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Investigation of the nontypical *Pasteurella multocida* strains obtained from multiple sources, regions, and times: an unexpected increase was detected

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Abstract: In this study, it was aimed to isolate *Pasteurella multocida* from 1250 lung samples (calf, sheep, and goat), with respiratory system infection, such as nasal discharge, cough, fever, and to investigate the phenotypic and genotypic properties of this bacteria. As a result, a total of 92 *P. multocida* were obtained, of which 66 (71.73%) were from calf, 19 (20.65%) from sheep, and 7 (7.6%) from goat. The A:3A type was the most common type with 34 isolates (36.95%), including 30 from calves (45.45%), and 4 from sheep (21.05%). An alternate mPCR protocol was developed to determine capB and capE genes and 5 from 41 nontypical strains were detected as type B (n = 3; 3.26%) and type E (n = 2; 2.17%) via this novel protocol. Twenty-six nontypical strains presented nonmucoid colonies. The serotype of 11 typical and 13 nontypical strains was not able to be determined by mPCR. The prevalences of nontypical *P. multocida* were significantly ($p < 0.01$) different by determinants. Nineteen virulence-associated gene profiles were determined, and the highest percentages of genes were ompA (70.65%), ptfA (69.56%), and tadD (64.13%). Also, there was a higher rate of similarity between calf and sheep strains. In conclusion, nontypical strains may commonly cause pneumonia in farm animals. These results may be considered for future vaccine studies.

Key words: Genotyping, *Pasteurella multocida*, pneumonia, virulence genes

1. Introduction

Pasteurella multocida causes various economically relevant diseases in animal species, including bovine hemorrhagic septicemia, enzootic pneumonia, snuffles in rabbits, avian fowl cholera, and swine atrophic rhinitis [1,2]. There are studies about prevalence and distribution of various virulence associated genes (VAGs) (outer membrane and porin proteins, adhesins, toxins, hyaluronidase, iron acquisition proteins, and sialidases) [3]. The most important VAGs are the capsule and lipopolysaccharide [4]. *P. multocida* is routinely classified into five serogroups (A, B, D, E, and F), and each is generally associated with, but not completely restricted to, a specific host [5]. It was reported that *P. multocida* strains with serotype A:3 or A:1 can especially cause pneumonia of cattle [6], sheep, and pigs [7–9]. Especially, capsular type A strains, which is an opportunistic pathogen that causes respiratory disease in cattle, can cause serious respiratory diseases in cattle and have especially caused epidemics in beef calves [2]. Infections have rarely been observed in humans related to cat or dog bites [10]. Owing to the broad host spectrum of *P. multocida* and high antigenic variability, there are enormous difficulties in producing vaccines. Therefore, although there are several commercial vaccines aiming to protect from this infection effectiveness is not at the desired level [2,3].

Capsular strains (typical) are more virulent than acapsular strains (nontypical) because capsules are the most important in the pathogenesis of *P. multocida* [11–13]. Although acapsular mutant (serogroup A) was unable to growe in chicken tissue, protective immunity was obtained in chickens vaccinated with high doses of this acapsular mutant [14]. Although a molecular method to the serological tests currently used for the classification of *P. multocida* capsular types represents [10], it has been reported that the incidence of nontypical *P. multocida* strains may change between approximately 0.5% and 10.6% in different sources and regions [2,8,15–

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17]. Although there are more studies related to typical *P. multocida* grouped into five capsular serogroups [2,4,18], actual and comprehensive data about nontypical strains could not be found.

It was aimed to determine the prevalence of VAG factors of *P. multocida* according to the source, region, and year, to determine the capsular types of all strains, and then to investigate the cause of the unexpected increase in nontypical strains.

2. Materials and methods

2.1.Bacterial strains and culture

A total of 1250 lung samples, collected between 2016 and 2019, from calves (850), sheep (300), and goats (100) with respiratory system infection from different farms located in four geographical regions (Southeastern Anatolia, Central Anatolia, Black Sea, Mediterranean) of Turkey, were sent to the Faculty of Veterinary Medicine, Microbiology Laboratory, Selçuk University (Turkey) for diagnosis. This research has been approved (grant number: 2020-69) by the Ethics Committee of the Faculty of Veterinary Medicine at the University of Selçuk in Konya, Turkey. All animals showed at least one of the clinical symptoms of respiratory infection, such as nasal discharge, cough, fever. Primary isolation of *P. multocida* from the lungs was carried out on a blood agar base supplemented with 5% sheep blood. Plated agar media was incubated for 24 h at 37 °C in 7% CO_2 atmosphere [2]. *P. multocida* isolates were identified by two-step procedure; standard biochemical procedures, (including oxidase, indol, production of catalase, and urease activity, and carbohydrate fermentation [5]), and PCR (for the detection of species specific gene fragment KMT [19]). *P. multocida* type strains ATCC 12945 (capA), NCTC10323 (capB, tbpA), ATCC 12948 (capD, toxA, nanH, hgbB), and ATCC 43020 (capE) were used as positive controls.

