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Determination of *Mannheimia haemolytica* and its macrolide antibiotic resistance genes in fibrinous pneumonia of cattle

Determinación de *Mannheimia haemolytica* y sus genes de resistencia a antibióticos macrólidos en neumonía fibrinosa del ganado bovino

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ABSTRACT

Early diagnosis and timely initiation of effective treatment are critical for the pneumonic pasteurellosis, which is mostly caused by *Mannheimia haemolytica*. But recently, strains of *M. haemolytica* resistant to antibiotics have begun to emerge, making the treatment of infections more difficult. Therefore, it is important to investigate the *erm (42), msr (E)* and *mph (E)* genes, which are held responsible for the resistance to antibiotics.The purpose of the present study was aimed to diagnose the presence of *M. haemolytica* by IHC method from lung tissue samples of young and adult cattle, and calves, also to detect *erm (42), msr (E)* and *mph (E)* genes, which are mostly detected in *Pasteurella multocida* but recently detected in *M. haemolytica*, by PCR method. In present study, paraffin blocks obtained from 100 young and adult cattle, and calves lung samples, and whose treatment was not responded, were used. Accordingly, the presence of *M. haemolytica* was detected by IHC in a total 52 cases of in 21 (38%) of the calves, 22 (69%) of the young cattle, and 9 (69%) of the adult cattle. At least one macrolide resistance gene was found by real–time PCR method in 75% of these positive cases. It was thought that the percentage frequency of these genes, which cause resistance by increasing the minimum inhibitory concentrations of macrolide group antibiotics used in the treatment, by high amounts, makes the treatment of infections difficult and causes serious economic losses by causing errors in effective antibiotic selection. Recently, strains of *M. haemolytica* resistant to antibiotics have begun to emerge, making the treatment of infections more difficult. For this reason, it is recommended that this type of prevalence studies should be renewed periodically due to possible resistance development and the repeated use of antibiotics with resistive shape should be avoided.

Keywords: *Mannheimia haemolytica*; fibrinous pneumonia; *erm (42)*; *msr (E)*; *mph (E)*; macrolide; antibiotic resistance; cattle

RESUMEN

El diagnóstico precoz y el inicio oportuno de un tratamiento eficaz son fundamentales para la pasteurelosis neumónica, causada principalmente por *Mannheimia haemolytica*. Pero recientemente, han comenzado a surgir cepas de *M. haemolytica* resistentes a los antibióticos, lo que dificulta el tratamiento de las infecciones. Por lo tanto, es importante investigar los genes *erm (42), msr (E)* y *mph (E),* que se consideran responsables de la resistencia a los antibióticos. El propósito del presente estudio tuvo como objetivo diagnosticar la presencia de *M. haemolytica* mediante el método IHC a partir de muestras de tejido pulmonar de bovinos jóvenes, adultos y terneros, también identificar los genes *erm (42), msr (E)* y *mph (E),* que se detectan principalmente en *Pasteurella multocida* pero recientemente en *M. haemolytica*, mediante el método PCR. En nuestro estudio se utilizaron bloques de parafina obtenidos de 100 muestras pulmonares de bovinos jóvenes, adultos y terneros, y cuyo tratamiento no tuvo respuesta. Por consiguiente, se detectó la presencia de M. haemolytica mediante IHC en un total de 52 casos, en 21 (38%) de los terneros, 22 (69%) de los bovinos jóvenes y 9 (69%) de los bovinos adultos.. Se encontró al menos un gen de resistencia a macrólidos mediante el método de PCR en tiempo real en el 75% de estos casos positivos. Se pensaba que la frecuencia de estos genes, que provocan resistencia al aumentar las concentraciones inhibitorias mínimas de antibióticos del grupo de los macrólidos utilizados en el tratamiento, en cantidades elevadas, dificulta el tratamiento de infecciones generando graves pérdidas económicas, al provocar errores en la selección efectiva de antibióticos. Recientemente, han comenzado a surgir cepas de *M. haemolytica* resistentes a los antibióticos, lo que dificulta el tratamiento de las infecciones. Por este motivo, se recomienda se renueven periódicamente por posible desarrollo de resistencias y se evite el uso repetido de antibióticos con forma resistiva.

Palabras clave: *Mannheimia haemolytica*; neumonía fibrinosa; *erm (42); msr (E); mph (E);* macrólido; resistencia a antibióticos; ganado bovino

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INTRODUCTION

Bovine respiratory disease (BRD) is a leading cause of illness and death in beef cattle (*Bos taurus*) worldwide. *Pasteurella multocida* and *Mannheimia haemolytica*, both of which are Gram–negative bacteria naturally residing in the upper respiratory tract, are the primary bacterial pathogens responsible for BRD. These bacteria can cause mild to severe clinical symptoms, especially when cattle are subjected to various stressors, including environmental, managemental, or infectious challenges [1, 2, 3].

