

Comparison of two different primer sets for detection of *Pasteurella caballi* in bronchoalveolar lavage fluids samples from thoroughbred Arabian foals, using PCR

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ABSTRACT

In the present study, *Pasteurella caballi* (*P. caballi*) was isolated and identified in bronchoalveolar lavage fluid and lung samples from thoroughbred Arabian foals using conventional microbiological methods. Subsequently, the ability of two different PCR primer sets was evaluated for detection and confirmation of *P. caballi*. Primer sets 1 and 2, targeting the 16S rRNA gene of *P. caballi*, were designed using the Primer 3 and Primer-BLAST programs, respectively. PCR was performed to confirm *P. caballi* strains and to detect it directly in the bronchoalveolar lavage fluid and lung samples. In total, 35 *Pasteurella* spp. were isolated from 25 (38.4 %) of 65 bronchoalveolar lavage fluid samples, and 10 (58.8 %) of 17 lung samples. These strains were identified as *P. caballi* based on conventional microbiological and biochemical characteristics. The sensitivities of primers 1 and 2 were determined to be 100 % to confirm cultured *P. caballi* strains. However, the specificity of *P. caballi* detection was lower with primer set-1 than primer set-2 in bronchoalveolar lavage fluid and lung samples. The sensitivity and specificity of primer set-2 were confirmed by gene sequence analysis. This study indicates that the 16S rRNA-PCR method, using primer set-2, provides a rapid and accurate tool for the detection and confirmation of *P. caballi* isolates in bronchoalveolar lavage fluid and lung samples from foals.

Key words: Arabian foals, *Pasteurella caballi*, polymerase chain reaction, primer-BLAST, primer 3, 16S rRNA

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Introduction

The *Pasteurella* species are members of the Pasteurellaceae family, which includes Gram-negative, oxidase-positive, nonmotile, fermentative, rod-shaped bacteria (WINN et al., 2006). Members of the genus *Pasteurella* are present on the mucous membranes of the respiratory and digestive tracts of mammals and birds (BADA et al., 1993). As a result of stress, these organisms may become invasive and play a significant role in the pathogenesis of a variety of infections in animals, such as: pneumonia, sinusitis, abortion, mastitis, and septicemia. The isolation of aerogenic *Pasteurella*-like organisms from horses was reported by Schlater in 1989. The same year, the name *Pasteurella caballi* (*P. caballi*) was proposed (SCHLATER et al., 1989) and this name was validated in 1990 (BADA et al., 1993). In horses, *P. caballi* is an inhabitant of the mucous membranes of the upper respiratory tract (SCHLATER et al., 1989).

Proper primer design is one of the most important steps for maximal specificity and efficiency of PCR (ABD-ELSALAM, 2003). Various software programs are available for the selection of primer pairs from a template sequence. Primer-BLAST and Primer 3 are the most popular non-commercial primer design software (ABD-ELSALAM, 2003; DIEFFENBACH et al., 1993). Detection of *P. caballi* in clinical samples is carried out by conventional microbiological methods, but bacterial culture takes a long time, normally more than 24 hours, or even days, and identification of isolated bacteria by classical bacteriological methods is difficult. There is currently no reported PCR detection of *P. caballi*. Thus, the current study aimed to detect *P. caballi* in bronchoalveolar lavage fluids (BALF) and lung samples from thoroughbred Arabian foals, using conventional microbiological methods and PCR. The ability of two different 16S rRNA-PCR primer sets, designed by different software programs, was evaluated for detection and confirmation of *P. caballi*.

Materials and methods

Sampling and culture. In the present study, bronchoalveolar lavage fluid (BALF) samples were collected five times at ten-day intervals from 13 thoroughbred Arabian foals, by inserting a nasotracheal tube and infusing 30 mL of sterile saline solution (MANSMANN and KNIGHT, 1972). Lung samples were collected from these 13 foals after necropsy, and from four other dead foals (ERGANIS et al., 2014). The samples were inoculated onto sheep blood (10 % vol/vol) agar (Oxoid, Basingstoke, UK) and incubated under aerobic conditions at 37 °C for 24 h. The conventional and biochemical characteristics of suspected *Pasteurella* colonies were determined (WINN et al., 2006).

