NOTE *Bacteriology*

Dominant Obligate Anaerobes Revealed in Lower Respiratory Tract Infection in Horses by 16S rRNA Gene Sequencing

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ABSTRACT. Obligate anaerobes are important etiological agents in pneumonia or pleuropneumonia in horses, because they are isolated more commonly from ill horses that have died or been euthanized than from those that survive. We performed bacterial identification and antimicrobial susceptibility testing for obligate anaerobes to establish effective antimicrobial therapy. We used 16S rRNA gene sequencing to identify 58 obligate anaerobes and compared the results with those from a phenotypic identification kit. The identification results of 16S rRNA gene sequencing were more reliable than those of the commercial kit. We concluded that genera *Bacteroides* and *Prevotella*—especially *B. fragilis* and *P. heparinolytica*—are dominant anaerobes in lower respiratory tract infection in horses; these organisms were susceptible to metronidazole, imipenem and clindamycin.

KEY WORDS: 16S rRNA gene sequencing, antimicrobial susceptibility test, lower respiratory tract infection, obligate anaerobe, Thoroughbred.

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Obligate anaerobes are normal inhabitants of the oral cavity and intestinal tract of horses, but they are also sometimes isolated from horses with signs of respiratory infection or enteritis, or with abscesses or other lesions [4, 13, 17]. In particular, obligate anaerobes are secondary etiological agents in lower respiratory tract disease, and survival rates are significantly lower in horses from which obligate anaerobes are isolated than in horses from which anaerobes are not isolated [20, 23]. Because the pathogenicity or antimicrobial susceptibility patterns of bacteria vary depending on the species and genus [12, 22], accurate bacterial identification should be performed for selection of appropriate agents and assessment of clinical significance.

Because not all veterinary bacteria are covered by commercial bacterial identification kits based on phenotypic methods, many veterinary isolates have not been correctly identified by these kits [11, 19]; such isolates are often identified instead by using 16S rRNA gene sequencing [6, 11].

Our objective was to use 16S rRNA gene sequencing to identify obligate anaerobes from horses with lower respiratory tract infection and to compare the results with those of a commercial phenotypic identification kit. In addition, we elucidated the antimicrobial susceptibility patterns of these obligate anaerobes to 11 antimicrobial agents. The overall goal was to enable better choices of antimicrobials to be made for the treatment of anaerobic lower respiratory tract infection in horses.

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The 58 obligate anaerobes used in this study were isolated between 2001 and 2010 from clinical specimens from 31 Thoroughbred horses with signs of lower respiratory tract infection. All specimens were incubated anaerobically on 5% horse blood agar at 37°C for 48 hr. When a few kinds of obligate anaerobes were isolated from a single horse, one to four kinds of dominant isolates from each specimen were selected for the experiment. Thirty-six (62.1%) of the 58 isolates were obtained from bronchoalveolar lavage fluid (BALF), 13 (22.4%) were from pleural effusions and five (8.6%) from lung abscesses. The remaining four (6.9%) were isolated from horses with signs of pneumonia, but their origin was not recorded.

The 16S rRNA gene sequencing was performed in accordance with published methods [15, 16]. The sequences obtained were compared with published 16S rRNA gene sequences in the database of the National Center for Biotechnology Information by using BLAST software (http://blast.ncbi.nlm.nih.gov/) [3]. Sequence-based identifications were interpreted according to the criteria of the Clinical and Laboratory Standards Institute (CLSI), namely species level, ≥99.0% identity to the type strain; genus level, ≥97.0% to<99.0% identity to the type strain; novel genus or species, or both, <97.0% identity to the type strain [9]. In addition, because the CLSI guideline recommends constructing a phylogenetic tree for a strain that has identity of <97.0% to the type strain, we used MEGA 5.03 software [26] to create a phylogenetic tree by using the neighbor-joining method with 1,000 bootstrap replicates.