The findings obtained support that outbreaks are often polymicrobial, that this disease occurs because of the disruption of the lung defense system and colonization by opportunistic pathogens by crossing the weakened defense mechanisms. The most prominent feature of severe pneumonia in cattle is intense fibrin exudation into the alveoli. Although direct effects of lipopolysaccharides on endothelial cells partly cause this condition, macrophage–derived inflammatory mediators such as TNF–α also play an important role. The use of aorebic and microaerophilic cultures to determine the specific etiology is usually successful unless the animals are treated with antimicrobial drugs. Subacute or chronic cases are often colonized by opportunistic bacteria, and the primary bacterial cause may no longer be found. Immunohistochemical (IHC) is used to diagnose *M. haemolytica*. The IHC method can also be used in cases where fresh tissue is not available, in archival tissues, in cases of autolysis and antimicrobial treatment. Serum antibody detection against *M. haemolytica* using leukotoxin neutralization test, ELISA method and bacterial agglutination test are common research tools. However, these are of limited use in field studies [4].

For years, minimal inhibition concentration (MIC) of antimicrobial drugs has been measured by tube dilution and disc diffusion (Kirby Bauer) techniques to determine antibiotic resistance [5]. However, the inability to isolate the target bacteria from the samples taken from antibiotic treated or autolytic tissues and the length of the test periods are considered as limiting factors for the use of these tests [6].

To determine the resistance of *M. haemolytica* against 14–, 15–, and 16–membered macrolides via the PCR method, specific resistance genes have been identified [7]. Whole genome sequencing of the bacteria has revealed that these resistance phenotypes result from the combination of at least three distinct macrolide resistance mechanisms. In the first group of isolates, the erythromycin resistance methyltransferase gene (*erm (42)*) is the determinant. This gene confers the MLSB (macrolide, lincosamide, and streptogramin B) type I phenotype, with high resistance to lincosamides and low to moderate resistance to macrolides and streptogramin B. Conformational changes in the P region of rRNA prevent macrolide binding and eliminate the inhibitory effect of macrolides on protein synthesis [8]. The second group of isolates contains neither an erm gene nor methylation at nucleotide A2058 and is resistant to macrolides without associated lincosamide resistance. This group harbors two resistance genes, *msr (E)* and *mph (E),* which are sequenced and expressed from the same source, encoding a macrolide efflux pump and macrolide inactivation phosphotransferase, respectively. The third group of isolates displays high resistance to a wide range of macrolides and carries three determinants: *erm (42), msr (E),* and *mph (E)* [7].

Studies conducted in many countries have shown that resistance to macrolide group antibiotics has developed in *M. haemoltica*, suggesting that this resistance may be regional. In our geography, it has been observed that many antibiotics, especially macrolide group antibiotics, are used for respiratory system diseases in cattle, but mostly no response to treatment is received. Although there have been some studies on the antibiotic sensitivity and resistance of *M. haemolytica* in our country, there has not been a study specifically investigating macrolide group antibiotics and the resistance genes formed against them. The fact that the anamnesis information obtained from the animal owners of the fibrinous pneumonia cases brought to pathology department indicates that there is no improvement despite antibiotic treatment also suggests that antibiotic resistance may develop.

In this study, it was aimed to diagnose the presence of *M. haemolytica* by IHC method from paraffin blocks prepared from lung tissue samples of young and adult cattle, and calves brought to pathology department and also to identify *erm (42), msr (E)* and *mph (E)* genes, which are mostly detected in *P. multocida* but recently detected in *M. haemolytica*, by PCR method. As a result of this study, it is intended to make an important contribution to the treatment plan of the disease by both diagnosing *M. haemolytica* and determining the macrolide resistance genes from the fibrinous pneumonia cases detected in cattle.

MATERIALS AND METHODS

Ethical statement

The ethics committee of the Faculty of Veterinary Medicine, Experimental Animal Production and Research Center, Selcuk University, Konya, Türkiye, approved this study (Approval no: 2017/64).

Animals, study area, period and design

The material of the study consisted of a total of 100 bovine animals (55 calves [less than 6 months old], 32 young cattle [6–24 months old] and 13 adult cattle [older than 24 months old]) that were brought to the Department of Pathology, Faculty of Veterinary Medicine, Selcuk University, Konya, Türkiye, between 2012 and 2017 for necropsy and diagnosed as fibrinous pneumonia. The diagnostic criteria of fibrinous pneumonia were determined according to the findings described in the Results. All animals were routinely necropsied, and tissue samples were taken for histopathological and IHC examinations after macroscopic inspection.

Histopathological method

Lung samples taken from necropsied ruminants were fixed in 10% buffered formaldehyde solution and embedded in paraffin after routine tissue processing procedures. The tissue samples were sectioned at a thickness of 5 μm on a microtome (Leica RM 2125RT, Germany), stained with Haemotoxylene&Eosin (H&E) and examined under a binocular light microscope (Olympus BX51, Tokyo, Japan). Photographs were taken of the lesions observed during microscopic examination (Olympus DP12, Tokyo, Japan).

