Application of loop-mediated isothermal amplification to detect seven groups of pathogens causing lower respiratory bacterial infection in Thoroughbred racehorses and its antimicrobial susceptibility patterns

(競走馬の細菌性下気道疾患の診断における LAMP 法の応用
および分離株の薬剤感受性に関する研究)

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CONTENTS

GENERAL INTRODUCTION

Bacterial infections of the lower respiratory tract (e.g., pneumonia and pleuropneumonia) are common in adult horses, and one of the causes of pneumonia in horses is transport for long periods [61]. The clinical signs vary depending on the disease severity; the most common signs are fever, anorexia, cough, tachypnea, bilateral nasal discharge, and respiratory distress [68]. Bacterial pneumonia or pleuropneumonia occasionally leads to a critical illness, with mortality rates as high as 56% [65, 66, 74, 75].

Various bacteria, including Gram-positive, Gram-negative, and anaerobic bacteria, can be isolated from respiratory tract specimens in affected horses, and mixed bacterial infection is commonly observed [74, 75]. The dominant pathogenic bacterium of pneumonia in adult horses is *Streptococcus equi* subsp. *zooepidemicus* [75], which is a beta-hemolytic Gram-positive Lancefield group C bacterium found in a wide range of species, including horses, pigs, monkeys, dogs, and humans [31, 64, 72, 79]. *Streptococcus zooepidemicus* is part of the normal bacterial microflora of the upper respiratory tract and caudal reproductive tract of horses [79], and is also an opportunistic pathogen associated with a wide variety of diseases in horses, including pneumonia, mastitis, placentitis, and endometritis [16, 39, 44, 52]. Previous studies have reported that *S. zooepidemicus* is not a homogeneous clonal population but consists of a wide diversity of strain types [7, 59], and furthermore that differences in genotype or phenotype of *S. zooepidemicus* might affect pathogenicity [45, 67]. Although *S. zooepidemicus* is a predominant pathogen in equine lower respiratory tract infection, microbial substitution occasionally occurs following the administration of antimicrobials. The other bacteria most frequently isolated before or after microbial

substitution include Pasteurellaceae, *Escherichia coli*, *Klebsiella* spp., and obligate anaerobes [65, 75]. In particular, obligate anaerobes and *E. coli* tend to be responsible for secondary bacterial pneumonia [65]. The fatality rate from pleuropneumonia caused by these agents and secondary to bacterial pneumonia is high [65, 74].

Previous studies have reported that the major obligate anaerobes isolated from horses with signs of lower respiratory tract infection are *Bacteroides* spp. and *Clostridium* spp. [75]; anaerobic cocci and *Eubacterium fossor* [65]; and *Clostridium perfringens*, *Bacteroides fragilis*, and *Bacteroides oralis* [11]. Tracheobronchial aspirates were used mainly in these previous works. However, many other types of bacteria, including transient bacteria, were found at tracheal sites in another study [34]. In other studies, 69 of 148 healthy Thoroughbred horses had bacteria in their tracheas at concentrations of 1 to 10^4 CFU/ml [18] and the average concentration of bacteria in tracheal washes from Standardbred horses with no clinical signs was 1.49×10^4 CFU/ml [43]. It is unclear whether transient bacteria were included in the tracheobronchial aspirates in those previous studies that found obligate anaerobes. Therefore, accurate identification of obligate anaerobes is needed in lower respiratory tract infection in adult horses to select appropriate antimicrobials.