Forty-four of 58 (75.9%) isolates were discriminated to genus level by 16S rRNA gene sequencing and phylogenetic analysis, and 37 of 58 (63.8%) isolates were identified to species level (Table 1). Although the isolates JAn-33 and JAn-35 to −39 were not discriminated as members of the genus *Prevotella* according to the CLSI criteria (identity to the type strain<97.0%), according to the phylogenetic analysis, these

Table 1. Comparison of identification results of 16S rRNA gene sequencing with those of Rapid ID 32A

Isolate	Derived from	16S rRNA gene sequencing (%) [a, b]	Rapid ID 32A (identification score [% id]) [a, c]
JAn-1	BALF [d]	***Bacteroides fragilis*** (99.0)	***Bacteroides fragilis*** (98.3)
JAn-2	BALF	***Bacteroides fragilis*** (99.6)	***Bacteroides fragilis*** (99.9)
JAn-3	BALF	***Bacteroides fragilis*** (99.7)	***Bacteroides fragilis*** (98.1)
JAn-4	BALF	***Bacteroides fragilis*** (99.3)	***Bacteroides fragilis*** (99.7)
JAn-5	BALF	***Bacteroides fragilis*** (99.5)	***Bacteroides fragilis*** (99.1)
JAn-6	BALF	***Bacteroides fragilis*** (99.7)	***Bacteroides fragilis*** (99.3)
JAn-7	BALF	***Bacteroides fragilis*** (99.6)	***Bacteroides fragilis*** (99.3)
JAn-8	BALF	***Bacteroides fragilis*** (99.7)	***Bacteroides fragilis*** (98.3)
JAn-9	BALF	***Bacteroides fragilis*** (99.6)	***Bacteroides fragilis*** (99.3)
JAn-10	BALF	***Bacteroides fragilis*** (99.6)	***Bacteroides fragilis*** (99.7)
JAn-11	BALF	***Bacteroides fragilis*** (99.6)	***Bacteroides fragilis*** (99.9)
JAn-12	pleural effusion	***Bacteroides fragilis*** (99.7)	***Bacteroides fragilis*** (93.5)
JAn-13	lung abscess	***Bacteroides fragilis*** (99.8)	***Bacteroides fragilis*** (96.9)
JAn-14	BALF	***Bacteroides fragilis*** (99.7)	***Bacteroides fragilis*** (97.0)
JAn-15	BALF	***Bacteroides fragilis*** (99.6)	***Bacteroides fragilis*** (99.9)
JAn-16	BALF	***Bacteroides thetaiotaomicron*** (99.6)	***Bacteroides thetaiotaomicron*** (99.8)
JAn-17	pleural effusion	***Bacteroides helcogenes*** (99.7)	***Bacteroides capillosus*** (96.9)
JAn-18	pleural effusion	***Bacteroides xylanisolvens*** (99.6)	***Bacteroides uniformis*** (94.6)
JAn-19	lung abscess	***Bacteroides pyogenes*** (99.7)	*Prevotella oralis* (55.0)/*P. denticola* (40.3)
JAn-20	BALF	***Bacteroides pyogenes*** (99.9)	*Prevotella denticola* (57.5)/*P. oralis* (36.6)
JAn-21	pleural effusion	***Bacteroides pyogenes*** (99.3)	***Prevotella melaninogenica*** (95.1)
JAn-22	BALF	***Bacteroides pyogenes*** (99.9)	***Prevotella denticolla*** (80.9)/*P. melaninogenica* (6.4)/*P. oralis* (6.4)/*P. loescheii* (5.9)
JAn-23	BALF	***Prevotella heparinolytica*** (99.7)	*Bacteroides uniformis* (79.8)/*B. ovatas* (18.7)
JAn-24	pleural effusion	***Prevotella heparinolytica*** (99.6)	*Bacteroides uniformis* (79.8)/*B. ovatas* (18.7)
JAn-25	BALF	***Prevotella heparinolytica*** (99.7)	*Bacteroides uniformis* (79.8)/*B. ovatas* (18.7)
