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Identification of Bacterial Pathogens from Nasal Cavity in Cattle with Pneumonia by MALDI-TOF MS

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The aim of our study was to show the etiological spectrum of pathogens in the nasal cavity of cattle with pneumonia using MALDI-TOF MS. The study material consisted of 64 dairy cows between the ages of 2 and 7 years, 39 calves aged between 30 and 85 days showing pneumonia symptoms and 20 healthy dairy cows and calves sampled from 24 farms in different districts of İzmir, Turkey. After clinical examination of all animals, nasal samples were taken using a swab with Charcoal Amies transport medium. The clinical examination revealed that the respiratory rate, heart rate, and rectal temperature were statistically higher in cases with pneumonia symptoms than in control cases ($p<0.001$). MALDI-TOF MS isolated 17 different bacteria from the dairy cow samples. The most frequently isolated bacterial pathogens were *Escherichia coli* (*E. coli* (32.8%), *Staphylococcus lentus* (*S. lentus)* (28.1%) and *Staphylococcus sciuri* (*S. sciuri)* (21.8%). Of the 11 different bacteria isolated from calves, the most frequent ones were *E. coli* (46.1%), *S. lentus* (41.0%), *Staphylococcus xylosus (S. xylosus)* (7.6%) and *S. sciuri* (7.6%). Culture and conventional PCR methods were used to determine the presence of Mycoplasma in the samples. In 29 of the samples taken from cattle and calves, *Mycoplasma spp. M. bovis* was isolated in 24. While *S. sciuri* (12.5%) and *E. coli* (12.5%) were detected the most among the factors causing mixed infection with *M. bovis*. While these findings reveal the spectrum of pneumonia agents in our region, they show that pathogens in the nasal cavity can be detected quickly and reliably with MALDI-TOF MS.

Key Words: *MALDI-MS, cattle, respiratory, pneumonia*

Pnömonili Sığırların Burun Boşluklarında Bulunan Bakteriyel Patojenlerin MALDI TOF MS ile İdentifikasyonu

Bu çalışmanın amacı, pnömonili sığırlarda MALDI-TOF MS kullanarak nazal kavitede bulunan patojenlerin etiyolojik spektrumunu belirlemektir. Çalışma materyalini İzmir'in farklı ilçelerinden 24 ayrı çiftlikten pnömoni semptomu gösteren 2 ile 7 yaş arasında 64 sığır, 30 ile 85 günlük yaştaki 39 buzağı ve 20 sağlıklı sığır ve buzağı oluşturdu. Tüm hayvanların klinik muayeneleri yapıldıktan sonra kömürlü amies transport besiyerli svap kullanılarak nazal numune alındı. Yapılan klinik muayene de solunum frekansı, kalp frekansı ve rektal sıcaklık pnömoni semptomu gösteren vakalarda kontrole göre istatistiksel olarak yüksek (<0.001) tespit edildi. MALDI-TOF MS ile sığır numunelerinden 17 farklı bakteri izole edildi. Sırasıyla; *Escherichia coli* (*E. coli*) (% 32.8), *Staphylococcus lentus (S. lentus)* (% 28.1), *Staphylococcus sciuri* (*S. sciuri)* (% 21.8) en fazla izole edilen patojenler oldu. Buzağılardan ise 13 farklı bakteri izole edildi. Sırasıyla; *E. coli* (% 46.1), *S. lentus* (% 41), *Staphylococcus xylosus (S. xylosus)* ve *S. sciuri* (% 7.6) en fazla izole edilen bakteriler oldu. Mycoplasma izolasyonu ve moleküler karakterizasyonu için ise PCR yapıldı. Sığır ve buzağılardan alınan örneklerin 29'unda *Mycoplasma spp.* 24'ünde ise *Mycobacterium bovis (M. bovis)* izole edildi. *M. bovis* ile mix enfeksiyon yapan etkenlerden en fazla *S. sciuri* (% 12.5) ve *E. coli* (% 12.5) tespit edildi. Bu bulgular bölgemizdeki pnömoni etkenlerinin spektrumunu ortaya koyarken, MALDI-TOF MS ile burun boşluğundaki patojenlerin hızlı ve güvenilir bir şekilde tespit edilebileceğini göstermektedir.