Standard therapies for bacterial pneumonia or pleuropneumonia are administration of antimicrobial and nonsteroidal anti-inflammatory agents, and pleural drainage and lavage[68]. From an antimicrobial perspective it is reasonable to treat the disease with β-lactams such as penicillin or cephalosporin, because *S. zooepidemicus*, being considered the most likely causative pathogen in the early stages of bronchopneumonia in adult horses, is generally susceptible to the β-lactams [73]. In some cases, a combination of trimethoprim and sulfonamide, gentamicin, and fluoroquinolones such as enrofloxacin can be used for bronchopneumonia caused by

aerobic or facultative anaerobic bacteria in adult horses [28, 51, 68]. Metronidazole is recommended for treatment of pneumonia caused by obligate anaerobes, because a numbers of obligate anaerobes produce β-lactamases and are potentially resistant to many β-lactams [68]. However, the antimicrobial susceptibility patterns of each bacterium could vary depending on factors such as geographic location or time of year [5, 29, 40]. Veterinary practitioners should therefore consider suitable regimens based on the antimicrobial susceptibility patterns in each country or region. Moreover, because microbial substitution occasionally occurs following the administration of antimicrobials, it is important to determine the primary causative agents or microbial substitutions, or both, rapidly and to select the antimicrobials used accordingly to suit the causative bacteria and thus prevent progression of infection.

Veterinary practitioners, however, are often faced with difficulties in conducting rapid diagnosis and choosing an appropriate antimicrobial. Isolating and identifying some bacteria such as obligate anaerobes is time consuming, and conventional polymerase chain reaction (PCR) assays, which can detect pathogens more quickly than can bacterial culture, need special equipment (e.g., a thermal cycler). Therefore, treatment of bacterial infections caused by obligate anaerobes is frequently empiric.

The loop-mediated isothermal amplification (LAMP) method was developed as a new nucleic acid amplification method [60]. This method employs a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA [60]. The LAMP reaction can be accelerated by using additional primers, termed loop primers [55]. The mechanism of the LAMP reaction consists of three steps: production of starting material, cycling amplification, and recycling [60, 80]. The LAMP reaction can be performed under isothermal conditions

with no special equipment, and its reaction temperature is between 60 and 65 °C—the optimal temperature for *Bst* DNA polymerase. Various methods are available to detect products amplified by LAMP methods (e.g., the naked eye or use of a UV trans-illuminator, gel electrophoresis, and intercalating dyes [27, 60, 86]). It is generally considered that LAMP amplifies nucleic acids with high speed, specificity, and efficiency [27, 86]. In recent years, LAMP has been applied clinically for rapid detection of various pathogens [56, 58], and the LAMP method is potentially valuable as a diagnostic tool for rapid diagnosis of bacterial infections of the lower respiratory tract in affected horses in less well-equipped laboratories.

The author conducted the following consecutive studies, the goal of which was to enable veterinary practitioners to identify causative bacteria promptly and to select appropriate antimicrobials. In CHAPTER 1, the author describes my use of 16S rRNA gene sequencing to precisely identify obligate anaerobes isolated from the lower respiratory tracts of ill horses. In CHAPTER 2, the author describes my development of five LAMP methods to detect major pathogens causing bacterial infections of the lower respiratory tract in Thoroughbred horses and compare the clinical efficacies of three simple DNA extraction methods. In addition, the author confirms the clinical efficacies of seven LAMP methods, namely the five new LAMPs and two previously described LAMPs. Last, in CHAPTER 3, the author reveals the antimicrobial susceptibility patterns of those pathogenic bacteria for which LAMP detection methods have been developed.

CHAPTER 1

Dominant obligate anaerobes revealed in lower respiratory tract infection in horses by 16S rRNA gene sequencing

Summary

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. The author performed bacterial identification for obligate anaerobes derived from ill horses with lower respiratory tract infection to reveal causative pathogens in Japan. The author 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. The author concluded that genera *Bacteroides* and *Prevotella*—especially *B. fragilis* and *P. heparinolytica*—are dominant anaerobes in lower respiratory tract infection in horses.

Introduction

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 [11, 30, 41]. 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 [65, 74]. Because the pathogenicity or antimicrobial susceptibility patterns of bacteria vary depending on the species and genus [29, 70], 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 [25, 63]; such isolates are often identified instead by using 16S rRNA gene sequencing [14, 25].

My objective was to identify obligate anaerobes from horses with lower respiratory tract infection by using 16S rRNA gene sequencing and to compare the results with those of a commercial phenotypic identification kit.