2.2.Determination of virulence factors by PCR

All DNA extracts of the isolates were obtained using the Wizard Genomic DNA Purification Kit (Promega, USA) by considering the kit protocol. The genotypes of the serogroup (*KMT1, capA, capB, capD, capE, capF*) was carried out prior protocols [18, 19]. Serotyping (*L1-8*, *L3A-L6A*) were carried out as previously described [4]. Also, toxin (*toxA*), adhesins (*ptfA, pfhA, tadD*), iron acquisition (*exbB, exbD, tonB, hgbA*), and protectins (*ompA, ompH, omp87, plpB*) among the isolates were determined using the PCR protocol [2] (Table 1).

Alternate capB and capE primers were designed by the NCBI program (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and the Primer-BLAST program (https://blast.ncbi.nlm.nih.gov/Blast) in NCBI to overcome some challenges encountered in field strains. The sequences of PcapBF (12863–12882) and PcapBR (13619–13600) primers matched the sequence of the serogroup B cap gene (accession number AF169324.1). The sequences of PcapEF (4408–4426) and PcapER (4901–4882) primers matched the sequence of the serogroup E cap gene (accession number AF302466.1). PcapB and PcapE primers were standardized using the mPCR protocol. The mPCR for PcapB and PcapE was performed according to the conditions listed in Table 1. All PCRs were carried out with each primer (10 pmol), DNA template (100 ng/µL), 5 µL 5 × FIREPol Master Mix (Solis Biodyne, Estonia), and 1 µL ultra pure water (negative control). Positive controls were used in each PCR series. PCR products were showed under UV illumination using ethidium bromide. For comparison of DNA sizes, a 50 bp DNA ladder (Thermo Scientific, SM0373) was used.

2.3. Random amplified polymorphic DNA-polymerase chain reaction

The single OPA11 primer was used to generate the profiles of *P. multocida* [20] (Table 1). PCR products were run on 1% agarose gel electrophoresis, and the variety of randomly amplified bands was determined.

2.4.Statistical analysis

The obtained results were compared using four procedures: the unweighted pair group method with arithmetic mean (UPGMA) [21]; hierarchical cluster analysis method (IBM SPSS Statistic 23 Program); the Hunter–Gaston discriminatory index (HGDI); and the multiple response (Monte Carlo) chi-square test, calculated with 95% confidence intervals by the program [22].

3.Results

In this study, a total of 92 *P. multocida* were isolated from 1250 lung samples, and it was confirmed both standard biochemical procedures and by PCR. These strains contained 66 (71.73%) calf strains, 19 (20.65%) sheep strains and 7 (7.6%) goat strains. The highest isolation rate was obtained from lung samples collected from calf farms in Central Anatolia in 2016. Sixty-three *P. multocida* isolates classified in one of three serogroups (A, B, and E) exhibited mucoid colony morphology. Colony formation of the 26 nontypical strains was nonmucoid, also 47.22% of them were recovered from the Southeastern Anatolia region (Table 2).

3.1.Typing of *Pasteurella multocida* isolates according to serogroup, and serotype

When serogroups of type strains were compared with band sizes as per previously described methods [18], the size of each PCR fragment corresponded to only two of ATCC 12945 capA (1044 bp), and ATCC 12948 capD (657 bp) from four reference strains was verified. Additionally, this PCR protocol resulted in the amplification of only *capA* and *capD* serogroup-specific regions of the biosynthesis

Table 1. The primer sequences and cycle conditions used in PCR, and band sizes expected.