Anahtar Kelimeler: *MALDI-MS, sığır, solunum, pnömoni*

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Introduction

Respiratory system diseases, which are the most important global problem in cattle breeding, cause serious economic losses due to high morbidity and mortality (1). Respiratory system diseases in cattle and calves are affected by type of microorganism, environmental factors, and individual immunity. While many bacteria are the primary cause of the disease, it may also be caused by many secondary factors (2). It is also etiologically complex because early weaning, poor transport, crowded, stuffy or unsuitable shelters, sudden climate changes, stress, and many microorganisms influence the disease’s emergence. Respiratory diseases cause annual losses of approximately 800-900 million dollars in the United States alone while treatment costs around 15.57 dollars per animal (3,4). While the incidence of respiratory system diseases in our country varies between 22-59.7%, it has been reported that 50-70% of the deaths in beef cattle are caused by the respiratory system (1, 5).

Four sampling techniques are commonly used for antemortem diagnosis of pathogens in cattle with respiratory disease: nasal swab, nasopharyngeal swab, bronchoalveolar lavage, and transtracheal wash (6). If more than one sampling is

required then nasal swabs are preferred to nasopharyngeal swabs because they are cheaper. In addition, for nasopharyngeal swabs, auxiliary elements are needed to restrain the animal while the process stresses the animal (7). Mcdaneld et al. analyzed the microbiome of nasal cavity samples from cattle with respiratory tract disease complex using a nasopharyngeal swab and a 6-inch swab (approximately 150 mm). They reported that both swabs gave similar results (7). Doyle et al. evaluated all four sampling techniques. They detected similar respiratory tract pathogens from nasal swabs and deep nasopharyngeal swabs in calves diagnosed with bovine respiratory system disease (6). Fast and accurate diagnosis of the causative agent is as important as taking samples. Respiratory pathogens in animals are detected by sampling culture and susceptibility testing. However, because diagnosis takes at least 48 hours, most antimicrobial infection treatments rely on experience-based antibiotic selection. Thus, it is critical to obtain rapid and accurate microbiological results to use antimicrobials correctly in treating respiratory tract infections. Accurate and rapid diagnosis is associated with the use of appropriate antibiotics and quicker patient recovery (8). Various studies have shown that matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) is a very valuable diagnostic method that produces satisfactory results. MALDI-TOF MS identifies gram-positive and gram-negative bacteria, aerobic and anaerobic bacteria, mycobacteria, and fungi with 90-100% agreement with the reference method (9). Nevertheless, despite the widespread use of modern herd management, vaccination programs, improved diagnostic techniques, and antibiotic treatments, yield and animal losses due to pneumonia continue (10).

This study used MALDI-TOF MS to identify the etiological spectrum of common pathogens in the nasal cavity of cattle and calves. This method provides accurate and rapid diagnosis in cattle and calves with respiratory system disease, thereby supporting greater awareness in use of antibiotics.

Material and Methods

Research and Publication Ethics: Before starting the study, ethical approval was obtained from Dokuz Eylul University University Animal Experiments Local Ethics Committee, dated 02.03.2022, decision number 11/2022.

The study material consisted of 64 cows aged between 2 and 7 years and 39 calves aged between 30 and 85 days. Nasal swabs were taken after clinical examination (respiration rate, heart rate, rectal temperature) for each calf and cows showing pneumonia symptoms (cough, fever, nasal discharge, respiratory distress) from 24 different farms in various districts of Izmir, Turkey. The control group consisted of 20 healthy dairy cows and 20 calves. An Amies transport media with charcoal (Transwab Pernasal, MW173C, Medical Wire & Equip. Co. Ltd, UK) were used to collect the nasal swab samples, which were sent to Fırat University, Faculty of Veterinary Medicine, Department of Microbiology for agent isolation under appropriate transport conditions. The samples were stored at -20 °C until analyzed.

Bacterial Isolation and Identification with MALDI TOF MS: For isolation, the samples were directly inoculated on blood agar with 5% sheep blood and incubated for 24-48 hours at 37°C in an aerobic environment. The colonies grown on the plates were then purified separately and typified using MALDI-TOF MS database v2.0 system (bioMerieux, France). The bacteria included in the typing were 99.9% similar to the bacterial names.

Mycoplasma Isolation and Molecular Characterization: For mycoplasma isolation from swabs, selective Mycoplasma broth and Mycoplasma agar were inoculated first. To avoid broth contamination, samples were diluted up to 10^{-4} and incubated in an oven with 5% CO_2 for three weeks. Typical mycoplasma colonies were identified by examining the agars under a stereomicroscope. Passages were then made from the broth with the last turbidity observed. The agar colonies grown were passaged three times before being put into sterile tubes with 50% mycoplasma broth and 50% horse serum, and stored at -20°C for genomic DNA extraction. Genomic DNA extraction with phenol and chloroform was performed on the isolates from the mycoplasma agar and broths (11). Target DNAs were first validated with Mycoplasma genus-specific primers before PCR analysis using primers specific for *M. bovis*, *M. alkalescens*, *M. arginini*, *M. dispar*, *M. bovirhinis*, and *M. canis* (Table 1). Then, PCR amplification was performed using separate protocols for each genomic DNA (12-17). A result of agarose gel electrophoresis of PCR products were evaluated.