Materials and Methods

Obligate anaerobes used in this study

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.

Identification by 16S rRNA gene sequencing

The 16S rRNA gene sequencing was performed in accordance with published methods [36, 37]. 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/) [4]. 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 [23]. In addition, because the CLSI guideline recommends constructing a phylogenetic tree for a strain that has identity of <97.0% to the type strain, the author used MEGA 5.03 software [77] to create a phylogenetic tree by using the neighbor-joining method with 1,000 bootstrap replicates.

Identification by phenotypic method

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.

Results

Identification results of 16S rRNA gene sequencing

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 1-1). 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 six isolates belonged to a cluster of *Prevotella* (Fig. 1-1). The author 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.

Identification results of phenotypic method

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

Comparison of two identification methods

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.

Discussion

As for bacterial identifications of obligate anaerobes, accordance rates between the result from 16S rRNA gene sequencing and those of phenotypic method are low in this study; 37.9% to genus level and 31.0% to species level. In agreement with my results, some identification kits have previously identified *P. heparinolytica*, including the type strain and clinical strains, as a strain associated with *B. uniformis* [3]. Phenotypic characterization by using identification test kits often results in unreliable identification, especially in the case of veterinary isolates [1, 14, 25, 63], and 16S rRNA gene sequencing has often provided reliable identifications of these otherwise unidentifiable strains [1, 14]. 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.

Previous studies have reported that the major obligate anaerobes obtained from horses with signs of lower respiratory tract infection are *Bacteroides* spp. and *Clostridium* spp. [74]; anaerobic cocci and *Eubacterium fossor* [65]; and *Clostridium perfringens*, *Bacteroides fragilis,* and *Bacteroides oralis* [11]. In agreement with my 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 [11, 65, 74]. Tracheobronchial aspirates were used mainly in these previous works [11, 65, 74]. In contrast, the specimens the author 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 [34], suggesting that samples from bronchial sites are more suitable than those from tracheal sites for detecting causative or secondarily invasive obligate anaerobes. Therefore, the author concluded that my identification results would be more reliable than those of the above-mentioned past studies in terms of the sampling sites used. The author 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.

To my 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 should result in better choices of antimicrobials for treating anaerobic lower respiratory tract infections in horses.

Table 1-1. 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." . f) According to the results of the phylogenetic analysis, strains related to *Prevotella* are described with dashed line. d) BALF: bronchoalveolar lavage fluid. e) These strains were isolated from horses with signs of pneumonia, but their origin was not recorded. 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. 1－1.