PCR types*	Primer sequence		Band (bp)	References
	KMT1F *KMT1R*	5'ATCCGCTATTTACCCAGTGG'3 5'GCTGTAAACGAACTCGCCAC'3	460	[18]
	*capA*F *capA*R	5'TGCCAAAATCGCAGTCAG'3 5'TTGCCATCATTGTCAGTG'3	1,044	[18]
mPCR1	*capB*F *capB*R	5'CATTTATCCAAGCTCCACC'3 5'GCCCGAGAGTTTCAATC'3	760	[18]
	*capD*F *capD*R	5'TTACAAAAGAAAGACTAGGAGCCC'3 5'CATCTACCCACTCAACCATATCAG'3	657	[18]
	*capE*F *capE*R	5'TCCGCAGAAAATTATTGACTC'3 5'GCTTGCTGCTTGATTTTGTC'3	511	[18]
	*capF*F *capF*R	5'AATCGGAGAACGCAGAAATCAG'3 5'TTCCGCCGTCAATTACTCTG'3	851	[18]
	*L1*F *L1*R	5'ACATTCCAGATAATACACCCG'3 5'ATTGGAGCACCTAGTAACCC'3	1307	[4]
	*L2*F *L2*R	5'CTTAAAGTAACACTCGCTATTGC'3 3'TTTGATTTCCCTTGGGATAGC'3	810	[4]
	*L3*F *L3*R	5'TGCAGGCGAGAGTTGATAAACCATC'3 5'CAAAGATTGGTTCCAAATCTGAATGGA'3	474	[4]
	*L4*F *L4*R	5'CTTTATTTGGTCTTTATATATACC'3 5'AGATTGCATGGCGAAATGGC'3	550	[4]
mPCR2	*L5*F *L5*R	5'CAATCCTCGTAAGACCCCC'3 5'TCTTTATAATTATACTCTCCCAAG'3	1175	[4]
	*L6*F *L6*R	5'AATGAAGGTTTAAAAGAGATAGCTGGAG'3 5'CCTATATTTATATCTCCTCCCC'3	668	[4]
	*L7*F *L7*R	5'CTAATATATAAACCATCCAACGC'3 5'CTAATATATAAACCATCCAACGC'3	931	[4]
	*L8*F *L8*R	5'GAGAGTTACAAAAATGATCGGC'3 5'TCCTGGTTCATATATAGGTAGG'3	255	[4]
	*L3A*F *L3A*R	5'TCCTTATCTGACATTGAAATCG'3 5'CTAGACATCTGGTGGTTGCG'3	415	[4]
	*L6A*F *L6A*R	5'AATATCTTTATAATTATACTCTCCC'3 5'AATGAAGGTTTAAAAGAGATAGC'3	668	[4]
sPCR1	*toxA*F *toxA*R	5'CTTAGATGAGCGACAAGG'3 5'GAATGCCACACCTCTATAG'3	846	[2]
sPCR2	*ptfA*F *ptfA*R	5'TGTGGAATTCAGCATTTTAGTGTGTC'3 5'TCATGAATTCTTATGCGCAAAATCCTGCTGG'3	488	[2]
sPCR3	*pfhA*F *pfhA*R	5'TTCAGAGGGATCAATCTTCG'3 5'AACTCCAGT TGGTTTGTCG'3	286	[2]
sPCR4	*tadD*F *tadD*R	5'TCTACCCATTCTCAGCAAGGC'3 5'ATCATTTCGGGCATTCACC'3	416	[2]
sPCR5	*OmpA*F *OmpA*R	5' CGCATAGCACTCAAGTTTCTCC'3 5'CATAAACAGATTGACCGAAACG'3	201	[2]
sPCR6	*OmpH*F *OmpH*R	5'CGCGTATGAAGGTTTAGGT'3 5'TTTAGATTGTGCGTAGTCAAC'3	438	[2]
sPCR7	*Omp87*F *Omp87*R	5'GGCAGCGAGCAACAGATAACG 5'TGTTCGTCAAATGTCGGGTGA'3	838	[2]

Table 1. (Continued).

sPCR8	*plpB*F *plpB*R	5'TTTGGTGGTGCGTATGTCTTCT 5'AGTCACTTTAGATTGTGCGTAG'3	282	[2]
sPCR9	*ExbB*F *tonBR*	5' GGTGGTGATATTGATGCGGC'3 5'GCATCATGCGTGCACGGTT'3	1144	[2]
sPCR10	*hgbA*F *hgbA*R	5'TGGCGGATAGTCATCAAG'3 5' CCAAAGAACCACTACCCA'3	419	[2]
sPCR11	*hgbB*F *hgbB*R	5'ACCGCGTTGGAATTATGATTG'3 5'CATTGAGTACGGCTTGACAT'3	788	[2]
sPCR12	*tbpA*F *tbpA*R	5'TTGGTTGGAAACGGTAAAGC'5 5'TAACGTGTACGGAAAAGCCC'3	728	[2]
mPCR3	*capB*F *capB*R	5'GCGATATCAATCTGCTTAAG'3 5'GGATTCTATCTTGACTGAAG'3	757	In this study
	*capE*F *capE*R	5'CTCTAGTATCAGGCGTACC'3 5'GCTTGCTGCTTGATTTTGTC'3	494	In this study
sPCR13	OPA11	5'-CAATCGCCGT-3'		[20]

*Cycle conditions of multiplex PCRs: mPCR1: 95 °C 300 s 30 (95 °C 30 s, 55 °C 30 s, 72 °C 30 s) 72 °C 300 s; mPCR2: 96 °C 300 s 30 (96 °C 30 s, 52 °C 30 s, 72 90 s) 72 °C 300 s; mPCR3: 95 °C 300 s 35 cyc (95 °C 30 s, 55 °C 45 s, 72 45 s) 72 °C 10 min.
Cycle conditions of simplex PCRs: sPCR1: 40 cyc (94 °C 30 s, 55 °C 30 s, 72 °C 30 s); sPCR2: 35 cyc (94 °C 60 s, 68 °C 60 s, 72 °C 60 s); sPCR3: 35 cyc (94 °C 60 s, 68 °C 60 s, 72 °C 60 s); sPCR4: 35 cyc (94 °C 60 s, 68 °C 60 s, 72 °C 60 s); sPCR5: 35 cyc (94 °C 30 s, 55 °C 30 s, 72 °C 60 s) 72 °C 300 s; sPCR6: 35 cyc (94 °C 30 s, 55 °C 30 s, 72 °C 60 s) 72 °C 300 s; sPCR7: 35 cyc (94 °C 30 s, 55 °C 30 s, 72 °C 60 s) 72 °C 300 s; sPCR8: 35 cyc (94 °C 30 s, 55 °C 30 s, 72 °C 60 s) 72 °C 300 s; sPCR9: 95 °C 5 min 35 cyc (94 °C 30 s, 53 °C 30 s, 72 45 s) 72 °C 10 min; sPCR10: 95 °C 300 s 35 cyc (94 °C 30 s, 53 °C 30 s, 72 45 s) 72 °C 10 min; sPCR11: 95 °C 300 s 35 cyc (94 °C 30 s, 54 °C 30 s, 72 45 s) 72 °C 10 min; sPCR12: 95 °C 300 s 35 cyc (94 °C 30 s, 53 °C 30 s, 72 45 s); sPCR13: 95 °C 300 s 35 cyc (94°C 30 s, 54°C 30 s, 72 45 s) 72°C 10 min