Immunohistochemical method

For the IHC method, sections taken from the lung tissues on 5 μm thick to adhesive slides were stained (Peroxidase Block, Protein Block, Post Primer, Polymer, DAB, DAB, Hematoxylin) in a fully automatic IHC (Leica Bondmax) kit procedure. Details of the staining protocols with each primary antibody and the applications are shown in TABLE I. Stained sections of IHC were examined under a binocular light microscope (Olympus BX51, Tokyo, Japan) and the criteria of positive IHC staining determined by pathologists.

TABLE I
IHC staining protocols according to primary antibodies

Primary antibody	Dilution	Antigen retrieval	Peroxidase block	Protein block	Antibody incubation	Post Primer	Polymer	DAB	Hem*
Mannheimia haemolytica antibody	1:1000	HIER1 – 100°C – 30 min	45 min	30 min	60 min	15 min	15 min	5 min	3 min

* Hem: Hematoxylin

Mannheimia haemolytica primary antibody production for IHC staining

Confirmation of *Mannheimia heamolytica* isolate

For DNA extraction, a colony of *M. haemolytica* isolates was transferred to brain heart infusion (BHI) liquid medium and incubated at 37°C for 1 night. Wizard® Genomic DNA Purification Kit (Promega, *Madison*, Wisconsin, USA) was used for DNA extraction according to the protocol. The polymerase chain reaction (PCR) (Gene Amp PCR System 2400, Applied Biosystems, USA) was performed for confirmation against 16S rRNA of suspected *M. haemolytica*. PCR mix 5 µL of 5x master mix (SolisBiodyne, Estonia), 50 ng·µL^{-1} DNA, 0.2 pmol of each primer: primer F5 5'–TGGGCAATACGAACTACTCGGG–3' and primer R5 5'–CTTTAATCGTATTCGCAG–3' were added to 25 µL of ultrapure water. For PCR, a protocol of 94°C 4 min pre–denaturation 40 cycles (94°C 30 s, 55°C 30 s, 72°C 30 s) and 72°C 10 min final extension was used. PCR products were run on agarose gel containing 10 µg·mL^{-1} ethidium bromide with a 100 bp ladder (Solisbiodyne, Estonia) and a band of 227 bp detected by UV light was considered positive (FIG 1).

Hyperimmune elicitation

One colony of *M. haemolytica* (BHI) confirmed by PCR for use in the hyperimmunisation study was transferred to 100 mL of liquid medium, incubated (M5040 BP, Elektromag, Türkiye) at 37°C for 48 h and inactivated with 0.4% formalin. Sterility controls were performed after inactivation. The turbidity of the culture was adjusted by McFarland Densitometer (DEN–1, Biosan, Türkiye) according to McFarland tube no:4 so as to have approximately 1.2 × 10^9 bacteria in 1 mL. Both antigen and vaccine were prepared from *M. haemolytica* for hyperimmune production. Antigen was obtained by not adding adjuvant to the inactive culture. Candidate vaccine was prepared by homogenizing (Ultratorrax, Isolab, Germany) the inactivated culture and Montanide ISA 206 Vegatable Grade (Seppic, France) adjuvant in equal proportions and sterility controls were performed (9 CFR§113.33). The prepared antigen and candidate vaccine were stored at 4°C until use. To produce hyperimmune serum, 4 swiss albino mice (*Mus musculus*) weighing between 16 and 18 g were used. During antigen and vaccine administration, study mice were monitored for 60 days (d) in terms of morbidity and mortality**.** The prepared adjuvant–free antigen was injected into the mice intramuscularly with 0.1 mL at 5 d intervals and 0.2 mL of vaccine was administered intramuscularly on the 30th d of immunisation, followed by the last intramuscular injection of 0.2 mL of vaccine on the 45th d and blood was taken from the heart of the mice on the 60th d. The blood was kept at room temperature for 30 min and then centrifuged (NF–200, Nüve, Türkiye) at 2800 G for 5 min to separate the serum and the serum was stored at -20°C until use.

