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Identification of *Pasteurella multocida* Strains Isolated from Respiratory Tract of Healthy and Diseased Cattle and Determination of Capsular Types by PCR in Van Region

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ABSTRACT The aim of this study was to identify *Pasteurella* (*P.*) *multocida* strains isolated from upper and lower respiratory tract of healthy and diseased cattle in Van region and to determine capsule type by PCR. The isolates were identified by standard bacteriological methods and PCR (PM-PCR) using *KMT1* gene-specific primer. From the total of 510 examined swab samples, 60 isolates were suspected as *P. multocida* by standard bacteriological methods and 53 (88.33%) of them were identified as *P. multocida* by PM-PCR. *P. multocida* was isolated from 31 (13.2%) of 222 healthy cattle and 19 (32.2%) of 59 diseased cattle. In all isolates, *CapA* gene-specific amplicons (1044 bp) were detected by PCR using capsular type specific primers. In conclusion, the findings of this study showed that standard bacteriological methods were important for preliminary identification of *P. multocida* isolates and the isolation rate of *P. multocida* from diseased cattle was higher than those of healthy cattle. It was also observed that *P. multocida* capsular type A strains were common in the respiratory tract of the cattle in Van region.

Keywords: *Pasteurella multocida, Capsular type, PCR, Cattle*

ÖZ

Van ve Yöresinde Sağlıklı ve Hasta Sığırların Solunum Yolundan İzole Edilen *Pasteurella multocida* Suşlarının İdentifikasyonu ve Kapsül Tiplerinin PCR ile Belirlenmesi*

Bu araştırmada, Van ve yöresinde sağlıklı ve hasta sığırların üst ve alt solunum yolundan alınan svap örneklerinden izole edilen *Pasteurella* (*P.*) *multocida* suşlarının identifikasyonu ve PCR yöntemi ile kapsül tiplerinin belirlenmesi amaçlandı. İzolatlar konvansiyonel bakteriyolojik yöntemler ve *KMT1* gen spesifik primerin kullanıldığı PCR (PM-PCR) ile identifiye edildi. İncelenen toplam 510 adet svap örneğinden bakteriyolojik yöntemle 60 adet *P. multocida* şüpheli izolat elde edildi. Şüpheli 60 izolatın 53 (%88.33)'ü PM-PCR ile *P. multocida* olarak identifiye edildi. Sağlıklı 222 sığırın 31 (%13.9)'inden, hasta olan 59 sığırın ise 19 (%32.2)'undan *P. multocida* izole edildi. Kapsül tip spesifik primerlerin kullanıldığı PCR ile tüm izolatlarda *CapA* gen spesifik 1044 bp'lik amplikonlar belirlendi. Sonuç olarak araştırmada elde edilen bulgular, konvansiyonel bakteriyolojik yöntemlerin *P. multocida* izolatlarının ön identifikasyonunda önemli olduğunu ve hasta sığırlardan *P. multocida* izolasyon oranının sağlıklı görünen sığırlara göre daha yüksek olduğunu gösterdi. Bununla birlikte Van yöresindeki sığırların solunum yolunda *P. multocida* kapsül tip A suşlarının yaygın olduğu gözlendi.

Anahtar Kelimeler: *Pasteurella multocida, Kapsül tip, PCR, Sığır*

INTRODUCTION

Pasteurella (*P.*) *multocida* was first identified by Louis Pasteur in the 1800s as agent of fowl cholera. About half a century after the study of Pasteur, the bacteria was named *P. septica* according to morphological and biochemical characteristics and then in 1939 it was renamed *P. multocida* (Dabo et al. 2008). *P. multocida* has already been classified as Gram negative, non-motile, non spore-forming, facultative anaerobic, oxidase and catalase positive small rod or cocobacilli in the family *Pasteurellaceae* (Mohamed and Abdelsalam 2008; Quinn et al. 2011).

P. multocida strains are divided into 5 serogroups as A, B, D, E, and F according to capsule antigens (Harper et al. 2006). Capsule type A and less common type D can cause cholera in poultry. Type F strains are often isolated from diseased poultry (especially from turkey) and from the cases of fatal fibrinous peritonitis in calves. Type A strains and toxigenic strains of capsule type D are isolated from cases of pneumonia and atrophic rhinitis in pigs, respectively. *P. multocida* capsular type B and E strains are

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** This study was summarized from in a part of PhD thesis of first author*

associated with the occurrence of hemorrhagic septicemia in cattle and water bubbles in the tropical regions of Africa and Asia. *P. multocida* capsular type A strains are considered to be one of the primary agent of bovine respiratory tract diseases causing significant economic loss in ruminant industry in worldwide (Dabo et al. 2008).