Statistical Analysis: Statistical analyses were performed using SPSS version 22. The variables were investigated using both visual (histograms, probability plots) and analytical methods (Kolmogorov-Simirnov) to determine whether or not they were normally distributed. The Student's t-test was used to compare respiratory rate, heart rate and rectal temperature levels between groups. Chi-square test was used to evaluate the microbial isolation rates from the samples. A p-value of less than 0.05 was considered as a statistically significant result (18).

Results

The clinical examination of all animals included in the study showed that respiratory rate, heart rate, and rectal temperature were statistically higher (<0.001) in cases with pneumonia symptoms than control animals (Table 2). Regarding the 64 cows showing pneumonia symptoms, MALDI-TOF MS analysis of the nasal swabs isolated 17 different bacteria species. The most frequent were *E. coli* (32.8%), *S. lentus* (28.1%), and *S. sciuri* (21.8%). Similarly, 11 different bacteria were isolated from calves, with most frequent being *E. coli* (46.1%), *S. lentus* (41.0%), *S. xylosus* (7.6%) and *S. sciuri* (7.6%) (Table 3). The rates of polymicrobial isolation were 61%

in cows and 58.3% in calves (Table 4). *Mycoplasma spp* were detected in 29 of 103 samples from cows and calves. Regarding mycoplasma genus, the PCR analysis indicated that 24 of 29 samples were *M. bovis*, while 5 could not be typed. No positivity was found for *M.alkalescens*, *M.arginini*, *M.dispar*, *M.bovirhinis*, or *M.canis* in any of the DNAs that were positive for *Mycoplasma spp. S. sciuri* and *E. coli* (12.5%) caused the most mixed infections with *M. bovis* while *E. coli* (20.8%) was found in calves (Table 5).

Table 1. Primer pairs used in PCR analyses

Name of Bacteria	Oligonucleotide sequence (5'-3')	Length (bp)	References
Mycoplasma spp	GGCGAATGGGTGAGTAACACG CGGATAACGCTTGCGACCTATG	464	12
M. bovis	TATTGGATCAACTGCTGGAT AGATGCTCCACTTATCTTAG	447	13
M. canis	TGATGATTAGCTGATAGTAGAACT GATTTGCTTGACGTCGCCGTT	434	14
M. alkalescens	GCTGTTATAGGGAAAGAAAACT AGAGTCCTCGACATGACTCG	704	15
M. arginini	GCATGGAATCGCATGATTCCT GGTGTTCTTCCTTATATCTACGC	545	16
M. dispar	GGCTGTGTGCCTAATACATGC CAGCGTGGACTACCAGGGTATC	583	17
M. bovirhinis	GGCTGTGTGCCTAATACATGC CAGCGTGGACTACCAGGGTATC	358	17

Table 2. Clinical findings of cows and calves showing symptoms of pneumonia

Parameters	Cow (n=64)	Cow control group (n=20)	P value	Calf (n=39)	Calf control group (n=20)	P value
Respiratory (rate/min)	52.5±2.18	32.6±3.13	.000	54.3±1.12	30.4±1.18	.000
Heart (rate/min)	97.4±4.31	68.4±6.80	.000	101.2±5.34	72.2±4.10	.000
Rectal temperature (ºC)	40.1±0.58	38.6±0.37	.000	40.9±0.50	38.8±00.4	.000

$P<0.001$

Table 3. The results of the detection of different pathogens in 103 nasal swabs samples collected from cows and calves with respiratory disease by MALDI-TOF MS

Name of Pathogens	Cow with Respiratory Disease			Calf with Respiratory Disease		
	Number of Tested Samples	Number of Positive Samples	% of Positive Samples	Number of Tested Samples	Number of Positive Samples	% of Positive Samples
Mycoplasma spp	64	18	28.1	39	11	28.2
Mycoplasma bovis	64	15	23.4	39	9	23.0
Pseudomonas aeruginosa	64	3	4.6	39	1	2.5
Staphylococcus saprophyticus	64	2	3.1	39	1	2.5
Escherichia coli	64	21	32.8	39	18	46.1
Serratia rubidaea	64	2	3.1	39	2	5.1
Staphylococcus lentus	64	18	28.1	39	16	41
Staphylococcus sciuri	64	14	21.8	39	3	7.6
Enterobacter cloacae	64	9	14	39	2	5.1
Bacillus clausii	64	1	1.5	39	-	-
Klebsiella oxytoca	64	1	1.5	39	1	2.5
Acinetobacter Iwofii	64	3	3.5	39	-	-
Bacillus cereus group	64	1	1.5	39	-	-
Staphylococcus xylosus	64	4	4.7	39	3	7.6
Stenotrophomonas maltophilia	64	1	1.5	39	2	5.1
Acinetobacter radioresistens	64	2	2.3	39	-	-
Enterococcus faecalis	64	1	1.5	39	-	-
Aerococcus viridans	64	1	1.5	39	-	-
Acinetobacter baumannii complex	64	-	-	39	1	2.5
Pantoea agglomerans	64	1	1.5	39	-	-