JAn-26	BALF	***Prevotella heparinolytica*** (99.6)	*Bacteroides uniformis* (67.3)/*B. ovatas* (31.8)
JAn-27	BALF	***Prevotella heparinolytica*** (99.7)	*Bacteroides uniformis* (79.8)/*B. ovatas* (18.7)
JAn-28	BALF	***Prevotella heparinolytica*** (99.6)	*Bacteroides uniformis* (79.8)/*B. ovatas* (18.7)
JAn-29	lung abscess	***Prevotella heparinolytica*** (99.7)	*Bacteroides ovatas* (53.6)/*B. uniformis* (44.2)
JAn-30	BALF	***Prevotella heparinolytica*** (99.7)	*Bacteroides ovatas* (71.2)/*B. eggerthii* (14.8)/*B. uniformis* (12.5)
JAn-31	pleural effusion	***Prevotella heparinolytica*** (99.5)	*Bacteroides ovatas* (53.6)/*B. uniformis* (44.2)
JAn-32	BALF	***Prevotella dentasini*** (99.9)	unidentifiable
JAn-33	BALF	*Prevotella salivae* (92.7) [f]	***Bacteroides capillosus*** (96.5)
JAn-34	BALF	*Prevotella heparinolytica* (97.0)	unidentifiable
JAn-35	BALF	*Prevotella baroniae* (91.0) [f]	*Prevotella loescheii* (55.4)/ *P. oralis* (40.0)
JAn-36	BALF	*Prevotella salivae* (91.6) [f]	***Bacteroides capillosus*** (99.9)
JAn-37	pleural effusion	*Prevotella oris* (92.1) [f]	unidentifiable
JAn-38	pleural effusion	*Prevotella salivae* (92.1) [f]	unidentifiable
JAn-39	BALF	*Prevotella bivia* (94.2) [f]	***Bacteroides ovatus*** (82.8)/ *B. uniformis* (11.5)
JAn-40	unplaceable [e]	***Clostridium argentinense*** (99.4)	unidentifiable
JAn-41	pleural effusion	*Clostridium coccoides* (93.2) [g]	unidentifiable
JAn-42	pleural effusion	*Paraprevotella clara* (91.7) [g]	unidentifiable
JAn-43	unplaceable [e]	***Clostridium perfringens*** (99.9)	***Clostridium perfringens*** (99.9)
JAn-44	unplaceable [e]	***Clostridium perfringens*** (99.9)	***Clostridium perfringens*** (99.9)
JAn-45	unplaceable [e]	*Eubacterium sulci* (93.0) [g]	unidentifiable
JAn-46	BALF	*Eubacterium saburreum* (93.1) [g]	unidentifiable
JAn-47	BALF	***Clostridium orbiscindens*** (100)	unidentifiable
JAn-48	BALF	*Clostridium coccoides* (93.3) [g]	***Clostridium clostlidiforme*** (86.0)/*C. beijerinckii*, *C. butyricum* (13.8)
JAn-49	pleural effusion	*Eubacterium sulci* (92.9) [g]	unidentifiable
JAn-50	lung abscess	*Eubacterium saburreum* (93.1) [g]	***Clostridium clostlidiforme*** (99.9)
JAn-51	BALF	*Clostridium coccoides* (93.1) [g]	unidentifiable
JAn-52	pleural effusion	*Eubacterium saburreum* (93.3) [g]	***Clostridium clostlidiforme*** (99.9)
JAn-53	BALF	*Clostridium coccoides* (93.1) [g]	unidentifiable
JAn-54	lung abscess	***Clostridium difficile*** (99.9)	*Clostridium difficile* (69.2)/ *C. bifermentans* (18.6)/ *C. glycolicum* (8.0)
JAn-55	BALF	*Clostridium aminophilum* (95.2) [g]	unidentifiable
JAn-56	BALF	*Eubacterium rectale* (93.1) [g]	unidentifiable
JAn-57	BALF	*Clostridium coccoides* (93.2) [g]	unidentifiable
JAn-58	pleural effusion	*Eubacterium saburreum* (91.2) [g]	***Clostridium histolyticum*** (95.0)