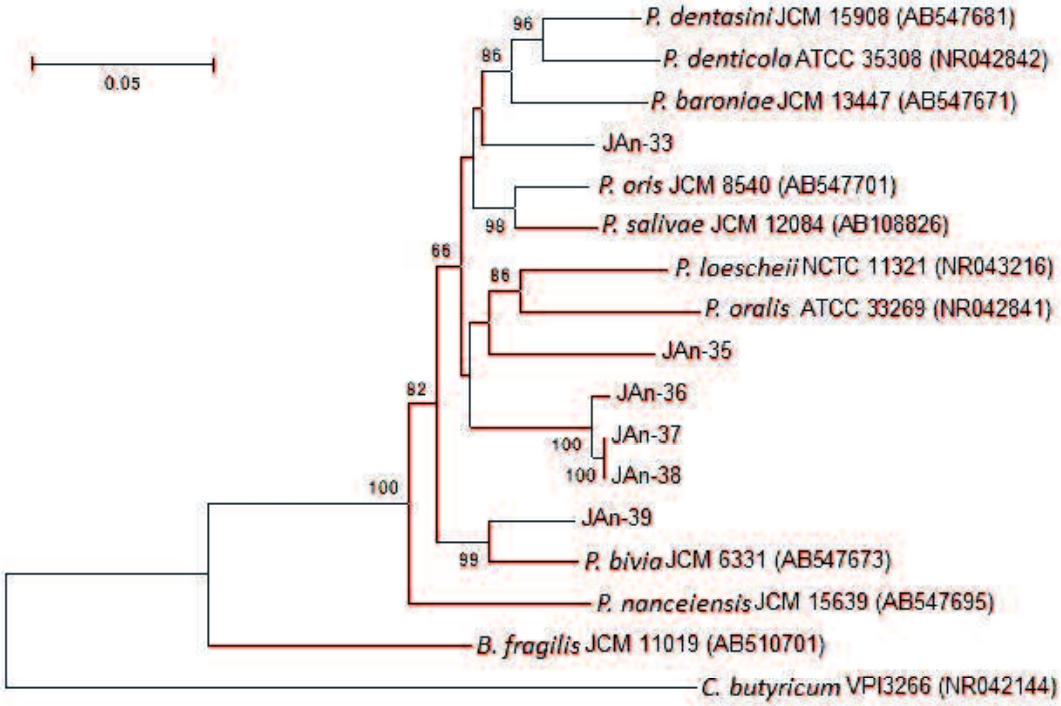

Fig. 1－1. Neighbor-joining phylogenetic tree derived from 16S rRNA gene sequences, showing the positions of JAn-33 and -35 to -39 within the genus *Prevotella*. Accession numbers of published sequences of *Prevotella* species are shown in parentheses. Bootstrap values (>50%) based on 1,000 replications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

CHAPTER 2

Development of loop-mediated isothermal amplification to detect seven groups of pathogens causing lower respiratory bacterial infection in horses and analysis of its use with three simple methods of extracting DNA from equine respiratory tract specimens

Summary

Although *Streptococcus equi* subsp. *zooepidemicus* is a dominant pathogenic bacterium in equine lower respiratory tract infection, microbial substitution occasionally occurs following the administrations of antimicrobials. The author developed five specific loop-mediated isothermal amplification (LAMP) assays to detect equine respiratory pathogens, e.g., *S. zooepidemicus*, strains of the *Bacteroides-Prevotella* group, *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia,* and *Staphylococcus aureus*. The author examined the clinical efficacies of LAMP assay in combination with each of three DNA extraction methods easily used by veterinary practitioners, namely the Loopamp PURE DNA Extraction Kit, InstaGene Matrix, and a conventional boiling method. The LAMP method plus the Loopamp PURE DNA Extraction Kit gave higher rates of positivity than the other combinations in both clinical and spiked samples containing clinically significant concentrations ($>1\times10^4$ CFU/ml) of *S. zooepidemicus*. The author then applied the five LAMP assays and two previously published LAMP assays targeting *Escherichia coli* or *Pseudomonas aeruginosa* to clinical respiratory specimens, showing high accordance rates between the results of LAMP assay and bacterial culture. Use of these LAMP assays could enable rapid detection of pathogenic bacteria and swift administration of the appropriate antimicrobials.

Introduction

Streptococcus equi subsp. *zooepidemicus* is a beta-hemolytic Gram-positive Lancefield group C bacterium found in a wide range of species, including horses, pigs, monkeys, dogs, and humans [31, 64, 72, 79]. It is part of the normal bacterial microflora of the upper respiratory tract and caudal reproductive tract of horses [79]. *Streptococcus zooepidemicus* is an opportunistic pathogen associated with a wide variety of diseases in horses, including pneumonia, mastitis, placentitis, and endometritis [16, 39, 44, 52]. In particular, *S. zooepidemicus* is a predominant pathogen in the bacterial pneumonia resulting from transport of horses for long periods [61]. Although *S. zooepidemicus* is a predominant pathogen in equine pneumonia, microbial substitution occasionally occurs following the administration of antimicrobials. In particular, obligate anaerobes and *Escherichia coli* tend to be the etiological agents of secondary bacterial pneumonia [65]. The fatality rate from pleuropneumonia caused by these agents and secondary to bacterial pneumonia is high [65, 74]. To prevent the progression of these infections it is important to diagnose causative pathogens and/or microbial substitutions rapidly and to select the antimicrobials used accordingly to suit the causative bacteria.

