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.

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 MEGA5.03 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 JAn-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...
| Isolate | Derived from | 16S rRNA gene sequencing (%) | Rapid ID 32A (identification score [% id]) |
|---------|--------------|------------------------------|-------------------------------------|
| JAN-1   | BALF         | 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 (98.3)         |
| JAN-8   | BALF         | Bacteroides fragilis (99.6) | Bacteroides fragilis (99.3)         |
| JAN-9   | BALF         | Bacteroides fragilis (99.6) | Bacteroides fragilis (99.9)         |
| JAN-10  | BALF         | Bacteroides fragilis (99.6) | Bacteroides fragilis (97.7)         |
| 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) | Bacteroides uniformis (94.6)       |
| JAN-20  | BALF         | Bacteroides pyogenes (99.9) | Bacteroides uniformis (94.6)       |
| JAN-21  | pleural effusion | Bacteroides pyogenes (99.3) | Bacteroides uniformis (94.6)       |
| JAN-22  | BALF         | Bacteroides pyogenes (99.9) | Bacteroides uniformis (94.6)       |
| JAN-23  | BALF         | Prevotella heparinolytica (99.7) | Bacteroides uniformis (99.7)       |
| JAN-24  | pleural effusion | Prevotella heparinolytica (99.6) | Bacteroides uniformis (99.7)       |
| JAN-25  | BALF         | Prevotella heparinolytica (99.7) | Bacteroides uniformis (99.7)       |
| JAN-26  | BALF         | Prevotella heparinolytica (99.6) | Bacteroides uniformis (99.7)       |
| JAN-27  | BALF         | Prevotella heparinolytica (99.7) | Bacteroides uniformis (99.7)       |
| JAN-28  | BALF         | Prevotella heparinolytica (99.6) | Bacteroides uniformis (99.7)       |
| JAN-29  | lung abscess  | Prevotella heparinolytica (99.7) | Bacteroides uniformis (99.7)       |
| JAN-30  | BALF         | Prevotella heparinolytica (99.7) | Bacteroides uniformis (99.7)       |
| JAN-31  | pleural effusion | Prevotella heparinolytica (99.5) | Bacteroides uniformis (99.7)       |
| JAN-32  | BALF         | Prevotella denticini (99.9) | Bacteroides uniformis (99.7)       |
| JAN-33  | BALF         | Prevotella salivae (92.7) | Bacteroides uniformis (99.7)       |
| JAN-34  | BALF         | Prevotella helicobacter (97.0) | Bacteroides uniformis (99.7)       |
| JAN-35  | BALF         | Prevotella baronae (91.0) | Bacteroides uniformis (99.7)       |
| JAN-36  | BALF         | Prevotella salivae (91.6) | Bacteroides uniformis (99.7)       |
| JAN-37  | pleural effusion | Prevotella oris (92.1) | Bacteroides uniformis (99.7)       |
| JAN-38  | pleural effusion | Prevotella salivae (92.1) | Bacteroides uniformis (99.7)       |
| JAN-39  | BALF         | Prevotella brevis (94.2) | Bacteroides uniformis (99.7)       |
| JAN-40  | unplaceable  | Clostridium argentinense (99.4) | Bacteroides uniformis (99.7)       |
| JAN-41  | pleural effusion | Clostridium coccoidei (93.2) | Bacteroides uniformis (99.7)       |
| JAN-42  | pleural effusion | Paraprevotella clara (91.7) | Bacteroides uniformis (99.7)       |
| JAN-43  | unplaceable  | Clostridium perfringens (99.9) | Bacteroides uniformis (99.7)       |
| JAN-44  | unplaceable  | Clostridium perfringens (99.9) | Bacteroides uniformis (99.7)       |
| JAN-45  | unplaceable  | Clostridium perfringens (99.9) | Bacteroides uniformis (99.7)       |
| JAN-46  | BALF         | Eubacterium salubris (93.0) | Bacteroides uniformis (99.7)       |
| JAN-47  | BALF         | Clostridium orbiscindens (100) | Bacteroides uniformis (99.7)       |
| JAN-48  | BALF         | Clostridium coccoidei (93.3) | Bacteroides uniformis (99.7)       |
| JAN-49  | pleural effusion | Eubacterium salubris (92.9) | Bacteroides uniformis (99.7)       |
| JAN-50  | lung abscess  | Eubacterium salubris (93.1) | Bacteroides uniformis (99.7)       |
| JAN-51  | BALF         | Clostridium coccoidei (93.1) | Bacteroides uniformis (99.7)       |
| JAN-52  | pleural effusion | Eubacterium salubris (93.5) | Bacteroides uniformis (99.7)       |
| JAN-53  | BALF         | Clostridium coccoidei (93.1) | Bacteroides uniformis (99.7)       |
| JAN-54  | lung abscess  | Clostridium difficile (99.9) | Bacteroides uniformis (99.7)       |
| JAN-55  | BALF         | Clostridium omniminutum (95.2) | Bacteroides uniformis (99.7)       |
| JAN-56  | BALF         | Eubacterium rectale (93.1) | Bacteroides uniformis (99.7)       |
| JAN-57  | BALF         | Clostridium coccoidei (93.2) | Bacteroides uniformis (99.7)       |
| JAN-58  | pleural effusion | Eubacterium salubris (91.2) | Bacteroides uniformis (99.7)       |

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.
Anaerobes from Equine Respiratory Tract

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* flossor [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...
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 (JA-n-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. These reliable identifications and antimicrobial susceptibility patterns should result in better choices of antimicrobials for treating anaerobic lower respiratory tract infections in horses.

Antimicrobial susceptibility patterns varied 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, cefotiofur, 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 cefotiofur. 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.

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

| Antimicrobial | 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 | 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 |
| Cefotiofur   | 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.
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