Preliminary identification of *P. multocida* isolates is performed by standart bacteriological methods including Gram staining, catalase and oxidase reaction, non-hemolysis in blood agar, no growth on MacConkey agar and fermentation of various carbohydrates. However, since the biochemical characteristics of *P. multocida* isolates obtained from different host and tissues vary, it is emphasized the necessity of confirming the identification by standart bacteriological methods, with moleculer methods (PCR) (Dziva et al. 2008).

For the identification of *P. multocida* isolates, PM-PCR method using *P. multocida* species-specific primers (*KMT1*) has been developed by Townsend et al. (1998) and this method has been used by many researchers up to date (Kumar et al. 2009; Hotchkiss et al. 2011; Ulker et al. 2012; Verma et al. 2013; Khamesipour et al. 2014; Sarangi et al. 2015; Al-Maary et al. 2017).

Besides the using of various serologic methods (Fillion et al. 1985) for the identification of capsular types of *P. multocida* isolates, a PCR method developed by Townsend et al. (2001) in recent years is being used for detecting capsular serotypes (Ewers et al. 2006; Katsuda et al. 2013; Jamali et al. 2014; Khamesipour et al. 2014).

Some studies performed in different region of our country on the identification of *P. multocida* isolates from the respiratory tract of sheep, goat and cattle by using biochemical tests and/or PCR method and their *in vitro* susceptibilities to various antimicrobial agents have already been reported (Kilic and Muz 2004; Ozbey and Muz 2004; Onat et al. 2010; Tel and Keskin 2010; Ulker et al. 2012; Guler et al. 2013).

To date, there are a small number of studies about bovine respiratory diseases in Van province. In some studies it has been reported that *P. multocida* isolates cultured from lung samples of slaughtered sheep and goats, were identified by conventional bacteriological methods (Yener et al. 2001; Solmaz and Ilhan 2011).

In this study, *P multocida* isolates cultured from the upper and lower respiratory tract of healthy and/or diseased cattle slaughtered in Van slaughterhouse or upper respiratory tract of cattle diseased in field condition, were identified by using standard bacteriological methods and PCR. In the research, capsule types of *P. multocida* isolates were determined by PCR to contribute for epidemiological investigations.

MATERIALS and METHODS

Samples

In this study, a total of 510 swab samples were examined and 458 of them were taken separately from the nazo-pharyngeal (NP) and tracheal-bronchial (TB) regions of 229 cattle slaughtered in Van province slaughterhouse between March 2016/March 2017. The other 52 swab samples were obtained from NP region of cattle, which were brought to Van Yuzuncu Yil University Veterinary Faculty Animal Hospital Clinics or private veterinary clinics in Van because of complaints of pneumonia.

Isolation and preliminary identification

The swab samples delivered to the Van Yuzuncu Yil University Veterinary Faculty Department of Microbiology Laboratory were centrifuged at 3,000 g for 3 minutes in 3 ml sterile physiological saline (FTS, pH:7.2). After the centrifugation, the supernatant was discarded and the sediment was suspended in 0.1 ml sterile FTS. 0.02 ml of the suspension was inoculated onto Columbia blood agar base (Oxoid, CM 03331) containing 5-7% defibrinated sheep blood and incubated at 37°C for 24-48 hours in aerobic conditions. Preliminary identification tests were performed on suspected colonies that was 1-2 mm in diameter, gray, smooth, round or mucoid, and non-hemolytic. The colonies found to be Gram negative and cocobacilli, oxidase and catalase positive, not grow on MacConkey agar medium, yellow bottom of triple sugar iron agar medium, non-motile and positive or negative indol reaction in SIM medium, were suspected as *P. multocida* (Carter 1984; Dziva et al. 2008; Onat et al. 2010; Quinn et al. 2011; Guler et al. 2013).

PCR

DNA extraction: The boiling method, previously reported by Ewers et al. (2006) was used for the extraction of suspected bacterial DNA. Genomic DNA was used to identify *P. multocida* suspected isolates and to determine capsule types by PCR.

Identification by PCR: The primer used for the identification of *P. multocida* by PCR that was previously reported by Townsend et al. (1998) were given in Table 1. The PCR mixture prepared in a final volume of 25 µl was consisted of 12.5 µl mastermix (Abm™ 2X PCR Taq Plus MasterMix, G014), 1 µl extracted DNA and 1 µl of each primer (10 µM). Amplification was carried out according to following protocol: initial denaturation at 95°C for 4 min; 35 cycles were denaturation at 95°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min. The final extension was at 72°C for 9 min.