Table 4. Microbial isolation rates from samples

Group	Number of Samples	Number of positive samples (%)	Number of samples contaminated with only one type of bacteria (%)	Number of samples contaminated with two different types of bacteria (%)	Number of samples contaminated with 3 or more types of bacteria (%)	Number of negative samples (%)	Polymicrobial Isolation Rate (%)
Cow	64	59 (92.1)	23 (35.9)	28 (43.7)	8 (12.5)	5 (7.8)	36 (61)
Calf	39	36 (92.3)	15(38.4)	16 (41.0)	5 (12.8)	3 (7.6)	21 (58.3)
Total	103	95(92.2)	38(36.9)	44(42.7)	13(12.6)	8 (7.7)	57 (60)

Table 5. Coinfection of M. bovis in Cow and calves

Etiologic agent	Proportion (%)
Mixed infection	24/24 (100)
Cow	
Staphylococcus sciuri	3 (12.5)
Escherichia coli	3 (12.5)
Staphylococcus lentus+Enterobacter cloacae+ Bacillus clausii	1 (4.1)
Staphylococcus lentus+Escherichia coli	1 (4.1)
Pseudomonas aeruginosa	1 (4.1)
Staphylococcus lentus+Enterobacter cloacae	1 (4.1)
Staphylococcus lentus+Acinetobacter Iwofii	1 (4.1)
Acinetobacter Iwofii+Enterobacter cloacae	1 (4.1)
Calf	
Escherichia coli	5 (20.8)
Staphylococcus lentus	3 (12.5)
Pseudomonas aeruginosa+Escherichia coli	1 (4.1)
Staphylococcus lentus+Staphylococcus xylosus	1 (4.1)
Staphylococcus lentus + Escherichia coli	1 (4.1)
Staphylococcus xylosus+Acinetobacter baumannii complex	1 (4.1)

Discussion

Respiratory diseases are one of the most leading problems in animals worldwide. Especially in cattle, they damage cattle industry and national economies by decreasing feeding efficiency and weight gain, and causing death in severe cases. Rapid and accurate diagnosis of the disease is thus critical to ensure animal health and welfare, reduce losses, and enable effective treatment and positive prognosis (19). Studies of bacterial isolation from slaughterhouse material (1, 20) and nasal cavities of healthy and ill cattle have been conducted in Turkey before (21-23). However, our study is the first to obtain data using nasal swabs obtained from cows with pneumonia using the MALDITOF MS.

In the present study, we performed clinical examinations of both the animals showing pneumonia symptoms and control-healthy animals. The clinical measurements (respiratory rate, heart rate, and rectal temperature) were higher in cows and calves showing pneumonia symptoms than control group animals and reference values (24). Hanzlicek et al. endoscopically infected calves with *Mannheimia haemolytica* and also detected changes from physical examination. While clinical measurements (respiratory rate, heart rate, and rectal temperature) varied in the following days, there were a general increase compared to reference values (25). Similarly, Van Donkersgoed et al. conducted an epidemiological study of enzootic pneumonia in calves. They reported an increase in respiratory rate and rectal temperature in pneumonia cases (26).

MALDI-TOF MS has gained popularity in recent years because it enables precise and rapid (i.e. on the same-day), species-level identification of microorganisms isolated from blood, urine, peritoneal, synovial, bronchoalveolar lavage fluid, body fluids like cerebrospinal, and from many sources (e.g., slaughterhouse material) (27). Van Driessche et al. used MALDI-TOF MS to determine the etiological spectrum of bacteria isolated from bronchoalveolar lavage fluid. They identified 45 different bacterial species from 100 samples. Furthermore, compared to traditional bacterial culturing to detect pathogens in the lower respiratory tract, they reported that this method reduced the diagnostic time from 24-48 hours to an average of 6.5 hours and gave more accurate results (28). Choudhary et al. applied MALDI-TOF to nasal swabs, tracheal swabs, and lung tissue samples from cattle and buffalo. They identified major pneumonia pathogens, such as *Pasteurella spp*, *Stenotrophomonas maltophilia* (3.1%), *S. sciuri* (0.79%), *E. coli* (20.63%), *Pseudomonas aeruginosa* (6.3%), and *Enterobacter cloacae* (3.1%) while 96% of samples were polymicrobial (29). Using the same method, our study isolated 19 different bacterial species from 103 samples. The most frequent bacterial species were *E. coli* (37.8%), *S. lentus* (33%), *Mycoplasma spp.* (28.1%), *S. sciuri* (16.5%), *E. cloacae* (10.6%), and *S. maltophilia* (2.91%). Polymicrobial isolation was observed in 60% of samples.