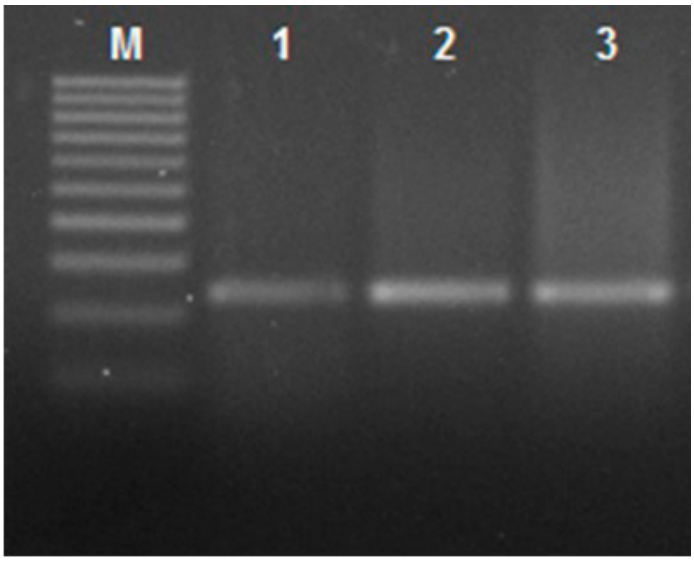

FIGURE 1. Agarose gel image of *Mannheimia haemolytica* confirmed by PCR. M: Marker (100 bp ladder), 1–3: *Mannheimia haemolytica* strains (227 bp)

Real–time PCR method and primer design

In *M. haemolytica*, *erm (42), msr (E), mph (E)* genes were isolated from paraffin blocks obtained from routine tissue follow–ups according to the DNA isolation kit procedure (QIAamp DNA FFPE Tissue Kit, Qiagen, Cat No./ID: 56404).

The plates were preincubated in Real–time PCR (LightCycler® 96 System, Roche, Germany) 96 Cycle device, 95°C by 10 min; 1 cycle, 95°C by 15 s for amplification and 64°C by 45 s, 45 cycles, cooling 37°C by 30 sec, 1 cycle. The results obtained were evaluated. For multiplex PCR, *erm (42), mph (E)* and *msr (E)* primers were designed as previously reported by Rose *et al*. [9] (TABLE II).

To each well using DNA master hydrolysis probes:

Component	Volume
Probe	1 µL
Primer Forward	1 µL
Primer Reverse	1 µL
Master	10 µL
H_2O	2 µL
Sample	5 µL
Total Volume	**20 µL**

TABLE II
Resistance gene sequences

Gen	Direction	Primary Sequence (5' – 3')	Fragment size (bp)	GenBank accession No
erm (42)	Forward	TGCACCATCTTACAAGGAGT	173	HQ888763
	Reverse	CATGCCTGTCTTCAAGGTTT		
mph (E)	Forward	ATGCCCAGCATATAAATCGC	271	JF769133
	Reverse	ATATGGACAAAGATAGCCCG		
msr (E)	Forward	TATAGCGACTTTAGCGCCAA	395	JF769133
	Reverse	GCCGTAGAATATGAGCTGAT		

RESULTS AND DISCUSION

Current knowledge on the frequency and antimicrobial resistance of bacterial respiratory pathogens is crucial to guide antimicrobial choice in the control and treatment of BRD. Etiological causes of bovine respiratory tract infections are quite numerous and complex. In fact, this infection can be successfully treated with many antibiotic groups including macrolide group. However, the emergence of *M.haemolytica* strains resistant to these antibiotics recently causes significant complications in the control and treatment of the disease [3, 10].

Macroscopic findings

The macroscopic findings seen in the lungs were red-brown hepatised areas usually scattered in the cranial and sometimes caudal lobes, enlargement of the interlobular septum, white-yellow fibrinous exudate in the pleura and areas of adhesion to the thorax. In addition, a purulent content (fibrinopurulent bronchopneumonia) or areas of necrosis of varying size (fibrinonecrotic pneumonia) were sometimes detected on the cut surface.

Histopathologic findings

Microscopically, an initial period characterized by active hyperaemia of the capillaries in the interalveolar septum and alveolar edema and the period of red hepatisation with neutrophil granulocytes, shed epithelial cells and dense fibrin threads in the alveoli were observed. Regions belonging to grey hepatisation periods with disappearance of hyperaemia but intraalveolar neutrophil granulocytes, shed epithelial cells, and abundant fibrin were also noted (FIG 2-A). In addition, enlargement of the interlobular septum was observed due to inflammation and fibrinous exudate (FIG 2-B). Necrotic areas surrounded by an inflammatory zone (FIG 2-C) and "oat cells" with elongated nuclei were occasionally observed (FIG 2-D).

The macroscopic and microscopic findings observed in present study are consistent with the findings of fibrinous, fibrinonecrotic