loci in 51 isolates (55.43%) (48 capA and 3 capD) (Table 2). The highest percentage of capA gene were determined in isolates obtained from calf samples in the Central Anatolia region in 2016. The type A:3A (36.95%) was the most common type with 34 isolates, including 30 from calf (45.45%), and 4 from sheep (21.05%), from different sources, geographical origin, and years. Although LPS types of 72 (75%) strains were precisely determined with the previously described mPCR [4].

The serogroup of forty-one isolates (44.56%) could not be defined with before described method [18]. According to the new mPCR results, the amplification products of *capB* and *capE* genes were respectively 757 bp and 494 bp for both reference strains and these isolates. Five (12.19%) of 41 nontypical strains were determined as *capB* or *capE* using a designed mPCR protocol. It had occurred from three type B (3.26%) and two type E (2.17%), also these serogroups appeared in calf samples from Central Anatolia and Mediterranean regions in 2016 (Table 3). Interestingly, the capsule type of 20 (n = 66) calf, 10 (n = 19) sheep, and 6 (n = 7) goat strains could not be determined using both PCR protocols (Table 2). Also, serotype of nontypical *P. multocida* strains (n = 12) could not detected by according to the previously reported mPCR [4].

According to multiple response chi-square test results, the highest isolation rate, referred to as nontypical, were determined in the different variables as goat samples 85.71% (6 in 7), Southeastern Anatolia region 47.22% (17 in 36), and in 2016 with 36.11% (13 in 36).

3.2. Distribution of virulence-associated genes

All strains had some genes from toxin (*toxA*), adhesins (*ptfA, tadD*), iron acquisition (*exbB, exbD, tonB, hgbA*), and protectins (*ompA, ompH, plpB*), while none of them possessed the *Oma87*, with *pfhA*. The highest percentages of all virulence genes were detected in strains obtained from calf samples in 2016. The highest percentages of ompA, ptfA, and tadD genes were 70.65%, 69.56%, and 64.13%, respectively (Table 2). VAG profiles were formed according to the presence of these genes, and 19 VAG profiles were determined in *P. multocida* strains (Table 4). Evaluated the virulence gene presence in both typical and nontypical strains, it was not observed a significant ($p < 0.01$) difference. The percentages of both VAG4 and VAG6 profiles, which were 20.65%, showed the highest values.

3.3. Random amplified polymorphic DNA-polymerase chain reaction results

A total of 96 strains were classified as 26 different subgroup. Profiles consisted of between 1 and 17 bands and the size

Table 2. Percentage of the serogroup, serotypes, toxin, adhesin, protectin and iron acquisition genes among the 96 *Pasteurella multocida* strains.

Sources →	**Calf**	**Sheep**	**Goat**	**Total**
Genes ↓				
Cap A	40	8	0	48
	81.6%	16.3%	0.0%	
Cap B	3	0	0	3
	100.0%	0.0%	0.0%	
Cap D	1	1	1	3
	25.0%	25.0%	25.0%	
Cap E	2	0	0	2
	100.0%	0.0%	0.0%	
Noncap	20	10	6	36
	54.1%	27.0%	16.2%	
L2	9	1	0	10
	81.8%	9.1%	0.0%	
L6	0	3	0	3
	0.0%	100.0%	0.0%	
L8	1	0	0	1
	100.0%	0.0%	0.0%	
L3A	41	7	5	53
	75.9%	13.0%	9.3%	
ToxA	2	0	3	5
	33.3%	0.0%	50.0%	
PtfA	53	10	1	64
	81.8%	15.2%	1.5%	
TadD	50	8	1	59
	85.0%	13.3%	1.7%	
OmpA	48	12	5	65
	72.7%	18.2%	7.6%	
OmpH	3	0	0	3
	75.0%	0.0%	0.0%	
PlpB	37	8	1	46
	79.2%	16.7%	2.1%	
HgbA	32	3	2	37
	80.5%	7.3%	4.9%	
HgbB	16	4	0	20
	80.0%	20.0%	0.0%	
TonB	26	1	2	28
	87.1%	3.2%	6.5%	
TbpA	29	3	2	34
	81.1%	8.1%	5.4%	