Because the bovine respiratory tract acts as a reservoir for pathogenic microorganisms, poor hygiene conditions, stress factors, and climate changes may trigger mixed infections in the lungs. In particular, some *Staphylococcus spp.* and *E. coli* induced pneumonias are thought to develop in this way (30). Our study detected high rates of *S. lentus*, *S. sciuri*, and *E. coli*. The first two of these are generally isolated from domestic, farm, and wild animals, and foods of animal origin. Hay et al. were the first to isolate *S. lentus* from the sinonasal cavity as a sinusitis agent, although it is under reported in humans (31). In humans, this bacterium causes serious infections, such as endocarditis, peritonitis, septic shock, endophthalmitis, pelvic inflammatory diseases, and wound infections (32). The fact that this bacterium was isolated at high rates in

our study suggests that the sampled animals had sinusitis in addition to pneumonia symptoms.

In recent years, it has been reported that caseonecrotic bronchopneumonia, a type of pneumonia characterized by chronic mycoplasma infection, is caused by *M. bovis*, which is more virulent than other mycoplasma species (33). In bovine respiratory system diseases, more than 70% of *Mycoplasma spp* pneumonia cases are generally seen as mixed infections, with only 20% reported as solitary (34, 35). Booker et al. identified microbiological agents in respiratory system diseases of beef cattle and investigated the relationship between these agents and pathological processes. It was found that 12/15 (80%) of animals positive for *Histophilus somni* were also positive for *M. bovis* (34). The prevalence of *M. bovis* in Denmark increased from 0.6% in 1983 (36) to 2% in 1999 (37) and 24% in the 2000s (38). Kusiluka et al. investigated the prevalence of mycoplasmas in pneumonic cattle lungs. They found that the most dominant (72.0%) mycoplasma species and Ureaplasma spp while 18.6% of samples had mixed *M. bovis* Ureaplasma infection (38). Soehnlen et al. reported a positivity rate for *M. bovis* as 40% from nasal swabs (39). Our study detected *M. bovis* in 23% of 103 animals while all samples with *M. bovis* also contained other bacteria. No bacterial growth was observed in the isolation performed on blood agar from samples containing the five *Mycoplasma spp*. Thus, the probability that suspected animals have mixed infections with *M. bovis* is around 80%. Bacterial identification using the isolation method from lower respiratory tract samples is the gold standard for diagnosing Mycoplasma induced pneumonia. Thomas et al. compared the isolation rates in different samples collected from cattle with clinical signs and reported much higher isolation rates from BAL fluids than nasal swabs (40). We could not take lower respiratory tract samples becuase the method is invasive, requires skilled extra labor and equipment, and, more importantly, is not acceptable by the animal owners. Karahan et al. examined a total of 148 samples (3 lung, 4 eye swab, 51 nasal swab, 90 milk) and found 23.5% positivity for *M. bovis* in nasal swabs (11). Akan et al. found 16 (12.5%) of 127 nasal swabs positive for *M. bovis* by PCR (41). In our study, the isolation rate for *Mycoplasma spp.* was 9.7% (10/103), which was quite low compared to the 28.1% (29/103) positivity rate according to the PCR analysis. Possible causes include the lengthy transport of samples after collection, freezing until processing, and using nasal swabs. This may also explain why five Mycoplasma spp. could not be typed due to the presence of contaminant bacterial and yeast DNA in their extracted DNA.

In conclusion, pneumonia is a common problem in dairy cattle and calves that harms animal welfare and causes significant economic losses every year. It has been a universal problem since the beginning of farm animal husbandry. Accurate diagnosis and treatment are as important as prophylactic measures for this disease. In our study, the samples were collected and the bacteria detected accurately and quickly using MALDI-TOF MS. This method is easy to apply in the field and causes minimal discomfort to the animals. The method successfully demonstrated in our study can contribute to preventing antibiotic resistance in both animals and humans by enabling the correct use of antibiotics and will contribute positively to the field.

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