Capsular typing by PCR: The primers used for the determination of capsule types of *P. multocida* strains by PCR that were previously reported by Townsend et al. (2001) were also given in Table 1. The PCR mixture used in capsular typing was prepared as indicated in the identification step by PCR method. Amplification was carried out according to following protocol: initial denaturation at 95°C for 5 min; 30 cycles were denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 30 sec. The final extension was at 72°C for 5 min. *P. multocida* subsp. *multocida* ATCC® 43137 capsule type A and *P. multocida* ATCC® 12948 capsule type D strains were used as positive controls and PCR water without genomic DNA was used as a negative control in all PCR amplification. The amplicons obtained by PCR were electrophoresed on 1.5% agarose jel containing gel-red at 80 V for 2 h and were examined in a gel imaging system (Genesis) compared to a 100 bp DNA ladder (Thermo Scientific, SM0321).

RESULTS

A total of 60 suspected isolates, 48 from NP and/or TB region of 229 slaughtered cattle and 12 from NP region of 52 cattle brought to Veterinary Faculty Animal Hospital Clinics or private veterinary clinics, were preliminary identified to be *P. multocida* by standard bacteriological tests and 53 (88.33%) of them in which is detected 460 bp amplicons, were identified as *P. multocida* by PM-PCR (Figure 1).

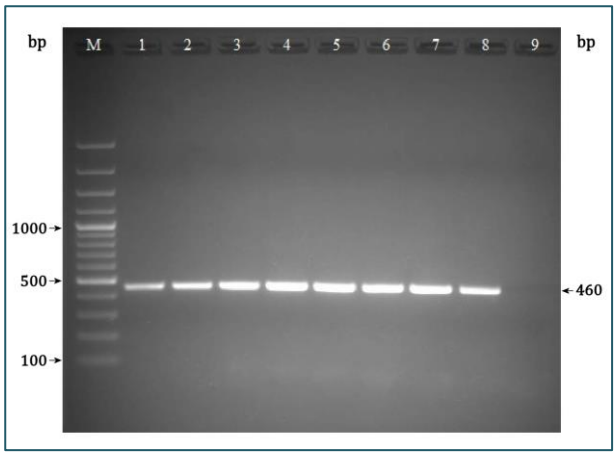

Figure 1. Amplicons were obtained from *P. multocida* isolates by PM-PCR on agarose gel (460 bp) (M: 100 bp DNA marker; 1: *P. multocida* ATCC® 12948 capsule type D; 2: *P. multocida* subsp. *multocida* ATCC® 43137 capsule A; 3-8: *P. multocida* isolates; 9: Negative control).

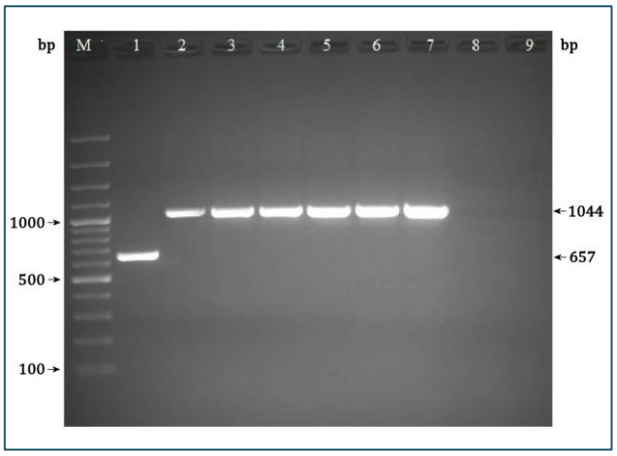

Figure 2. Amplicons were obtained from *P. multocida* isolates by PCR using specific primers to *CapA* and *CapD* genes (*CapA*: 1044 bp, *CapD*: 657 bp); M: 100 bp DNA marker; 1: *P. multocida* ATCC® 12948 capsule type D; 2: *P. multocida* subsp. *multocida* ATCC® 43137 capsule type A; 3-7; *P. multocida* capsule type A isolates; 8: Negative control for *CapD*; 9: Negative control for *CapA*.

Table 1. Primers and amplicon size for identification of *P. multocida* (PM) and their capsular types by PCR

Genes	Primers (5' - 3')	Amplicon size (bp)
PM specific		
KMT1	**F:** ATC CGC TAT TTA CCC AGT GG **R:** GCT GTA AAC GAA CTC GCC AC	460
Capsular type specific		
hyaD-hyaC (Cap A)	**F:** TGC CAA AAT CGC AGT CAG **R:** TTG CCA TCA TTG TCA GTG	1044
bcbD (Cap B)	**F:** CAT TTA TCC AAG CTC CAC C **R:** GCC CGA GAG TTT CAA TCC	760
dcbF (Cap D)	**F:** TTA CAA AAG AAA GAC TAG GAG CCC **R:** CAT CTA CCC ACT CAA CCA TAT CAG	657
ecbJ (Cap E)	**F:** TCC GCA GAA AAT TAT TGA CTC **R:** GCT TGC TGC TTG ATT TTG TC	511
fcbD (Cap F)	**F:** AAT CGG AGA ACG CAG AAA TCA G **R:** TTC CGC CGT CAA TTA CTC TG	851

