

Biotyping and Serotyping of *Mannheimia (Pasteurella) haemolytica* Isolated from Lung Samples of Slaughtered Sheep in the Van Region

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Abstract: A total of 584 lung samples of slaughtered sheep having clinical symptoms of pneumonia were investigated microbiologically. Out of the 584 samples, 66 (11.3%) *Mannheimia (Pasteurella) haemolytica* strains were isolated on the basis of cultural, morphological and biochemical characteristics. Sixty-six isolates of *M. haemolytica* were biotyped by carbohydrate fermentation tests and serotyped by coagglutination and indirect haemagglutination (IHA) tests. Out of the 66 *M. haemolytica* strains, 57 (86.3%) were detected as biotype A, and 9 (13.6%) were biotype T. The 66 *M. haemolytica* isolates were serotyped as follows by coagglutination test: 13 (19.6%) A2, 11 (16.6%) A1, 9 (13.6%) A6, 7 (10.6%) A9, 4 (6.0%) A5, 4 (6.0%) A7, 4 (6.0%) A12, 3 (4.5%) A8, 1 (1.5%) T4, 1 (1.5%) A13, and 1 (1.5%) T15. Of the *M. haemolytica* strains, 14 (21.2%) belonged to serotype A2, 11 (16.6%) to serotype A1, 10 (15.1%) to serotype A6, 7 (10.6%) to serotype A9, 4 (6.0%) to serotype A5, 4 (6.0%) to serotype A7, 4 (6.0%) to serotype A12, 4 (6.0%) to serotype A8, 1 (1.5%) to serotype T4, 1 (1.5%) to serotype A13 and 1 (1.5%) to serotype T15 by IHA test. Eight (12.1%) and 5 (7.5%) isolates were unserotyped by coagglutination and IHA tests, respectively. T3, T10, A11, A14 and A16 serotypes were not determined.

Key Words: Sheep, *Mannheimia haemolytica*, biotyping, serotyping

Van Bölgesindeki Koyunlardan İzole Edilen *Mannheimia (Pasteurella) haemolytica* Suşlarının Biotip ve Serotiplendirilmesi

Özet: Bu çalışmada, pnömoni semptomları gösteren ve kesimi yapılan toplam 584 adet koyuna ait akciğer örneği mikrobiyolojik olarak incelendi. Kültürel, morfolojik ve biyokimyasal özellikleri temel alınarak 584 örneğin 66 (% 11,3) adedinden *Mannheimia haemolytica* izole ve identifiye edildi. İzole edilen *M. haemolytica* suşlarının karbonhidrat fermantasyon testleriyle biyotiplendirilmeleri, koaglutinasyon ve indirekt hemaglutinasyon (IHA) testleriyle de serotiplendirmeleri yapıldı. Suşların 57 (% 86,3)'sinin biyotip A, 9 (% 13,6)'unun da biyotip T olduğu belirlendi. Koaglutinasyon testiyle izolatların 13 (% 19,6)'ü serotip A2, 11 (% 16,6)'i A1, 9 (% 13,6)'u A6, 7 (% 10,6)'si A9, 4 (% 6,0)'ü A5, 4 (% 6,0)'ü A7, 4 (% 6,0)'ü A12, 3 (% 4,5)'ü A8, 1 (% 1,5)'i T4, 1 (% 1,5)'i A13 ve 1 (% 1,5)'i de T15 olarak belirlendi. IHA testiyle suşların 14 (% 21,2)'ünün A2, 11 (% 16,6)'inin A1, 10 (% 15,1)'unun A6, 7 (% 10,6)'sinin A9, 4 (% 6,0)'ünün A5, 4 (% 6,0)'ünün A7, 4 (% 6,0)'ünün A12, 4 (% 6,0)'ünün A8, 1 (% 1,5)'inin T4, 1 (% 1,5)'inin A13 ve 1 (% 1,5)'inin ise T15 olduğu saptandı. Koaglutinasyon testiyle 8 (% 12,1), IHA test ile 5 (% 7,5) izolat serotiplendirilemedi. Çalışmada, T3, T10, A11, A14 ve A16 serotipleri saptanamadı.