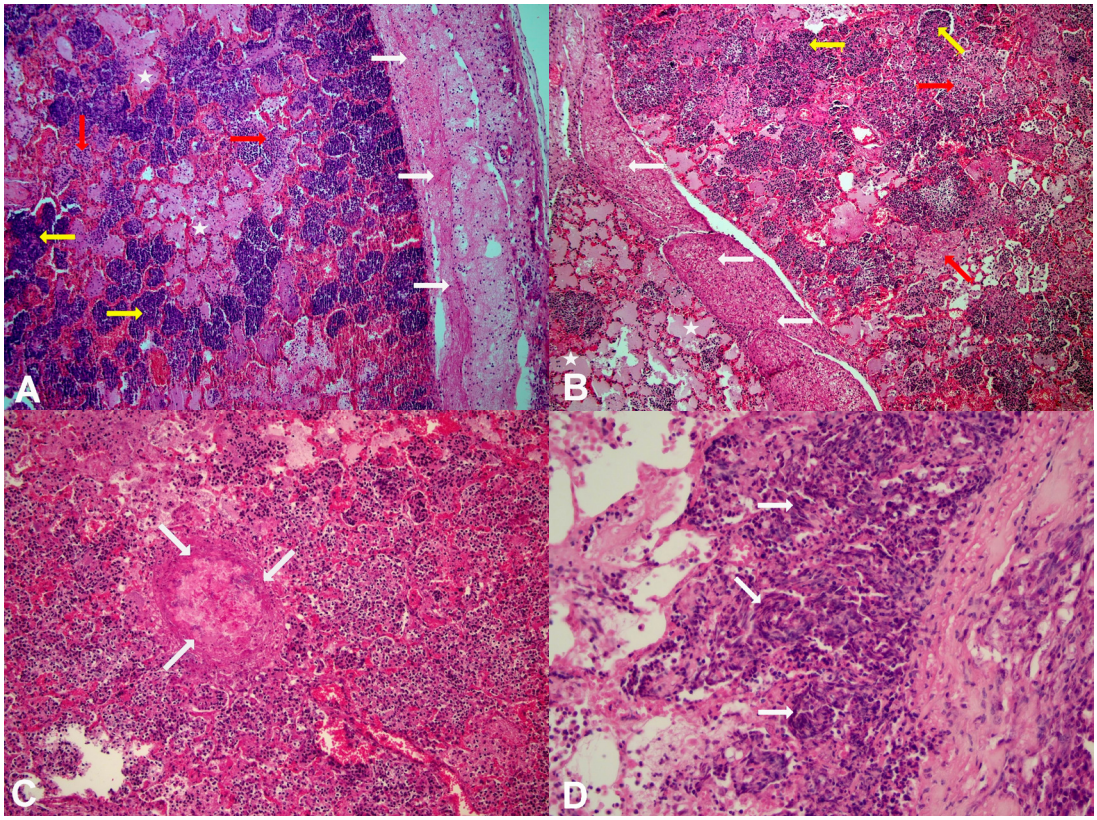

FIGURE 2. Stages of fibrinous pleuropneumonia. A: Active hyperaemia (asterisks), red hepatisation (red arrows), grey hepatisation (yellow arrows) and expansion of the pleura due to fibrin and inflammatory edema (white arrows), Lung, H&E, 100×. B: Active hyperaemia (asterisks), red hepatisation (red arrows), grey hepatisation (yellow arrows) and enlargement of the interlobular septum due to fibrin and inflammatory edema (white arrows), H&E, Lung, 100×. C: Fibrinonecrotic pneumonia, stages of fibrinous pneumonia and necrosis (arrows), Lung, H&E, 200×. D: Fibrinous pneumonia, the oat cells with elongated nuclei (arrows) in the alveolar lumen, Lung, H&E, 400×

or fibrinopurulent bronchopneumonia previously reported [2, 4, 11]. Dorso *et al.* [2] reported that the microscopic findings of fibrinous pneumonias are characterized by fibrinous pleuritis, luminal exudate composed of neutrophils, fibrinous alveolitis, "oat cells", necrotic foci and blood vessel or lymphatic thrombosis. The researchers also recorded that the most frequently detected pathogens among the 26 lungs sampled for histological and PCR examinations with macroscopic lesions were *M. haemolytica* (69.2%) and *P. multocida* (61.5%).

Immunohistochemical findings

Accordingly, the presence of *M. haemolytica* was detected by IHC in 21 of 55 calves (38%), 22 of 32 young cattle (69%) and 9 of 13 adult cattle (69%), totalling 52 cases (52%) (FIG 3).

Immunoreactivity was found in the lumens of alveoli, bronchi and bronchioles, in the cytoplasm of neutrophil granulocytes and alveolar macrophages in areas of inflammation and sometimes in bronchial and bronchiolar epithelium (FIG 4 A–B–C–D)

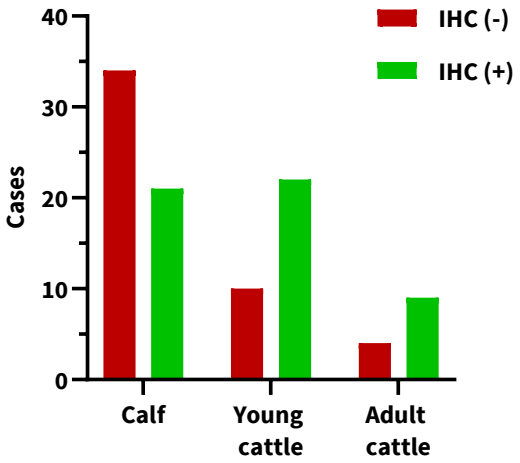

FIGURE 3. Graphical representation of *Mannheimia haemolytica* positive or negative cases by IHC