Table 2. Distribution of *P. multocida* isolates in diseased and healthy cattle

Source/ Health Situation	No. of Animals	No. of Isolates		Total No. of Isolates	Isolation Rates (%)
		NP	TB		
Slaughterhouse/Healthy	222	31	0	31	13.9
Slaughterhouse /Diseased*	7	3	7	10	32.2
Field/Diseased	52	12	-**	12	

*: *P. multocida* was isolated from both NP and TB regions of 3 cattle. **: Sample was not taken.

P. multocida was isolated from 31 (13.9%) NP swab samples of 222 healthy cattle slaughtered and from 19 (32.2%) NP and/or TB samples of 59 diseased cattle including all 7 slaughtered cattle with pneumonic lung lesions and 12 of 52 cattle diseased in field condition (Table 2). *CapA* gene spesific amplicon (1044 bp) were determined by PCR in all *P. multocida* isolates (Figure 2)

DISCUSSION AND CONCLUSION

Bovine respiratory tract diseases cause significant economic losses in most of the countries of the world. It is accepted that stress factors play an important role in the emergence of the disease as well as infectious agents (Horwood and Mahony 2011). *P. multocida*, known as the opportunistic pathogen of the bovine upper respiratory tract, play a role as primer or secondary pathogen in cases of hemorrhagic septicaemia, enzootic pneumonia, bovine pasteurellosis (shipping fever) and bovine respiratory tract disease complex (Harper et al. 2006; Dziva et al. 2008; Quinn et al. 2011).

Various studies have already been reported on the isolation and identification of *P. multocida* from respiratory tract of healthy and/or animals diseased in field condition by conventional and/or molecular methods in our and other countries (Kilic and Muz 2004; Ewers et al. 2006; Onat et al. 2010; Ulker et al. 2012; Katsuda et al. 2013; Verma et al. 2013; Jamali et al. 2014; Khamesipour et al. 2014; Al-Maary et al. 2017; Cucco et al. 2017).

In a study *P. multocida* was isolated from 30 (6%) of 500 pneumonic bovine lung samples (Kilic and Muz 2004). In a similar study conducted in Hatay region, *P. multocida* was isolated and identified by bacteriological and PM-PCR methods from 3 of the 122 lung samples taken from cattle slaughtered and it is suggested that a low isolation rate could be associated with semi-open cultivation due to the hot climatic conditions in the region (Ulker et al. 2012). In another study (Onat et al. 2010), *P. multocida* was isolated from the bilateral nasal swab samples of 27 (57.4%) of 47 healthy cattle.

Khamesipour et al. (2014) reported that *P. multocida* was isolated from 25 (11.4%) pneumonic and 5 (4.4%) healthy bovine lung samples and most of them were identified as capsular type A. Similarly, 105 of 157 *P. multocida* isolates from diseased calves, sheep and goat samples were determined as capsular type A (Al-Maary et al. 2017).

In many studies, it has also been reported that *P. multocida* isolates, from different bovine samples, were mostly identified as capsular type A by PCR in a rate of 92.3% (Ewers et al. 2006), 93.7% (Katsuda et al. 2013), 89.4% (Jamali et al. 2014) and 100% (Cucco et al. 2017).

In another study, it was reported that out of 16 (70%) and 7 (30%) *P. multocida* isolates cultured from diseased and healthy cattle, respectively, 4 (17.4%) isolates were found to be capsular type A whereas 19 (82.6%) isolates were identified as capsular type B (Verma et al. 2013).

Studies have shown that the isolation rate of *P. multocida*, known as the opportunistic pathogen of the upper respiratory tract of cattle, is higher in disease cases and capsular type A can be found in the majority of *P. multocida* isolates from healthy or diseased cattle. Similar to the findings reported in national and international research, in this study, *P. multocida* was commonly found in the respiratory tract of the diseased cattle (32.2%) and all the examined isolates were determined as a capsular type A.

In conclusion, in this study, 88.33% of *P. multocida* suspected isolates, preliminary identified by conventional bacteriological methods, were confirmed by PCR as *P. multocida* and this finding indicated the importance of the preliminary bacteriological identification procedure. The results of this study also showed that isolation rate of *P. multocida* from apparently healthy cattle was lower than those from diseased cattle and the capsular type A strains were common in *P. multocida* isolates obtained from respiratory tracts of cattle in Van region.

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