Anahtar Sözcükler: Koyun, *Mannheimia haemolytica*, biyotiplendirme, serotiplendirme

Mannheimia haemolytica is the etiological agent of pneumonic pasteurellosis of sheep and cattle, which is an infection that causes considerable financial losses in the sheep and cattle industries (1-3). *M. haemolytica* is divided into 2 biotypes based on arabinose, xylose, and trehalose fermentation patterns (4,5), and 17 serotypes

based on surface antigens (6). Two biotypes of *M. haemolytica* have been traditionally recognised: biotype A and biotype T (4). Isolates of biotype A of *M. haemolytica* are associated with respiratory disease in sheep and cattle and septicaemia in young lambs, while biotype T isolates have been associated with septicaemia in young adult

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sheep. All serotypes can be involved in pneumonic pasteurellosis in sheep, but serotype A2 is the most commonly isolated serotype from cases of ovine pneumonic pasteurellosis. Furthermore, a wider range of serotypes is associated with disease in sheep, particularly serotypes A1, A6 to A9, A11 and A12, although they are recovered much less frequently than serotype A2 isolates (1,2,7).

Several methods have been used for the serologic typing of *M. haemolytica* strains (8-11). The indirect haemagglutination (IHA) test became the most widespread method for the examination of *M. haemolytica* serotypes, and the coagglutination test proved to be reliable and suitable for serotyping including *Pasteurella* species (10).

In Turkey, it was reported that the prevalence of the serotypes of *M. haemolytica* isolated from pneumonic pasteurellosis of sheep has a wide variation (5,12). For this reason, we aimed to determine the prevalence of *M. haemolytica* serotypes isolated from pneumonic lungs of slaughtered sheep in the Van region and to compare coagglutination and IHA tests for the biotyping of *M. haemolytica* strains.

In this study, samples were collected from female sheep that showed respiratory symptoms, namely coughing, nasal discharge and dyspnoea. These sheep had no history of being vaccinated with *Mannheimia (Pasteurella)* vaccines and were 2-10 years old. A total of 584 lung samples obtained from Akkaraman and Morkaraman sheep from 46 different flocks slaughtered in a local abattoir were the materials of the present study. The lung samples were examined and subjected to bacteriological examinations and 10-20 g lung pieces were taken with a sterile bistoury. These samples were transferred to the laboratory within 2 h. Briefly, the samples were inoculated in 5% sheep blood agar (Merck, KGaA 64271, Darmstadt, Germany) and incubated aerobically at 37 °C for 72 h. Smears were prepared from a single colony and Gram stained. Cultural, morphological, biochemical and sugar fermentation tests were performed as described elsewhere (4,13).

In this research, a total of 66 (11.3%) *M. haemolytica* strains were isolated from the lung samples of 584 sheep and these strains were biotyped and serotyped.

The biotypes of *M. haemolytica* strains (A and T) were determined using fermentation of arabinose, xylose and trehalose in nutrient broth (Difco, Detroit, MI 48232-7058, USA) containing 1% specific carbohydrates with bromothymol blue as indicator at 37 °C for 14 days (1,4,5,13).

Antisera against the type strains of 16 *M. haemolytica* were kindly donated by Dr. Donachie, Moredun Research Institute, Edinburgh, UK, to Dr. İhsan Keleş. The antisera were specific for biotype A (serotypes 1, 2, 5, 6, 7, 8, 9, 11, 12, 13, 14, 16) and biotype T (serotypes 3, 4, 10, 15).