In pneumonia cases in cattle in our country; Haziroglu *et al.* [12] reported that *M .haemolytica* was isolated in 42%, *P. multocida* in 8%, *Histophilus somni* in 10%, both *M. haemolytica* and *H. somni* in 7%, and *M. haemolytica* and *P. multocida* in 2% of 100 calf lungs with pneumonia. Kilic and Muz [13] detected pneumonia in 500 (6%) of a total of 8,222 cattle lungs slaughtered in an abattoir and isolated *P. multocida* in 30 (6%) and *M. haemolytica* in 9 (1.8%) of them. As can be understood from these studies, *M. haemolytica* is one of the most important bacterial agents in bovine pneumonia in our country. However, it was observed that not only fibrinous pneumonias but also cases with pneumonia in general and even healthy lungs were included in a study [14]. In addition, it is inevitable that there may be deficiencies in diagnosis and treatment due to the inability to

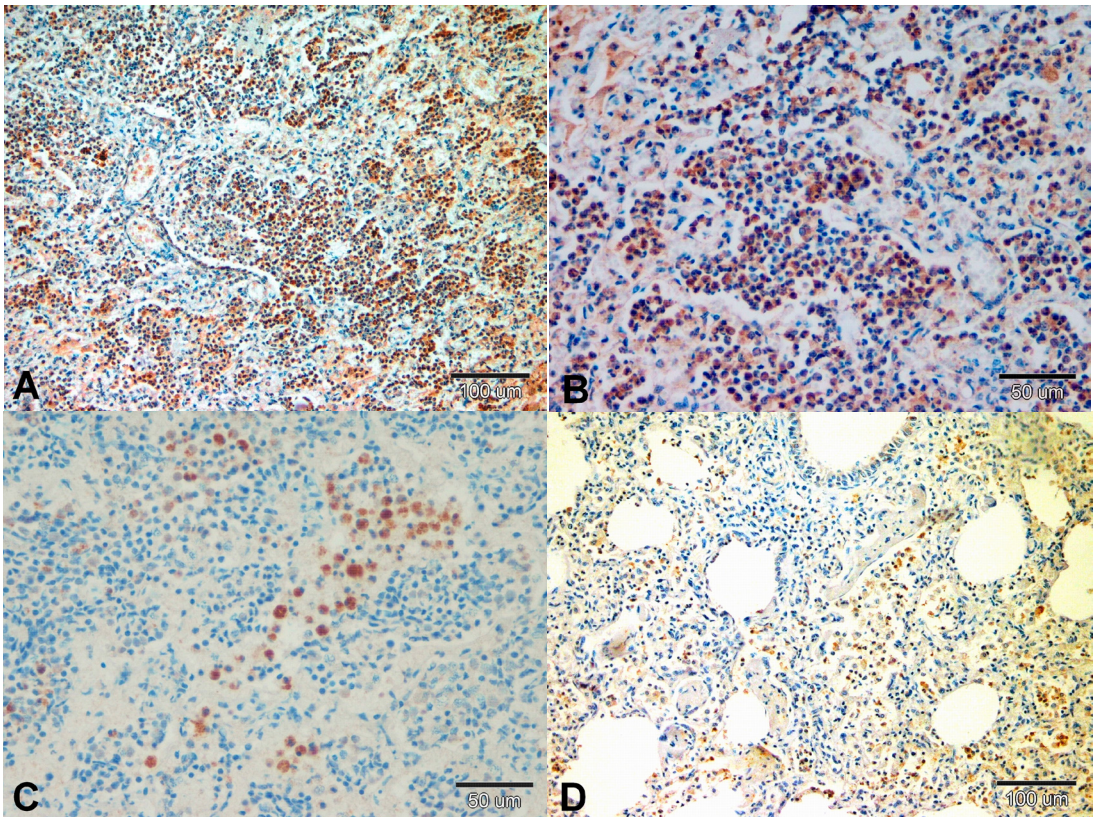

FIGURE 4. A: Anti–*Mannheimia haemolytica* positive immunoreactivity in inflammatory cells, desquamated epithelial cells and alveolar macrophages in alveolar lumens, Lung, IHC, bar: 100 µm. B: Anti–*Mannheimia haemolytica* positive reaction in desquamated epithelium and inflammatory cells in alveolar lumens, IHC, Lung, bar: 50 µm. C: Anti–*Mannheimia haemolytica* positive reaction in inflammatory cells, IHC, bar: 50 µm. D: IHC, bar: 100 µm

produce *M. haemolytica* in bacterial cultures both in these studies and in laboratory analyses, especially in cases where antibiotics are used. As a matter of fact, in present study in which only 100 different fibrinous bronchopneumonia cases were evaluated and which differed from other studies in this respect, *M. haemolytica* was found positive in 52 cases by IHC method. When the distribution of these cases according to age groups was analysed, the rate of the causative agent, which was around 38% in calves, could reach up to 69% in young cattle and adult cattle (FIG 3). It can be thought that this situation may be because this agent is less common in calves and that calves are not exposed to predisposing factors such as transportation, cramped housing, inadequate nutrition, etc.