a) Strains identified to species and genus level are shown in bold and underlined, respectively. b) 16S rRNA sequence identity to the type strain submitted to GenBank. c) "Low discrimination", "Not reliable" and "Unacceptable" results are described here as "Unidentifiable." d) BALF: bronchoalveolar lavage fluid. e) These strains were isolated from horses with signs of pneumonia, but their origin was not recorded. f) According to the results of the phylogenetic analysis, these double-underlined isolates are strains related to *Prevotella*. g) These strains were not identifiable to genus level according to either identity to the type strain or the results of the phylogenetic analysis.

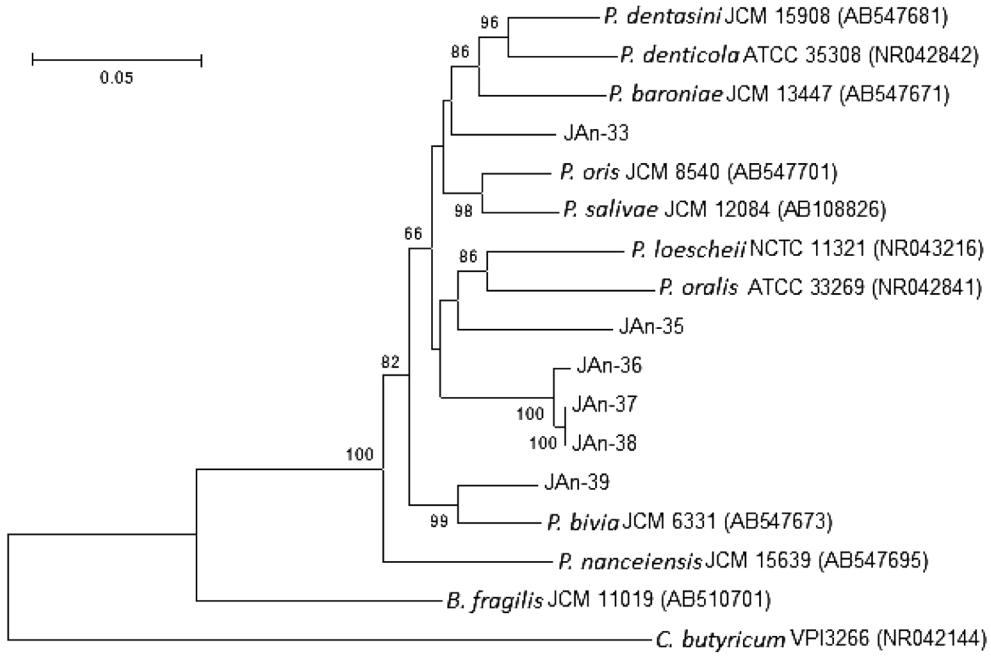

Fig. 1. Neighbor-joining phylogenetic tree derived from 16S rRNA gene sequences, showing the positions of JAn-33 and −35 to −39 within the genus *Prevotella*. Accession numbers of published sequences of *Prevotella* species are shown in parentheses. Bootstrap values (>50%) based on 1,000 replications are shown at branch nodes. Bar, 0.05 substitutions per nucleotide position.

