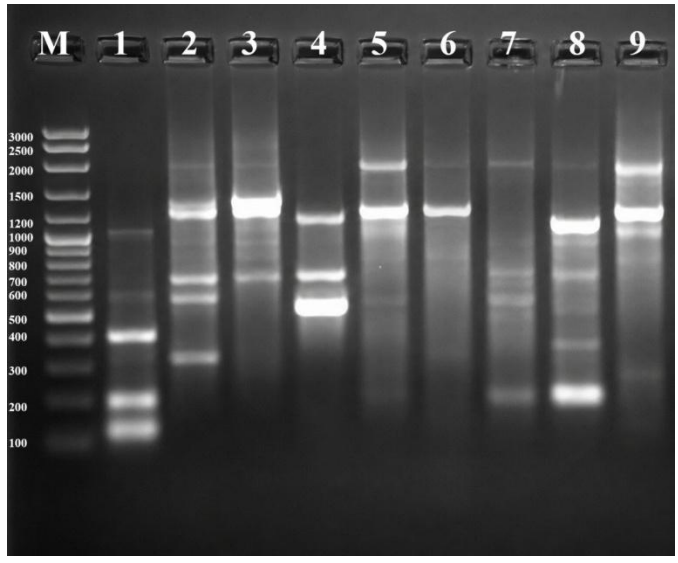

Figure 9. Agarose gel image of amplicons obtained from *M. haemolytica* isolates using the BOXA1R primer in BOX-PCR (M: Marker; 1–9: M. haemolytica isolates).

Recent approaches have increasingly turned to molecular methods for more accurate serotyping. Klima et al.[11] and Andrés-Lasheras et al.[21] used multiplex PCR and slide agglutination to detect serotypes A1, A2, and A6 in field and reference isolates. In Klima's study, both methods were deemed effective, but multiplex PCR was favored for its speed and reliability. Andrés-Lasheras et al.[21] identified 48% A1, 16% A2, and 16% A6 among 98 isolates, though 18% remained untypable. Despite using the same primers as these studies, the current investigation observed differing results. These discrepancies may stem from species-specific serotype determinants or regional variations in nutrition, climate, and management practices. Overall, these findings reinforce the need for broader, standardized molecular surveillance to improve serotype identification and inform regional vaccination strategies.

Studies on the genetic diversity of *M. haemolytica* are limited. Most have focused on isolates obtained from

cattle[10,11,31,32] while research specifically targeting ovine-derived isolates remains limeted.[33,34]

The most commonly used methods for determining genotypic differences among *M. haemolytica* strains include pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), repetitive sequence-based PCR (BOX-PCR), enterobacterial repetitive intergenic consensus (ERIC)-PCR, and poly-trinucleotide $(GTG)_5$ PCR fingerprinting.[10,31-34]