On the other hand, the detection of the presence of the agent in the cytoplasm of epithelial and inflammatory cells in the lesioned areas (FIG 4) is in agreement with the findings of other studies [15, 16]. In addition, by revealing the presence of the causative agent with IHC, disadvantages such as antibiotic use, autolysis, and failure to isolate the causative agent in culture were prevented and more reliable results were obtained.

Real–Time PCR and resistance genes findings

The details of the resistance gene investigations performed by multiplex real time PCR method on *M.haemolytica* positive cases (52) by IHC are summarised in TABLE III.

While *erm (42)* gene alone was not found in any case, only *msr (E)* resistance gene was found in 8 cases (4 calves, 2 young cattle, 2 adult cattle) and only *mph (E)* gene was found in 2 cases (1 calf and 1 adult cattle). While *msr (E)* and *mph (E)* genes were expressed together in 17 cases (7 calves, 7 young cattle, 3 adult cattle), three of *msr (E)*, mph *(E)* and *erm (42)* genes were expressed together in 12 cases (5 calves, 5 young cattle, 2 adult cattle). In 13 cases (4 calves, 8 young cattle, 1 adult cattle), no resistance gene was found (TABLE III).

There are some studies on antibiotic susceptibility and resistance of *M. haemolytica* in our country and in the world. In the wide ranging study of Portis *et al.* [8] no significant change was observed in the distribution of minimal inhibitory concentration (MIC) distributions for penicillin and ceftiofur among *M. haemolytica* isolates, while decreased susceptibility was observed for enrofloxacin and macrolide group antibiotics tilmicosin and tulathromycin. As a result of this study, it was emphasised that cross–resistance occurred in macrolide group antimicrobials. In presented study, *M. haemolytica* was detected in 52% of cattle with fibrinous pneumonia and resistance gene was observed in 75% of the cases.

In a study [10] conducted in healthy (n:107) and feedlot cattle with pneumonic pasteurellosis (n:210), the prevalence and antimicrobial susceptibility of three disease–associated bacteria (*M. haemolytica*, *P. multocida* and *H. somni*) were analyzed. In diseased cattle, *P. multocida* was isolated most frequently (54.8%), followed by *M. haemolytica* (30.5%) and *H. somni* (22.9%). It was reported that *M. haemolytica* was isolated more frequently in healthy cattle compared to others. While high resistance levels (>70%) to tulathromycin, one of the macrolide group antibiotics, were determined in *M. haemolytica* isolates. Researchers [10] stated that this resistance may be caused by macrolide resistance genes [*msr (E), mph (E), erm (42)*], but additional studies should be carried out in this regard.

All of the antibiotic susceptibility or resistance tests used in these studies are based on the technique of measuring MIC values of drugs by tube dilution or disc diffusion (Kirby Bauer) techniques [17]. However, these tests have disadvantages such as the inability to grow bacteria in culture medium from samples taken from living organisms treated with antibiotics or the lack of sufficient bacteria or the long duration of culture and sensitivity tests. For this reason, it is very important to determine antibiotic resistance genes in bacteria by using PCR method, which is a shorter and more reliable test to determine antibiotic resistance in bacteria.

In a study conducted by Michael *et al.* [18] to determine the macrolide resistance genes in *M. haemolytica,* they determined *erm (42)* genes in 1 of 29 *M. haemolytica* isolates and *erm (42)+msr (E)+mph (E)* genes in 21 of them by PCR method. They found that the MIC value of isolates carrying these three genes increased by 32–64 times for both gamithromycin and tildiprosin, while the MIC value of gamithromycin increased by 4 times and the MIC value of tildiprosin increased by 32 times in those with *erm (42)* alone. Rose *et al*. [9] in their study to determine the resistance to tulathromycin and tilmicosin, which are macrolides used in the veterinary field, and gamithromycin and tildoprosin, which are new compounds; *erm (42)* in 6, *msr (E)–mph (E)* in 5 and *erm (42)+msr (E) –mph (E)* in 4 of 20 *M.haemolytica* isolates were detected by multiplex PCR method. It was determined that the MIC values of tildiprosine, tilmicosin, tulathromycin and gamithromycin increased 8 – ≥128 times in bacterial isolates containing only *erm (42)* or *msr (E) –mph (E)* genes or all three genes together. Thus, they demonstrated that these three genes [*erm (42), msr (E), mph (E)*] were responsible for resistance to macrolide antibiotics in *M.haemolytica* isolates.