Veterinary practitioners, however, are often faced with difficulties in conducting rapid diagnosis and choosing an appropriate antimicrobial. Isolating and identifying some bacteria such as obligate anaerobes is time consuming, and conventional polymerase chain reaction (PCR) assays, which can detect pathogens more quickly than bacterial culture, need special equipment such as thermal cycler.

Loop-mediated isothermal amplification (LAMP) method was developed as a new type of nucleic acid amplification method [60]. LAMP amplifies nucleic acids with high speed, specificity, and efficiency, and it can be performed under isothermal

conditions with no special equipment. In recent years, LAMP has been applied clinically as a method for rapid detection of various pathogens [33, 57]. Here, the author developed novel LAMP methods specific to the pathogenic bacteria found in equine lower respiratory tract infection, namely, *S. zooepidemicus*, the *Bacteroides-Prevotella* group, *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia,* and *Staphylococcus aureus*. The author then applied these five newly developed LAMP assays and two previously described ones targeting *Escherichia coli* [35] and *Pseudomonas aeruginosa* [32] to clinical samples to assess the efficacy of the assays in diagnosing lower respiratory tract infection in horses.

The process of DNA extraction from clinical samples is necessary for various genetic tests, including LAMP. However, veterinary practitioners are unable to use most DNA extraction methods, because they require complicated processes that are time-consuming and labor-intensive. Here, the author also compared the results obtained when LAMP was used with each of three DNA extraction methods that veterinary practitioners could apply easily to clinical samples.

Materials and Methods

Primer designs

All LAMP primers were designed by using PrimerExplorer V4 software (Fujitsu Limited, Tokyo, Japan). The primer sets included five or six primers: two outer primers (F3 and B3), two inner primers (FIP and BIP), and one or two loop primer (Loop B, or Loop B and Loop F), and the primer sequences for the LAMP methods are shown in Table 2-1. Primers for species-specific LAMPs to detect *S. zooepidemicus* (Sz-LAMP), *K. pneumoniae* (Kp-LAMP), *S. maltophilia* (Sm-LAMP), and *S. aureus* (Sa-LAMP) were designed on the basis of published sequences of *sorD*, tyrosine aminotransferase, *smeT*, and nuclease gene, respectively. The GenBank accession numbers were showed in Table 2-2. The LAMP primers for detecting obligate anaerobes were focused on *Bacteroides* spp. and *Prevotella* spp. (Bac-Pre-LAMP); these species were revealed to be dominant obligate anaerobes in equine respiratory tract infection in CHAPTER 1. To select the specific region of the 16S rRNA gene for the *Bacteroides-Prevotella* group, the author compared the 16S rRNA gene sequences of 58 obligate anaerobes previously isolated from the equine respiratory tract in CHAPTER 1 and 24 gene sequences of various bacteria registered in GenBank (Table 2-2). These 16S rRNA gene sequences were also used to create a phylogenetic tree by using the neighbor-joining method with 1,000 bootstrap replicates using MEGA 5.03 software [77].

LAMP methods

The reaction mixture was prepared by using a Loopamp DNA Amplification Kit (Eiken Chemical Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's

instructions. In brief, 25 μl of reaction mixture was prepared to contain 12.5 μl of 2× reaction mix buffer, 0.2 μM of each outer primer (F3 and B3), 1.6 μM of each inner primer (FIP and BIP), 0.8 μM of loop primer (Loop B, or Loop B and Loop F), 1.0 μl of *Bst* DNA polymerase (8 units/μl), and 2.0 μl of sample DNA. The seven LAMP assays (the five new developed assays and the two previously described assays targeting *E. coli* (Ec-LAMP) [35] and *P. aeruginosa* (Pa-LAMP) [32]) were separately performed at 65 °C for 60 min and then terminated by heating the mixtures at 80 °C for 5 min. LAMP products were detected by monitoring the turbidity with a real-time turbidimeter (LA-320C, Eiken Chemical Co., Ltd.).