of these bands was in size between approximately 250 bp and 1250 bp. Two major groups were detected using cluster analysis, and the group I consisted of thirteen isolates (16, 23, 28, 58, 76, 78–80, 83, 84, 92, 95, and 96). The similarity of strains belonging to the group I was at least 18%. The group II occurred from 76 isolates (1–15, 17–20, 22, 24–27, 29–57, 59–64, 66–75, 77, 81, 82, 85–91, and 94) and the reference strains. The similarity of strains within this group was at least 54%. The following rates of calf, sheep, and goat strains took into the of cluster II (subgroup A), respectively: 81.81% (n = 54), 84.21% (n = 16), and 85.71% (n = 6) (Figure). When the groups of all strains were obtained from calves, sheep, and goats, there was a higher rate of similarity of calf and sheep strains. Additionally, 16 nontypical isolates took part in subgroup A-I of cluster II. HGDI was detected to be 0.90, and the similarity of Jaccard's coefficient was determined to be 0.884 by UPGMA.

4.Discussion

There are previous studies reported related to the level of genetic diversity in the *P. multocida* strains obtained from the same host or region [2,6–9,13,14,23]. But, significant virulence factors of *P. multocida* have recently been reviewed [3] but no host-specific factors have been identified as yet [20]. It can be associated with population disease, host, structure, and control system [24]. This infection is more common in cattle than in other farm animals. Stress plays an important factor in the inhibition of the normal physical defense mechanisms to infections caused by especially *Pasteurella* spp., *Mannheimia* spp., *Mycoplasma* spp., facilitating the invasion of lung tissue and development of pneumonia [3]. As a result of alveolar macrophage dysfunction by viral pneumonia in calves, a decreased clearance of inhaled bacterial pathogens, and then allowing them to become established [23]. Pasteurella and Mannheimia pneumonias can develop with same path in sheep and goats, in same [9,10,17]. In this study, we hypothesized that the prevalence of *P. multocida* infection could change according to the source, region, and years because more *P. multocida* was isolated from calf samples from the Central Anatolia region in 2016. We presumed that the harsher climate of the Central Anatolia region might be the primary factor in this case, and calves may be more sensitive to this bacterium than other farm animals.

P. multocida, an opportunistic pathogen, with capsular type A causes respiratory disease in cattle [25]. *CapA* - positive strains among calf and sheep samples were thought to be linked to *capD*, and it was found a similar result with the reported study [26]. Type A:3A (56.25%) is a common serogroup: serotype in farm animals in our country, and this result is similar to that of a previous study [27,28]. None of the isolates harbored *capF* [2], while *capB*,

Table 3. Evalution of the *Pasteurella multocida* strains according to various determinants.

Isolates number	Year	Source*	Region**	Colony morphology***	Cluster	Serogroup: Serotype	VAG profile****
1-C1	2016	C	MD	M	II-A-II-II	E:3A	VGP1
2-C2	2016	C	CA	M	II-A-II-II	E:8	VGP 1
3-C3	2016	C	MD	M	II-A-I-II	A:3A	VGP2
4-C4	2016	C	MD	M	II-A-I-II	A:2-3A	VGP2
5-C5	2016	C	CA	M	II-A-II-II	A:2	VGP 3
6-C6	2016	C	MD	M	II-A-I-II	A:3A	VGP 4
7-C7	2016	C	B	M	II-A-I-II	A:3A	VGP 5
8-C8	2016	C	MD	M	II-A-I-II	A:3A	VGP 4
9-C9	2016	C	MD	M	II-A-I-II	A:3A	VGP 4
10-C10	2016	C	MD	M	II-A-I-II	A:3A	VGP 6
11-C11	2016	C	B	M	II-A-I-II	A:3A	VGP 5
12-C12	2017	C	MD	M	II-A-II-II	A:2	VGP 7
13-C13	2016	C	CA	M	II-A-I-II	A:2	VGP 6
14-C14	2016	C	MD	M	II-A-I-II	A:3A	VGP 6
15-C15	2016	C	SA	M	II-A-I-II	A:3A	VGP 4
16-C16	2016	C	CA	M	I-A	A:2	VGP 6
17-C17	2016	C	MD	M	II-A-I-II	A:3A	VGP 6
18-C18	2016	C	CA	M	II-A-I	A:3A	VGP 6
19-C19	2016	C	MD	M	II-A-I-II	A:3A	VGP 6
20-C20	2016	C	CA	M	II-A-I-II	A:3A	VGP 6
21-C21	2016	C	MD	M	II-B	A:2	VGP 6
22-C22	2016	C	MD	M	II-A-I-II	A:3A	VGP 4
23-C23	2016	C	CA	M	I-B	A:3A	VGP 6
24-C24	2016	C	CA	M	II-A-I-II	A:3A	VGP 4
25-C25	2016	C	MD	M	II-A-I-II	A:3A	VGP 6
26-S1	2016	C	CA	M	II-A-I-II	A:3A	VGP 6
27-C26	2016	C	MD	M	II-A-I-II	A:3A	VGP 6
28-C27	2016	C	CA	M	I-B	A: Nontype	VGP 6
29-C28	2016	C	B	M	II-A-I-II	A:3A	VGP 5
30-C29	2019	C	CA	M	II-A-I-II	A:2	VGP 4
31-C30	2018	C	MD	M	II-A-I-II	A:3A	VGP 4
32-C31	2017	C	MD	M	II-A-I-II	A:3A	VGP 4
33-Type A		C		M	II-A-I-II	A: Nontype	VGP 8
34-C66	2018	C	CA	M	II-A-I-II	A:3A	VGP 1
35-Type B		C		M	II-A-II-II	B: Nontype	VGP 9
36-C32	2016	C	MD	M	II-A-II-II	A:3A	VGP 4
37-C33	2018	C	CA	M	II-A-I-II	A:3A	VGP 10
38-C60	2017	C	MD	M	II-A-I-II	A:3A	VGP 4
39-C34	2018	C	MD	M	II-A-I-II	A:3A	VGP 4
40-C35	2016	C	MD	M	II-A-II-II	A:3A	VGP 4
41-C61	2016	C	CA	M	II-A-I-II	A:3A	VGP 10