six isolates belonged to a cluster of *Prevotella* (Fig. 1). We therefore considered that the six isolates were strains related to *Prevotella*. Twenty-two of 58 (37.9%) isolates belonged to the genus *Bacteroides*, and members of *Bacteroides* were the obligate anaerobes most commonly isolated. Of the *Bacteroides*, *B. fragilis* (15 isolates) was the predominant species. Seventeen of 58 (29.3%) isolates belonged to the genus *Prevotella*, including the six related isolates. Members of *Prevotella* were the second most commonly isolated obligate anaerobes. Among members of *Prevotella*, *P. heparinolytica* (nine isolates) was the dominant species. Five of 58 (8.6%) isolates belonged to the genus *Clostridium*. As for the remaining 14 isolates, 16S rRNA gene sequences of seven, six and one isolates were the most similar to those of *Eubacterium*, *Clostridium* and *Paraprevotella*, respectively. However, the identities of the 14 isolates (91.2−95.2%) were lower than the CLSI criteria (<97.0%). Unlike the strains related to *Prevotella*, the 14 strains did not belong to certain cluster according to the phylogenetic analysis (data not shown). Therefore, these 14 strains were not identifiable to genus level in terms of both identity to the type strain and the results of the phylogenetic analysis.

Previous studies have reported that the major obligate anaerobes obtained from horses with signs of lower respiratory infection are *Bacteroides* spp. and *Clostridium* spp. [24]; anaerobic cocci and *Eubacterium fossor* [20]; and *Clostridium perfringens*, *Bacteroides fragilis* and *Bacteroides oralis* [4]. In agreement with our findings, these studies reported that strains of *Bacteroides* were among the dominant anaerobes in horses with signs of lower respiratory tract infection. However, in disagreement, they did not consider *Prevotella* a major causative anaerobe [4, 20, 24]. Tracheobronchial aspirates were used mainly in these previous works [4, 20, 24]. In contrast, the specimens we used were predominantly BALFs, which are generally considered sterile in healthy horses. Unlike at bronchial sites, many bacteria, including transient bacteria, have been found at tracheal sites [14], suggesting that samples from bronchial sites are more suitable than those from tracheal sites for detecting causative or secondarily invasive obligate anaerobes. Therefore, we concluded that our identification results would be more reliable than those of the above-mentioned past studies in terms of the sampling sites used. We conclude that strains of *Bacteroides* and *Prevotella*—in particular *B. fragilis* and *P. heparinolytica*—are the most important obligate anaerobes in lower respiratory tract infection in horses.

A commercial bacterial identification test kit (Rapid ID 32A, SYSMEX bioMérieux, Tokyo, Japan) was used in accordance with the manufacturer's instructions to compare the identification results from 16S rRNA gene sequencing with those from the identification test kit. This kit uses, for species level, identification score (% id) ≥80 and for genus level, % id of each bacterial species <80 and total % id of some bacterial species belonging to the same genus≥80.

Rapid ID 32A identified 42 of 58 (72.4%) isolates to genus level and 29 of 58 (50.0%) isolates to species level (Table 1). Thirty, seven and five of the 42 isolates were identified as genus *Bacteroides*, genus *Clostridium* and genus

Table 2. Antimicrobial susceptibilities of 58 obligate anaerobes to 11 antimicrobial agents

Antimicrobial	Percent (%)								
	Total anaerobes (n=58)			*Bacteroides* spp. (n=22)			*Prevotella* spp. and related strains (n=17)		
	S [a)]	I [a)]	R [a)]	S	I	R	S	I	R
Penicillin	48.3	5.2	46.6	9.1	0	90.9	76.5	5.9	17.6
Ampicillin	51.7	3.4	44.8	9.1	0	90.9	82.4	0	17.6
Cephalothin	58.6	1.7	39.7	13.6	0	86.4	82.4	5.9	11.8
Ceftiofur	48.3	6.9	44.8	9.1	13.6	77.3	88.2	5.9	5.9
Imipenem	98.3	0	1.7	100	0	0	100	0	0
Clindamycin	93.1	0	6.9	100	0	0	100	0	0
Metronidazole	93.1	0	6.9	100	0	0	100	0	0
Minocycline	91.4	8.6	0	100	0	0	76.5	23.5	0
Doxycycline	82.8	15.5	1.7	86.4	13.6	0	70.6	23.5	5.9
Enrofloxacin	19	46.6	34.5	13.6	72.7	13.6	5.9	58.8	35.3
Moxifloxacin	82.8	15.5	1.7	100	0	0	94.1	5.9	0

a) S: susceptible; I: intermediate; R: resistant.