Specificities

To confirm the specificities of the Sz-LAMP, the author examined 172 strains, all of which except for a type strain of *S. zooepidemicus* (ATCC 43079^T) were isolated from diseased horses between 1980 and 2013. The 172 strains comprised 51 strains of *S. zooepidemicus*, 50 strains of *Streptococcus equi* subsp. *equi*, 23 strains of *Streptococcus dysgalactiae* subsp. *equisimilis*, 18 strains of other *Streptococcus* species including *Streptococcus pneumonia*, which is generally human pathogenic bacterium, and 30 strains belonging to other genera frequently isolated from horses (Table 2-3). The author examined 115 bacterial strains to confirm the specificity of the Bac-Pre-LAMP. The 115 strains comprised 58 obligate anaerobes isolated from the respiratory tracts of horses in CHAPTER 1, 12 reference strains purchased from the American Type Culture Collection (ATCC) and 45 strains belonging to other genera frequently isolated from horses (Table 2-4). The author used 185 bacterial strains to examine specificities for Kp-LAMP (Tables 2-5), 82 strains for Sm-LAMP (Tables 2-6), and 168 strains for Sa-LAMP (Tables 2-7). Each bacterial DNA was extracted from fresh bacterial culture

by using InstaGene Matrix (Bio-Rad Laboratories, Tokyo, Japan) in accordance with the manufacturer's instructions.

Detection limits of each LAMP assays

To measure the detection limits of the seven LAMP assays, 10-fold serial dilutions of a suspension of each bacterium were prepared, and the colony forming units (CFUs) in the suspensions were counted: *Bacteroides fragilis* ATCC 25285^{T} was used as a representative obligate anaerobe because *B. fragilis* is a dominant target of Bac-Pre-LAMP. In the case of the other six LAMP assays (Sz-, Kp-, Sm-, Sa-, Ec-, and Pa-LAMPs), the author used the respective type strains, namely *S.zooepidemicus* ATCC 43079^{T}, *K. pneumoniae* ATCC 13883^{T}, *S. maltophilia* ATCC 13637^{T}, *S. aureus* ATCC 12600^{T}, *E. coli* ATCC 11775^{T}, and *P. aeruginosa* ATCC 10145^{T}. Bacterial DNA in the suspensions was then extracted by using a Loopamp PURE DNA Extraction Kit (Eiken Chemical Co., Ltd.) in accordance with the manufacturer's instructions. Sensitivity tests were performed three times for each LAMP method; the lowest bacterial concentrations that yielded positive results at least twice were regarded as the detection limits (CFU/ml).

Three different DNA extraction methods for applying to clinical samples

Three DNA extraction methods were selected, namely a Loopamp PURE DNA Extraction Kit, InstaGene Matrix or a boiling method, because they could be performed with only a few steps and within 30 min. The Loopamp PURE DNA Extraction Kit and InstaGene Matrix were used in accordance with the manufacturer's instructions. With the boiling method, samples were boiled at 100 °C for 10 min and were centrifuged at 13,000 g for 3 min. The aliquots obtained from the supernatant were then subjected to

LAMP analysis.

Comparison of efficacies of three different DNA extraction methods

To measure the detection limits of the LAMP method in combination with each of three different DNA extraction methods, 10-fold serial dilutions of a suspension of *S. zooepidemicus* were prepared, and the colony forming units (CFUs) in the suspension were counted. Bacterial DNA in the suspensions was then extracted by using one of the three DNA extraction methods. Sensitivity tests were performed three times for each DNA extraction method.