Table 3. (Continued).

42-C62	2016	C	CA	M	II-A-I-II	A:3A	VGP 10
43-C36	2019	C	CA	M	II-A-II-II	A:3A	VGP 6
44-C37	2019	C	CA	M	II-A-II-II	A:2	VGP 11
45-Type D		Pig		M	II-A-II-II	D:2	VGP 12
46-C38	2016	C	CA	M	II-A-I-II	Nontypical:3A	VGP 6
47-C39	2017	C	CA	M	II-A-I-II	B: Nontype	VGP 9
48-C40	2016	C	CA	M	II-A-II-I	B: Nontype	VGP 13
49-C41	2016	C	CA	M	II-A-I-II	B: Nontype	VGP 3
50-G1	2016	G	CA	NM	II-A-I-II	D:3A	VGP 12
51-C42	2016	C	MD	NM	II-A-I-II	D:3A	VGP 4
52-S13	2016	S	CA	NM	II-A-I-II	A:3A	VGP 6
53-S2	2019	S	B	NM	II-A-I-II	A:3A	VGP 11
54-S14	2018	S	MD	NM	II-A-I-II	A:3A	VGP 6
55-S3	2016	S	CA	NM	II-A-I-II	Nontypical:2	VGP 5
56-S4	2016	S	MD	NM	II-A-II-I	Nontypical:3A	VGP 6
57-G3	2018	G	CA	NM	II-A-I-II	Nontypical:3A	VGP 14
58-C59	2016	C	B	NM	I-A	Nontypical:3A	VGP 5
59-C43	2018	C	CA	M	II-A-I-II	Nontypical:3A	VGP 5
60-S16	2016	S	CA	NM	II-A-I-II	Nontypical:3A	VGP 11
61-C49	2016	C	B	M	II-A-II-II	Nontypical:3A	VGP 9
62-S18	2016	S	SA	M	II-A-I-II	Nontypical:2-3A	VGP 4
63-S17	2016	S	SA	M	II-A-I-II	Nontypical:6	VGP 4
64-C44	2016	C	MD	M	II-A-I-II	Nontypical:3A	VGP 4
65-S19	2017	S	CA	NM	II-B	Nontypical:3A	VGP 9
66-S5	2016	S	CA	M	II-A-I-II	A:3A	VGP 14
67-G2	2017	G	CA	NM	II-A-I-II	Nontypical:3A	VGP 7
68-S6	2017	S	SA	NM	II-A-I-II	Non-typical:6	VGP 14
69-G7	2018	G	CA	NM	II-A-I-II	Nontypical:3A	VGP 15
70-G6	2016	G	B	NM	II-A-I-II	Nontypical:3A	VGP 2
71-C45	2016	C	SA	NM	II-A-I-II	Nontypical:3A	VGP 16
72-S7	2018	S	SA	NM	II-A-I-II	Nontypical:3A	VGP 1
73-C64	2017	S	CA	M	II-A-II-II	A:6	VGP 1
74-C46	2016	C	SA	NM	II-A-I-II	Nontypical:3A	VGP 3
75-C47	2016	C	SA	NM	II-A-II-II	Nontypical:3A	VGP 5
76-C48	2019	C	SA	NM	I-A	Nontypical	VGP 1
77-Type E		Bovine	Africa	M	II-A-II-II	E:3A	VGP 17
78-C50	2019	C	SA	NM	I-A	Nontypical	VGP 18
79-C51	2017	C	SA	NM	I-A	Nontypical	VGP 4
80-G4	2018	G	SA	NM	I-A	Nontypical	VGP 15
81-G5	2019	G	CA	NM	II-A-II-II	Nontypical	VGP 15
82-C65	2017	C	SA	NM	II-A-I-II	Nontypical	VGP 17
83-C52	2019	C	B	NM	I-A	Nontypical	VGP 5
84-C58	2018	C	SA	NM	I-A	Nontypical:3A	VGP 1
85-S8	2019	S	CA	NM	II-A-I-II	Nontypical	VGP 1
86-C53	2019	C	SA	NM	II-A-II-I	Nontypical	VGP 3

Table 3. (Continued).