Prevotella, respectively. Twenty-two of 58 (37.9%) isolates had the same identification results to genus level by both identification methods, and 18 of 58 (31.0%) isolates had the same identification results to species level by the two methods. The identification results for the remaining 36 (62.1%) isolates differed between the two methods. In particular, all isolates identified as *P. heparinolytica* by 16S rRNA gene sequencing (JAn-23 to −31) were identified as strains associated with *B. uniformis* by Rapid ID 32A.

In agreement with our results, some identification kits have previously identified *P. heparinolytica*, including the type strain and clinical strains, as a strain associated with *B. uniformis* [2]. Phenotypic characterization by using identification test kits often results in unreliable identification, especially in the case of veterinary isolates [1, 6, 11, 19], and 16S rRNA gene sequencing has often provided reliable identifications of these otherwise unidentifiable strains [1, 6]. These findings combined indicate that 16S rRNA gene sequencing is a more reliable tool for identifying obligate anaerobes derived from horses than are kits based on phenotypic characterization. Hereafter, unless otherwise noted, the species names used here for isolates are those from the 16S rRNA gene sequencing.

Antimicrobial susceptibility patterns vary depending on the species or genus [22], and appropriate antimicrobial therapy should be conducted on the basis of the antimicrobial susceptibility patterns of each bacterium. Therefore, to elucidate the antimicrobial susceptibility patterns of the obligate anaerobes, we measured the minimum inhibitory concentrations (MICs) of antimicrobials by using a customized commercial panel (Eiken Chemical Co., Ltd., Tokyo, Japan) including penicillin, ampicillin, cephalothin, ceftiofur, imipenem, clindamycin, metronidazole, minocycline, doxycycline, enrofloxacin and moxifloxacin. The results were interpreted according to CLSI guidelines [8, 10]. The CLSI guideline recommends using one or more strains in four recommended anaerobes for quality control of the procedure [8]. In this study, we used *Bacteroides fragilis* ATCC 25285 and *Bacteroides thetaiotaomicron* ATCC 29741 as quality control strains. MICs of the antimicrobial agents for these two strains were within the quality control limits published in CLSI standard M100-S22 [10].

Antimicrobial susceptibility patterns differed among the species (Table 2). *Bacteroides* isolates tended to be resistant to most of the β-lactam antimicrobials, including penicillin, ampicillin, cephalothin and ceftiofur. In contrast, most *Prevotella* isolates were susceptible to these antimicrobials. All *Bacteroides* and *Prevotella* isolates, including the related strains, were susceptible to imipenem, clindamycin and metronidazole.

In agreement with previous studies in human medicine [21], most of our *Bacteroides* isolates were resistant to most β-lactams. In contrast, most *Prevotella* isolates were susceptible to β-lactams, although previous studies have reported that many *Prevotella* isolates in human medicine are resistant to penicillin [5, 21]. Therefore, β-lactams may be effective against equine respiratory tract infections caused by *Prevotella* strains. Although metronidazole has been regarded as a useful antimicrobial for the treatment of obligate anaerobes in equine medicine [7, 18, 25], metronidazole-resistant *Bacteroides* and *Prevotella* strains have recently been reported in humans [22]. All of our *Bacteroides* and *Prevotella* isolates were susceptible to metronidazole, suggesting that this drug may still be useful against obligate anaerobes in horses.

To our knowledge, this is the first report to identify the obligate anaerobes in lower respiratory tract infections in horses by using 16S rRNA gene sequencing. The genera *Bacteroides* and *Prevotella*—especially *B. fragilis* and *P. heparinolytica*—were revealed to be the dominant obligate anaerobes. These reliable identifications and antimicrobial susceptibility patterns should result in better choices of antimicrobials for treating anaerobic lower respiratory tract infections in horses.

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