Coagglutination test was carried out as described by Mittal et al. (14). Field isolates of *M. haemolytica* were grown in 7% sheep blood agar (Merck) (diameter 9 cm) aerobically at 37 °C for 18 h and the bacteria were suspended in 3 ml of formaldehyde saline containing 10% bacterial cells. The suspension was left at room temperature for 5 min and centrifuged at 8000 xg for 15 min. The whole cell suspension was used for serotyping. *Staphylococcus aureus strain Cowan I* (NCTC 8330) was grown on tryptic soy agar (Merck) overnight at 37 °C. The bacteria were collected with phosphate buffered saline solution (pH 7.4, PBSS) and washed twice with PBSS. The bacteria were suspended containing 0.5% formaldehyde in PBSS and kept at room temperature for 3 h. Then they were washed once in PBSS, and adjusted to a concentration of 10% (v/v). The suspension was treated at 80 °C in a water bath for 5 min. To 1 ml of this Cowan I suspension was added 0.025 ml of 16 serotype specific antisera, followed by mixing. The suspension was kept at room temperature for 30 min and then washed twice with PBSS. After the final washing, the bacteria were resuspended in a concentration of 10% (vol/vol) in PBSS containing 0.05% sodium azide and 0.1% bovine serum albumin (Sigma, St. Louis, MO 63178, USA). Staphylococcal coagglutination reagent (0.05 ml) was mixed on a glass slide with an equal volume of bacterial suspension. The slide was rotated and examined against a dark background. If agglutination did not occur within 2 min, the result was considered negative. The reaction was scored as 0, +, 2+, 3+ or 4+, based on the degree of agglutination. Any antigen giving a 2+ reaction was evaluated as positive. Staphylococcal cell suspensions coated with normal rabbit serum were used as negative controls.

The IHA test was performed by a method previously described (8). *M. haemolytica* strains were cultivated on to blood agar (Merck) containing 7% sheep blood that had been incubated aerobically at 37 °C for 24 h. One colony from this medium was transferred into 5 ml of brain heart infusion (BHI) broth (Oxoid, Basingstoke, RG24 OPW, UK) tubes and incubated at 37 °C for 18 h. Bacteria in BHI broth were inactivated at 56 °C in a water bath for 30 min. After being inactivated, 0.05 ml of 1% suspension of bovine red cells that were prepared by washing 3 times with PBSS (pH: 7.2) under sterile conditions was added to each BHI broth tube. The tubes were placed at 37 °C in a water bath for 60 min and the tubes were occasionally shaken. Following incubation, the suspensions were washed 3 times by successive centrifugation at 2000 rpm for 10 min with PBSS containing 0.5% formaldehyde. The supernatant was discarded and the pellet was resuspended in 10 ml of sterile physiological saline solution (pH 7.2), and 0.5% of modified bovine erythrocyte solution was prepared. The test was performed on U-bottomed polystyrene plates. For the test, 16 antisera were diluted 1/10 in PBS (pH 7.2). Sterile PBS (25 µl) was added to each well and equal volumes of the antiserum were added. Then 50 µl of modified bovine erythrocyte solution was added to each well and incubated for 2-3 h at room temperature. Sterile PBS was used for negative serum control.

In this study, both biotype A and biotype T were recovered from lung samples of sheep according to fermentation pattern of trehalose, arabinose and xylose. Out of the 66 *M. haemolytica* strains, 57 (86.3%) were biotype A and 9 (13.6%) were biotype T. Trehalose was fermented by all *M. haemolytica* strains and all of them biotyped as biotype T. Concerning the biotype T, only 2 (3.0%) strains fermented xylose and 5 (7.5%) isolates utilised arabinose. Arabinose and xylose were also fermented by 58 (87.8%) and 52 (78.7%) *M. haemolytica* strains of biotype A, respectively. None of the 57 (86.3%) *M. haemolytica* strains that biotyped as biotype A fermented trehalose.

Out of the 66 *M. haemolytica* field strains, 56 (84.8%) were of the same serotype as determined by both coagglutination and IHA tests, and 3 (4.5%) were not serotyped by either method. Fifty-eight (87.8%) isolates were serotyped by coagglutination, but 8 (12.1%) strains were not serotyped. A total of 61 (92.4%) strains were serotyped by IHA, but 5 (7.5%)

isolates were not serotyped. Five (7.5%) strains were serotyped by IHA, but not by coagglutination. Two (3.0%) isolates were unserotypable by IHA, but were serotyped by coagglutination. Six and 2 unserotypable strains by coagglutination were biotype A and biotype T, respectively. Three and 2 unserotypable strains by IHA were biotype A and biotype T, respectively. The results of coagglutination and IHA tests examining 66 field strains of *M. haemolytica* are summarised in the Table.

Table. Serotyping of *M. haemolytica* strains isolated from lung samples of sheep by coagglutination and IHA tests.