Extraction of DNA. DNA was extracted, using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) in accordance with the manufacturer's protocol, from

BALF and lung samples, and overnight cultures of *Pasteurella* isolates identified as *P. caballi* on the basis of biochemical tests.

PCR analysis. Primer sets 1 and 2 were designed using the Primer 3 (University of Massachusetts Medical School, U.S.A., <http://bioinfo.ut.ee/primer3/>) and Primer-BLAST programs (National Center for Biotechnology Information (NCBI), <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) to amplify a 203 bp and a 178 bp fragment of the 16S rRNA gene sequence from *P. caballi* strains, respectively (Table 1). The NCBI-BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to check the specificity of primer set-1. PCR was performed on a thermocycler (Eppendorf, Hamburg, Germany) as follows: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 sec, annealing at 56 °C for 45 sec, and extension at 72 °C for 45 sec; and a final extension at 72 °C for 10 min. The total reaction volume was 50 µL with 25 µL of PCR master mix (2x), (Fermentas, Burlington Ontario, Canada), 1 µM of each primer, 1 µg of template DNA, and nuclease-free water to reach a volume of 50 µL. A 10 µL sample of the PCR product was separated using agarose gel (1.5 %, w/v) stained with ethidium bromide (5 µg/mL) electrophoresis, (Sigma-Aldrich, Missouri, USA) and a 100 bp DNA ladder (Solis BioDyne, Tartu, Estonia). The gels were visualized and photographed using an ultraviolet transilluminator (Carestream, Rochester, China). *Pasteurella multocida* (*P. multocida*) and *Mannheimia haemolytica* (*M. haemolytica*), isolated from the lungs of calves, and *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Rhodococcus equi* (*R. equi*), *Staphylococcus aureus* (*S. aureus*), and *Mycoplasma* spp., isolated from the lungs of foals, were used as negative controls.

Table 1. PCR primer sets used in this study

Primer name	Sequence (5'-3')	Amplicon size	Genetic target	Primer source
Primer set-1	F-CAGCCACACTGGAAGTGAAGA	203 bp	16S rRNA	Primer-3
	R-TTAGCCGGTGCTTCTTCTGT			
Primer set-2	F-ACGGGTGAGTAATGCTTGGG	178 bp	16S rRNA	NCBI Primer-BLAST
	R-GAGATCGTCGGCTTGGTAGG			

Gene sequence analysis. For the gene sequence analysis of *P. caballi* isolates, the 178 bp fragment of the 16S rRNA gene, amplified by the primer-2 set, and sequences of the amplified PCR products were determined with forward and reverse primers using an ABI 3130 XL (USA) automated sequencer and a BigDye Terminator v3.1 cycle sequencing kit (Perkin-Elmer Applied Biosystems, Warrington, United Kingdom), according to the kit protocol. Basic Local Alignment Search Tool (BLAST) analysis was used to identify the DNA of the sequence data (www.ncbi.nlm.nih.gov/BLAST). For confirmation of the sensitivity and specificity of primer set-2, gene sequence analysis was repeated on PCR-2

products obtained from 10 of the *P. caballi* culture positive BALF samples, and from 10 culture negative samples.

Results

In total, 35 *Pasteurella* spp. were isolated from 25 (38.4 %) of 65 BALF samples and 10 (58.8 %) of 17 lung samples at the end of the culture. The isolates grew on sheep blood agar and produced grey-white, non-hemolytic colonies containing bacteria that are small Gram-negative rods, non-motile, oxidase-positive, and catalase, urease-negative and that ferment glucose, lactose, maltose, and mannitol. Additional characteristics are presented in Table 2. These strains were identified as *P. caballi* based on their biochemical characteristics.