87- C54	2018	C	SA	M	II-A-II-I	Nontypical	VGP 4
88-S9	2017	S	B	NM	II-A-II-III	Nontypical	VGP 5
89-C55	2017	C	SA	M	II-A-II-I	Nontypical	VGP 17
90-C56	2019	C	CA	M	II-A-II-I	E: Nontype	VGP 17
91-C57	2019	C	SA	M	II-A-II-II	E: Nontype	VGP 17
92-S10	2019	S	CA	M	I-A	A: Nontype	VGP 5
93-C63	2019	C	CA	NM	II-C	Nontypical	VGP 1
94-S11	2019	C	SA	NM	II-A-II-III	A: Nontype	VGP 1
95-S12	2019	S	CA	NM	I-A	D:Nontype	VGP 19
96-S15	2016	S	SA	M	I-A	A:Nontype	VGP 19

* C: Calf, S: Sheep, G: Goat.
** MD: Mediterranean, SA: Southeastern Anatolia, CA: Central Anatolia, B: Black Sea.
*** M: Mucoid, NM: Nonmucoid.
**** VGP: Virulence-associated genes profiles.

Table 4. Composition of virulence-associated genes profiles (VGP) for typing of the *Pasteurella multocida* strains.

Virulence genes →	**Toxin**	**Adhesins**			**Protectins**				**Iron acquisition**			
VAG profiles ↓	**toxA**	**ptfA**	**pfhA**	**tadD**	**ompA**	**ompH**	**Omp87**	**plpB**	**hgbA**	**hgbB**	**tonB**	**tbpA**
VGP1	-	-	-	+	-	-	-	-	-	-	-	-
VGP 2	-	-	-	-	-	-	-	+	+	-	+	+
VGP 3	-	+	-	+	+	-	-	-	-	-	-	-
VGP 4	-	+	-	+	+	-	-	+	+	-	+	+
VGP 5	-	+	-	+	+	-	-	+	-	-	-	-
VGP 6	-	+	-	+	+	-	-	-	-	+	-	-
VGP 7	+	+	-	+	+	-	-	+	-	-	-	-
VGP 8	-	+	-	-	-	+	-	+	+	-	-	+
VGP 9	-	+	-	+	-	-	-	+	+	-	+	+
VGP 10	-	+	-	-	-	+	-	+	+	-	-	+
VGP 11	-	-	-	-	-	-	-	+	-	-	-	-
VGP 12	-	-	-	-	+	-	-	-	+	-	+	+
VGP 13	-	-	-	-	+	-	-	+	+	-	+	+
VGP 14	-	-	-	-	+	-	-	-	-	-	-	-
VGP 15	+	-	-	-	+	-	-	-	-	-	-	-
VGP 16	-	+	-	+	+	-	-	+	-	-	+	+
VGP 17	-	-	-	-	-	-	-	-	+	-	-	-
VGP 18	+	+	-	+	+	-	-	+	+	-	+	+
VGP 19	-	+	-	-	+	-	-	+	+	-	+	+

capD, and *capE* occurred less frequently, and this result was similar to those in previous studies [2–4].

P. multocida strains present considerable genetic diversity in calf, sheep, and goat farms. However, calf and sheep strains were identified as similar according to subgroup and VAG profiles. It was thought that this similarity occurred due to the animals feeding together on farms or grazing on the same pasture [2,24,29]. It was