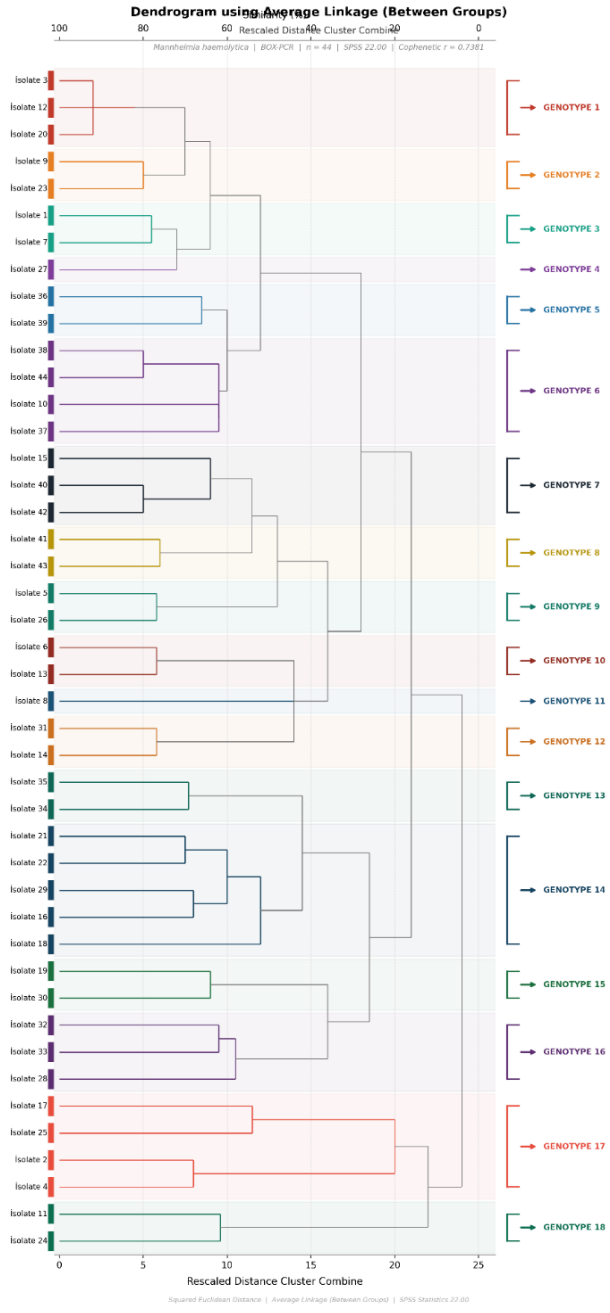

Figure 10. Dendrogram analysis of data obtained from *M. haemolytica* isolates using the BOXA1R primer in BOX-PCR.

Several studies have employed various molecular typing techniques to assess the genetic diversity of *M. haemolytica* isolates. Katsuda et al.[33], analyzed 130 *M. haemolytica* serotype A1 strains isolated from 13 different regions in Japan using PFGE and RAPD methods. PFGE revealed six distinct genotypes with 15 subtypes, while RAPD analysis identified four different genotypes. The present study's RAPD-PCR results using the OPA4 primer showed similar levels of polymorphism, although minor differences were observed. In another study conducted in Canada, genotyping of 40 bovine *M. haemolytica* isolates revealed that BOX-PCR produced 25 profiles and PFGE 37 profiles, both clustering all isolates into a single group.[10] The authors suggested BOX-PCR as a viable alternative to PFGE in epidemiological studies. In the current study, BOX-PCR produced genotypic profiles with differing fragment sizes despite the use of the same primers, possibly due to regional genetic heterogeneity. Supporting this, a study in Egypt comparing *M. haemolytica* and *P. multocida* isolates from sheep and goats using RAPD and ERIC primers also reported distinct genotypes, with fragment sizes varying by species and primer type.[34]

Collectively, these findings highlight the importance of regional surveillance and the use of multiple genotyping methods to evaluate the genetic diversity of *M. haemolytica*. In this study, RAPD-PCR with the OPA9 primer generated only three genotypes, indicating limited discriminatory power. In contrast, the OPA4 primer identified 18 genotypes, and BOX-PCR with the BOXA1R primer revealed 15 distinct genotypes. Although BOX-PCR required longer processing time, it produced more consistent and informative banding profiles, suggesting its suitability for epidemiological investigations.

No statistically significant association was found between the genotypes and the serotypes (A1, A2, A6) in this study. This emphasizes the need for broader studies including additional serotypes to better understand the genetic structure and epidemiology of *M. haemolytica*.

Real-Time PCR proved to be a rapid, reliable, and reproducible method for detecting the major serotypes A1, A2, and A6. Among the isolates examined, serotype A2 was the most prevalent. However, the observed potential cross-reaction with serotype A7 during assay optimization suggests that gel electrophoresis should be used to confirm results prior to routine application.

In conclusion, identifying dominant *M. haemolytica* serotypes and genotypes at local, national, and international levels is essential for developing effective control and prevention strategies. These findings indicate that the prevalent serotypes and genotypes should be taken into account in future vaccine development efforts targeting respiratory diseases in small ruminants.

Ethics Committee Approval: This study was reviewed by the Van Yüzüncü Yıl University Animal Researches Local Ethics Committee and, in accordance with Article 8-19k of the Regulation on the Working Procedures and Principles of Animal Experiments Ethics Committees, was deemed not to require ethics committee approval (Decision No. 2025/10-17).

Participant Consent: As the study was conducted on bacteria in the culture collection of the Department of Microbiology, Faculty of Veterinary Medicine, Van Yüzüncü Yıl University, no participant consent form was required.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept- C.Ö.; Design- C.Ö., İ.H.E.; Data Collection or Processing- C.Ö.; Analysis or Interpretation- C.Ö., İ.H.E.; Literature Search- C.Ö., İ.H.E.; Writing – C.Ö.; Critical Review - İ.H.E., Ö.G.

Conflict of Interest: The authors declare that there is no conflict of interest.

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