Table 2. Biochemical characteristics of *P. caballi* strains

Characteristic	<i>P. caballi</i>	Acid produced from	<i>P. caballi</i>
Hemolysis	-	Glucose	+
Motility	-	Arabinose	-
MacConkey agar growth	-	Lactose	+
Catalase	-	Maltose	+
Oxidase	+	Mannitol	+
Beta (β)-Galactosidase	+	Raffinose	+
Indole	-	Sorbitol	-
Urease	-	Trehalose	-
Esculin hydrolysis	-	Xylose	+
Nitrate reduction	+	Sucrose	+
Voges-Proskauer	-	Saccharose	+
Phosphatase	+	Mannose	+
Gelatinase	-	Fructose	+
		Galactose	+

- = negative; + = positive

In the PCR analysis of these strains, all of them (100 %) were confirmed as *P. caballi* using primer set-1 and 2. In PCR-1 (using primer set-1) analysis of 65 BALF and 17 lung samples, a 203 bp fragment specific for *P. caballi* was amplified in 40 (61.5 %) BALF and 14 (82.3 %) lung samples (Fig. 1). For PCR-2 (using primer set-2) analysis, 31 (47.9 %) BALF and 11 (64.7 %) lung samples exhibited positive bands for the 178 bp fragment specific for *P. caballi* (Fig. 2).

Eleven (16.9 %) BALF and 3 (17.6 %) lung samples, which exhibited positive bands in PCR-1 analysis, gave negative results in PCR-2. Conversely, PCR-1 positive 1 (1.5 %) BALF sample exhibited positive bands in PCR-2.

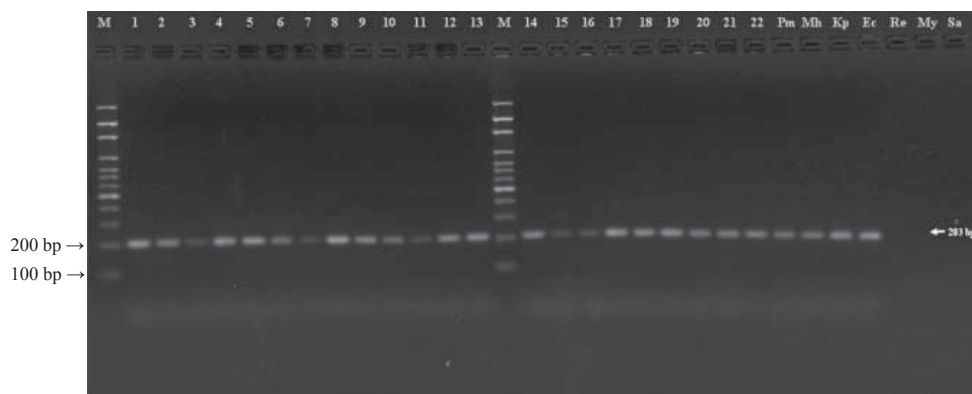


Fig. 1. Ethidium bromide-stained agarose gel electrophoresis of 16S rRNA-PCR-1 amplified products from BALF and lung samples, amplicon size of 203 bp (M: 100 bp size marker, lanes: 1-13: BALF samples, lanes 14-22 lung samples, Pm: *P. multocida*, Mh: *M. haemolytica*, Kp: *K. pneumoniae*, Ec: *E. coli*, My: *Mycoplasma* spp., Re: *R. equi*, Sa: *S. aureus*).