TABLE III
Distribution of resistance genes according to cases

	Calf (< 6 month) (n = 55)	Young cattle (> 6 month – < 2 year) (n = 32)	Adult cattle (> 2 year) (n = 13)	Total (n = 100)
IHC (+)	21 (38%)	22 (69%)	9 (69%)	52
erm (42)	–	–	–	–
msr (E)	4	2	2	8 (15%)
mph (E)	1	–	1	2 (4%)
msr (E) + mph (E)	7	7	3	17 (33%)
msr (E) + mph (E) + erm (42)	5	5	2	12 (23%)
No expression	4	8	1	13 (25%)

Accordingly, at least one resistance gene was found in 75% of these positive cases

As stated in all these studies, it is observed that *erm (42), msr (E), mph (E)* genes increase the MIC values against macrolide antibiotics and therefore they are responsible for the resistance against them. In the present study, at least one of these resistance genes was detected as a result of multiplex PCR analyses in 75% of 52 cases found positive for *M. haemolytica* by IHC method. This resulted in an increase in the MIC values of macrolide group antibiotics to various degrees during the treatment process and consequently, deaths occurred as a result of the failure to achieve the desired success in treatment. It is important to consider antimicrobial resistance and the spread of antimicrobial resistance genes in treatment decisions associated with common diseases such as BRD. Understanding the epidemiology of antimicrobial resistance in BRD pathogens in healthy cattle, as well as treated and untreated sick cattle, is crucial to understanding the role of resistance in BRD treatment failure [19].

In these studies, it is also stated that the macrolide resistance mechanism formed in bacteria may be caused by at least 3 different combinations. The *erm (42)* gene is determinant in the first group of isolates. In this group, MLS_B (macrolide, lincosamide and streptogramin B) phenotype with high resistance to lincosamides and low to medium resistance to macrolide and streptogramin B is formed. The second group of isolates was macrolide resistant without lincosamide resistance and contained two resistance genes, *msr (E)* and *mph (E),* encoding a macrolide efflux pump and a macrolide inactivation phosphotransferase, respectively, expressed from the same source. The third group of isolates is highly resistant to a comprehensive set of macrolides and includes all three determinants *erm (42), msr (E)* and *mph (E)* [7, 8]. Regarding these issues, Owen *et al.* [19] stated that their study showed that antimicrobial susceptibility phenotypes are necessary to complement genomically predicted antibiotic resistance gene genotypes in order to better understand how the presence of antibiotic resistance genes in a particular bacterial species can potentially affect optimal treatment of bovine respiratory disease and morbidity/mortality outcomes.

From this point of view, no isolate was found in the first group in which only *erm (42)* was found in present study. The second group, i.e. 17 isolates with *msr (E)* and *mph (E)* genes together (7 calves, 7 young cattle, 3 adult cattle), and the third group, in which *msr (E), mph (E)* and *erm (42)* genes were found together, was 12 (5 calves, 5 young cattle, 2 adult cattle) (TABLE III). It was reported that the MIC value of tulathromycin, gamithromycin, tilmicosin and clindamycin increased 16–128 times in group 2 isolates and 128 – >1024 times in group 3 isolates [7]. According to Alhamami *et al.* [20] noting that most of the isolates were obtained from BRD-affected animals with a history of antimicrobial therapy, they suggested possible treatment failure due to resistance in causative pathogens. The researchers also suggested that continuous monitoring is necessary to support the antimicrobial management programs recently developed by the industry to ensure the effectiveness of veterinary antimicrobial drugs. These results show how important the presence of resistance genes is in treatment.

CONCLUSION

The etiological diagnosis of *M. haemolytica* by IHC method in only fibrinous pneumonia cases in Konya and its surrounding provinces and the presence of resistance genes against macrolide group antibiotics were determined for the first time in Türkiye. It is thought that the use of IHC method in cases where fresh tissue is not available, autolysis and antimicrobial treatment is applied makes a significant contribution to the etiological diagnosis and thus this method can be safely transferred to routine studies, especially in cases where bacteriological analyses are not appropriate. There may be many reasons for the failure of fibrinous pneumonia treatment. It was concluded that one of these reasons may be *M. haemolytica* isolates containing *erm (42), msr (E), mph (E)* genes responsible for resistance to macrolide antibiotics commonly used in the treatment of fibrinous pneumonia. It is known that these genes can be found regardless of age or disease/health status, that they are responsible for cross-resistance between this group of antibiotics, and that resistance may even develop against macrolide group antibiotics that have not yet been introduced to the field. Therefore, the frequency and antibiotic susceptibility profiles of *M. haemolytica* should be periodically renewed due to the possible development of resistance as it will help to guide the choice of antimicrobials in the control and treatment of such diseases. As can be seen from the presented study, repeated and unlimited use of these antibiotics should be avoided in cases where the frequency of resistance is high.

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Conflict of interest

There is no conflict of interest between the authors.

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