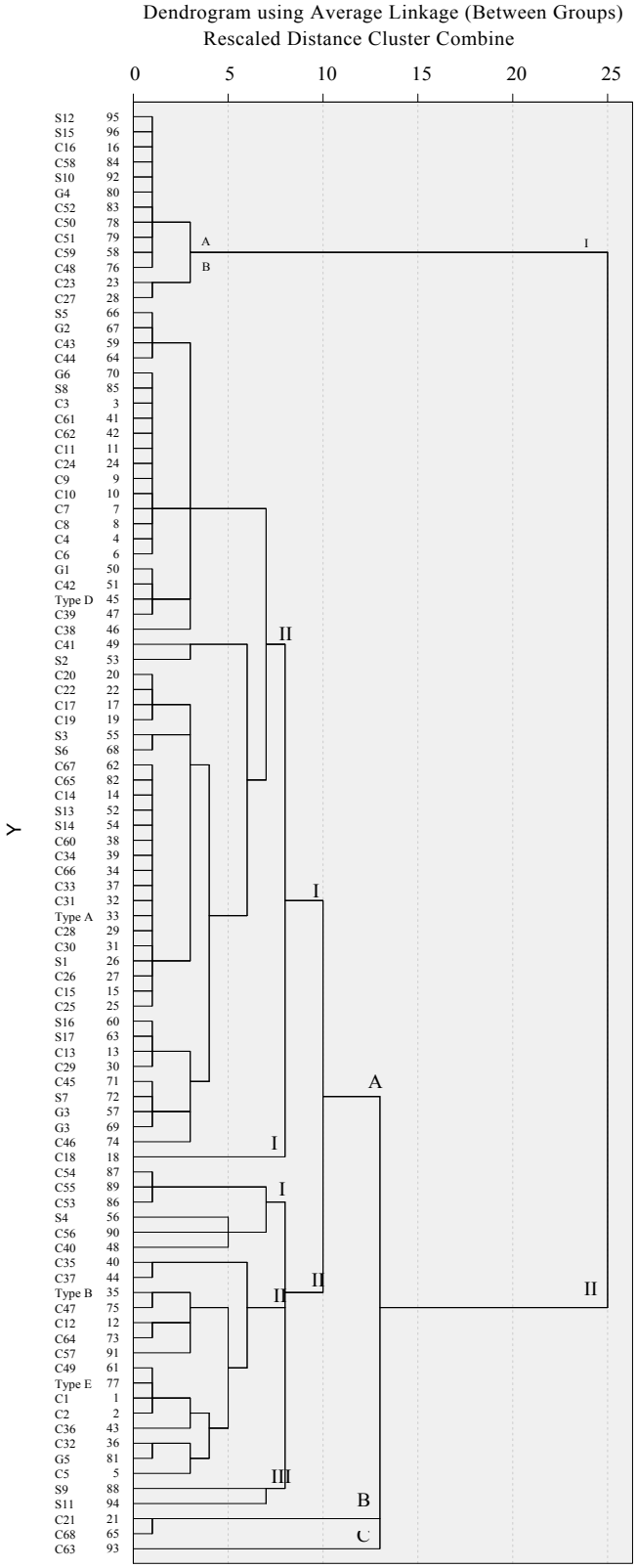

Figure. Similarity analysis of the profiles produced by RAPD cluster analysis. C1–C66, *Pasteurella multocida* obtained from calve samples; S1–S19, *Pasteurella multocida* obtained from sheep samples; G1–G7, *Pasteurella multocida* obtained from goat samples; 33, Type A; 35, Type B; 45, Type D; 77, Type E.

found that virulence factors were not associated with host-specific or certain capsule types except toxA, and this result was similar to those in previous studies [24]. Although the toxA is mainly related to capsular type D *toxA* + pig isolates [29], *toxA* was detected in higher percentages in the capsular type D strains obtained from goats in this study.

mPCR was developed for capsular typing instead of traditional indirect hemagglutination assays. Although it has been reported that this mPCR is easy, this protocol does not work for typing five capsular antigens. Nucleotide sequences of capB and capE primers were not found in the accession number registered in the NCBI gene bank in a previous study [18]. However, it has been reported that this protocol could not type between approximately 0.5% and 10.6% strains as *P. multocida* by PCR [2,8,15,16,17]. It was thought that some of these strains might be capB or capE type. In this study, five (12.19%) of 41 nontypical strains were determined as capB or capE using a new mPCR protocol. Therefore, this protocol can be useful for obtaining more reliable results for related future studies.

LPS types of 72 (75%) strains were also found to be precisely determined with the previously described mPCR [3]. Additionally, the LPS types of 24 strains, which included 11 typed and 13 nontyped strains, were not determined (Table 3). For typing the LPS structure of strains, Heddleston serotyping, multilocus sequence typing (MLST), LPS genotyping, and repetitive element PCR fingerprinting (rep-PCR), and mPCR are used for epidemiological investigations [30,31]. However, none of these techniques are discriminatory enough to provide the required LPS structure of isolates. Therefore, it has been reported that genomics analysis is important to provide more detailed information on the possible structure of the outer LPS [3,6,11,32].

In this study, the incidence of nontypical strains is interestingly higher than that reported so far, which may be owing to several reasons including, new capsular type, regional differences, and deletion of the capsular gene. However, this variation has been ignored until now. In the colony morphology of *P. multocida*, it can be major variations; a mucoid colony structure is generally observed in the pneumonic lesions of pigs, rabbits, and cattle, while nonmucoid colonies are obtained from poultry [24]. However, nontypical strains presented nonmucoid colonies despite originating from calves, sheep, and goats. Therefore, *P. multocida* may have a new or unknown capsular type/subtype. Our results were different from those of previous studies [1–10]. If 47.22% of the strains obtained from Southeastern Anatolia region were nontypical, this might be due to its border and the excessive animal mobility in this region.

In conclusion, it was determined that there was a difference in the occurrence of *P. multocida* as per the source, region, and year. The incidence of nontypical strains showed a higher and unexpected rate. Additionally, *P. multocida* may change its capsule and virulence gene forms. These unexpected differences may be considered for future genotyping or vaccine studies.

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