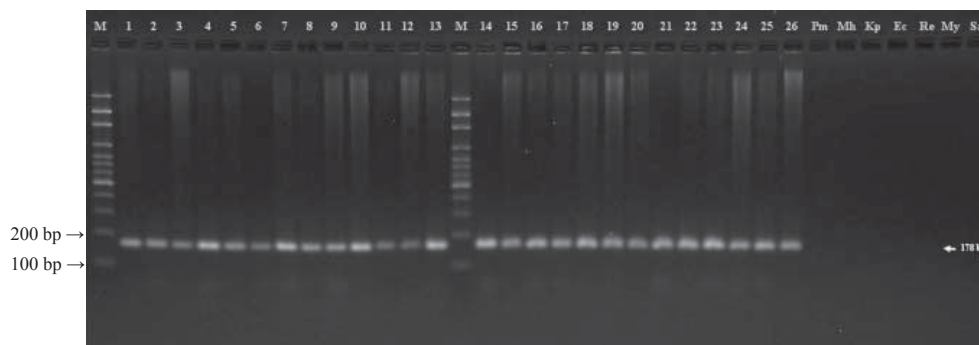


Fig. 2. Ethidium bromide-stained agarose gel electrophoresis of 16S rRNA-PCR-2 amplified products from BALF and lung samples, amplicon size of 178 bp (M: 100 bp size marker, lanes: 1-13: BALF samples, lanes 14-26 lung samples, Pm: *P. multocida*, Mh: *M. haemolytica*, Kp: *K. pneumoniae*, Ec: *E. coli*, My: *Mycoplasma* spp., Re: *R. equi*, Sa: *S. aureus*).

The sequence of primer set-1 matched with the genes of numerous bacteria species, such as *P. multocida*, *M. haemolytica*, *K. pneumoniae*, and *E. coli*, in NCBI-BLAST analysis. PCR-1 amplification with DNA from these bacteria amplified a 203 bp fragment. Amplification was not observed in PCR-2 analysis of the bacteria used as negative controls.

The 16S RNA gene sequences of *P. caballi* strains were 99 % identical to sequences deposited in the GenBank under accession numbers: DQ381154.1 (*P. caballi* strain NVSL 84679), NR_042881.1 (*P. caballi* strain ATCC 49197), AY362918.1 (*P. caballi* strain ATCC 49197), AY634654.1 (*P. caballi*), AY634649.1 (*P. caballi* isolate 23), AY178044.1 (Bisgaard Taxon 42), and AF224291.1 (*P. caballi* MCCM 00841). The same results were determined in gene sequence analysis of *P. caballi* culture positive BALF samples, but no matches were observed in amplicons culture-negative samples.

Discussion

Pneumonia is very common in 8 to 9-month-old foals. This infection is typically caused by bacteria that are found as opportunistic pathogens of the respiratory tract, such as *P. caballi*, *Streptococcus zooepidemicus*, *S. pneumoniae*, *Actinobacillus equuli*, and *Mycoplasma* spp. Most foals exhibit mixed infections that involve one or more of these bacteria (CHAPMAN et al., 2000).

P. caballi was initially isolated from horses (SCHLATER et al., 1989). Most strains were isolated from the respiratory tracts of horses (BISGARD, 1993; HAYAKAWA et al., 1993; BLACKALL et al., 1997; CHURCH et al., 1998). In addition, this organism has been isolated from the female reproductive tract (SCHLATER et al., 1989) and from aborted horse fetuses (BLACKALL et al., 1997), from horse-bite wounds in humans (BISGARD et al., 1991; ESCANDE et al., 1997), from clinical specimens of pigs (CHRISTENSEN et al., 2005 and 2006), from sheep with pleuropneumonia (EZZI et al., 2007), and from nasopharyngeal swabs from donkeys (GUTEMA et al., 2009). The isolation of this species suggests that *P. caballi* could play a significant role in the pathogenesis of those infections (BADA et al., 1993).

In the present study, 35 strains of *Pasteurella* spp. isolated from BALF and lung samples were identified as *P. caballi* based on their biochemical characteristics. All *P. caballi* isolates presented the same phenotypic characteristics as the strains reported in previous studies (SCHLATER et al., 1989; BISGARD et al., 1991; ESCANDE et al., 1997).

16S rRNA gene sequencing has been used as the gold standard for precise molecular identification and phylogenetic relationship studies of Pasteurellaceae species (DEY et al., 2007). In the current study, all isolates that were identified as *P. caballi* on the basis of their biochemical characteristics were confirmed using 16S rRNA-PCR-1, and PCR-2.

In culture-negative samples, PCR amplification of bacterial 16S rRNA gene has significantly improved the identification of bacterial agents (CLARIDGE, 2004). In this work, *P. caballi* was detected by PCR-1 in 40 and 14 *P. caballi* culture-negative BALF and lung samples, respectively, and 31 BALF and 11 lung samples by PCR-2. Thus, PCR-1 amplified the 16S rRNA gene sequence of *P. caballi* in 9 BALF and 3 lung samples which were *P. caballi* culture and PCR-2 negative. These amplifications may be due to

non-specific amplification of other bacterial DNA present in the samples. Also, non-specific amplifications were observed in PCR-1 analysis of negative control bacteria such as *P. multocida*, *M. haemolytica*, *K. pneumoniae*, and *E. coli*.