Serotype	No. of positive strains			
	Coagglutination test	(%)	IHA test	(%)
A2	13	(19.6)	14	(21.2)
A1	11	(16.6)	11	(16.6)
A6	9	(13.6)	10	(15.1)
A9	7	(10.6)	7	(10.6)
A5	4	(6.0)	4	(6.0)
A7	4	(6.0)	4	(6.0)
A12	4	(6.0)	4	(6.0)
A8	3	(4.5)	4	(6.0)
T4	1	(1.5)	1	(1.5)
A13	1	(1.5)	1	(1.5)
T15	1	(1.5)	1	(1.5)
Not typeable	8	(12.1)	5	(7.5)

In this study, none of the serotypes T3, T10, A11, A14 and A16 were determined. Two strains that serotyped as A1 cross-reaction appeared between immune serum anti-A6 by coagglutination test.

M. haemolytica in particular is recognised as an important cause of pneumonia in small ruminants. It has been reported that biotype A *M. haemolytica* isolates ferment arabinose, and biotype T isolates ferment trehalose (1,15). In this research, trehalose was fermented by all *M. haemolytica* strains and all of them biotyped as biotype T. However, arabinose was fermented by 58 (87.8%) *M. haemolytica* isolates biotype A. This result indicated that the trehalose fermentation test is more reliable than the arabinose fermentation test for biotyping of biotype A (16).

In Turkey, a total of 119 *M. haemolytica* strains have been isolated from sheep and goats (5). Of the 119 strains, 110 (92.4%) were reported as biotype A, and 9 (7.5%) were biotype T. Barbour et al. (1) stated that biotype A (88.0%) and biotype T (12.0%) were recovered from the respiratory tracts of unhealthy sheep and calves. In this study, out of the 66 *M. haemolytica* isolates, 57 (86.3%) isolates were biotype A and 9 (13.6%) isolates were biotype T. This result has shown that biotype A is more frequently isolated than biotype T in sheep with pasteurellosis (17).

Frank and Wessman (11) reported that 23 field isolates of *M. haemolytica* were serotyped from sheep using the IHA and rapid agglutination tests. Out of 23 isolates, 20 (86.9%) were the same serotype as determined by both methods and 3 (13.0%) were not serotyped. Güler et al. (5) stated that 12 (10.0%) strains of *M. haemolytica* were not typeable by IHA test. In the present research, 8 (12.1%) and 5 (7.5%) field isolates of *M. haemolytica* were not serotyped by coagglutination and IHA tests, respectively. In addition to isolates that express these serotypes, approximately 10% of disease isolates recovered from sheep and cattle have also been reported to be unserotypeable (6). While some unserotypeable isolates are closely related to serotype A1, A2 or A11 isolates and probably represent either new capsular types or capsule deficient strains, others represent distinct species. On the other hand, concerning serotype A2, in some cases, this can be explained by the low capsule content of serotype A2 strains. The capsule

has been demonstrated to contain the type-specific antigens (18,19).

Coagglutination and IHA tests have been used widely for the serotyping of *M. haemolytica* strains (5,10). In the present study, there was total agreement between the 2 methods in 59 (89.3%) out of the 66 isolates tested. However, we observed that the IHA test was a more reliable method than the coagglutination test for serotyping of *M. haemolytica* strains isolated from sheep, because neither cross-reaction appeared between tested strains, and serotyped strain numbers were higher than in the coagglutination test.

Some information is available on the serotype distribution of *M. haemolytica* in Turkey. The predominant serotype isolated from sheep (5,12) and goats (5) is type A2. However, serotypes A1, A5, A7, A8, A9, T10, A11, A12, T4 (5, 12), A13, T16, A6 and T3 have also been isolated (5). In this study, the most frequently isolated serotype was type A2. Serotypes A1, T4, A5, A6, A7, A8, A9, A12, A13 and A15 were also determined.

In conclusion, serotypes A2, A1, A6 and A9 of *M. haemolytica* were the most commonly isolated serovars from pneumonic sheep in the Van region. In all countries where pasteurellosis occurs, vaccinations are considered an effective means of controlling this disease. Local isolates are recommended for vaccine preparation (9). Therefore, future investigations on the development of vaccine preparations with local strains are thought to be necessary.

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