The most critical parameter for successful PCR is that designed primers must not amplify other genes in the mixture (ABD-ELSALAM, 2003; YE et al., 2012). The widely used Primer-BLAST offers a number of features that are not available in other software tools. The Primer-BLAST program consists of a module for generating candidate primer pairs and a module for checking the target specificity of the generated primer pairs. Another advantage of Primer-BLAST is its high detection sensitivity (ABD-ELSALAM 2003; DIEFFENBACH et al., 1993; YE et al., 2012). Since Primer 3, the other software used in this study, does not perform target analysis, it is typically necessary to test primer specificity using additional tools.

The gene sequences of *P. caballi* strains in this study were 99 % identical to 6 different *P. caballi* strains in the NCBI database. According to the gene sequence analysis of *P. caballi* culture positive and negative samples, primer set-2 is very sensitive and specific for direct use on clinical samples.

Conclusions

Our results demonstrated that *P. caballi* is very common on the mucus membranes of the respiratory tract of Arabian foals. The presence of *P. caballi* in the respiratory tract of foals does not describe the infection. However, as a result of stress factors, these bacteria can cause infection or predispose the animal to other bacteria and viruses infections. The primer set-2, which was designed to amplify a segment of the 16S rRNA gene region of *P. caballi*, is very useful for the confirmation of *P. caballi* isolates by PCR. Also, primer set-2 is appropriate for directly detecting *P. caballi* in BALF and lung samples from foals, but primer set-1 is not suitable for this.

Ethical Statement

This research was approved (date 15/02/2008 and 2008/010) by the Ethics Committee of the Faculty of Veterinary Medicine at the University of Selcuk in Konya, Turkey.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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SAŽETAK

Pasteurella caballi (*P. caballi*) izdvojena je i identificirana u uzorcima bronhoalveolarnog ispirka i tkiva pluća ždrebadi čistokrvnoga arapskoga konja uobičajenim mikrobiološkim postupcima. Potom je istražena vrijednost dvaju različitih kompleta početnica za dokaz i potvrdu vrste *P. caballi* lančanom reakcijom polimerazom. Početnica 1 za dokaz gena 16S rRNA *P. caballi* bila je pripremljena upotrebom programa Primer 3, a početnica 2 upotrebom programa Primer-BLAST. Lančana reakcija polimerazom rabljena je za potvrdu sojeva vrste *P. caballi* i za njezin izravni dokaz u uzorcima bronhoalveolarnog ispirka i plućnoga tkiva. Ukupno je bilo izdvojeno 35 izolata *Pasteurella* spp. iz 25 (38,4 %) od 65 pretraženih uzoraka bronhoalveolarnog ispirka i 10 (58,8 %) izolata iz 17 uzoraka plućnoga tkiva. Ti su sojevi bili identificirani na osnovi poznatih mikrobioloških i biokemijskih značajki. Početnice 1 i 2 pokazale su 100 %-tnu osjetljivost za identifikaciju uzgojenih sojeva. Međutim, početnica set-1 za dokaz vrste *P. caballi* bila je slabije specifičnosti od početnice set-2 pri pretrazi uzoraka bronhoalveolarnog ispirka i plućnoga tkiva. Osjetljivost i specifičnost početnice 2 bila je potvrđena analizom genske sekvencije. Istraživanje pokazuje da početnica set-2 za lančanu reakciju polimerazom za dokaz 16S rRNA pruža brz i točan alat za dokazivanje i potvrdu izolata vrste *P. caballi* u bronhoalveolarnom ispirku i plućnom tkivu ždrebadi.

Ključne riječi: arapski konj, ždrebac, *Pasteurella caballi*, lančana reakcija polimerazom, Primer-BLAST, Primer 3, 16S rRNA