Fifty-four clinical samples and 34 spiked samples which were prepared experimentally were used in this study to compare the efficacies of the three DNA extraction methods. Of the 54 clinical samples obtained from the respiratory tracts of Thoroughbred horses, one was obtained in 2010, 25 in 2012, and 28 in 2013. The 54 clinical samples consisted of 33 bronchoalveolar lavage (BAL) fluids, 12 tracheal washes, 8 pleural effusions, and one guttural pouch lavage specimen. The clinical samples were suspended in equal amount of Cary-Blair medium and were transported to my laboratory. One hundred μl of the mixtures were incubated aerobically on 5% horse blood agar at 37 °C for 24 hr. The remaining samples were stored at –20 °C until DNA isolation. Thirty-four of 54 clinical samples yielded *S. zooepidemicus* at less than 20 CFU/ml, which is detection limit in this study. The concentration of *S. zooepidemicus* was between 20 CFU/ml and 1×10^4 CFU/ml in 6 of 54 samples and greater than 1×10^4 CFU/ml in 14 of 54 samples. To prepare the spiked samples, 34 clinical samples with *S. zooepidemicus* concentrations of less than 20 CFU/ml were spiked at one-tenth their volumes with a suspension in which the concentration of *S. zooepidemicus* (ATCC 43079^T) was 1×10^7 CFU/ml. The final concentration of *S. zooepidemicus* in each spiked

sample was at least 1×10^6 CFU/ml. Bacterial DNA in the 54 clinical samples or 34 spiked samples was extracted by using one of the three DNA extraction methods, and then each DNA was applied to Sz-LAMP.

Application for clinical and spiked samples

The author used 67 clinical samples to assess the clinical efficacies of the seven LAMP assays. The 67 clinical samples comprised 54 samples described above and additional 13 samples collected in 2013 to 2014. The 67 clinical samples consisted of 41 bronchoalveolar lavage fluids (BALFs), 14 tracheal washes, 11 pleural effusions, and one guttural pouch lavage fluid. Clinical samples were prepared in the same manner as described above. The author then incubated 100 μl of each mixture aerobically at 37 °C on 5% horse blood agar and MacConkey agar for 24 hr and anaerobically on 5% horse blood agar for 48 hr. Bacteria were identified by using Gram staining and a commercial identification kit (API Systems, SYSMEX bioMérieux, Tokyo, Japan). The remaining samples were stored at –20 °C until DNA isolation.

Bacterial DNA was extracted from clinical samples by using a Loopamp PURE DNA Extraction Kit. The extracted DNA was then used in each LAMP assay. To confirm the clinical specificity of each LAMP method, specific PCRs that separately detect the *Bacteroides fragilis* group and *Prevotella* group [50], *E. coli* [10], *K. pneumoniae* [46] and *S. maltophilia* [84] were used on the clinical samples that gave positive results in each LAMP assay. The rates of accordance between the results of bacterial culture and those of the LAMP assays were calculated separately on the basis of the clinically important bacterial culture value (accordance rate 1: 1×10^4 CFU/ml) and the detection limit of bacterial culture (accordance rate 2: 20 CFU/ml), as below: Accordance rate 1 (%) = 100 × (numbers of samples with both $\geq1\times10^4$ CFU/ml and

LAMP positive + numbers of samples with both $<1\times10^4$ CFU/ml and LAMP negative) / (total numbers of clinical samples), Accordance rate 2 (%) = 100 × (numbers of samples with both ≥20 CFU/ml and LAMP positive + numbers of samples with both <20 CFU/ml and LAMP negative) / (total numbers of clinical samples).

Results

Phylogenetic tree

According to the phylogenetic tree derived from 16S rRNA gene sequences of various bacteria (Figure 2-1), the strains of *Bacteroides* and *Prevotella*—dominant obligate anaerobes associated with respiratory disease in horses— formed a *Bacteroides-Prevotella-Porphyromonas* group and were obviously separated from the aerobes, facultative anaerobes and obligate anaerobes except for *Bacteroides-Prevotella-Porphyromonas* group.

Specificities

Amplification in the Sz-, Bac-Pre-, Kp-, Sm-, and Sa-LAMPs was confirmed for all strains of *S. zooepidemicus*, *Bacteroides* and *Prevotella*, *K. pneumoniae*, *S. maltophilia,* and *S. aureus*, respectively. No amplification of DNA from non-target bacteria by the five LAMP products was confirmed except for strains of *Porphyromonas* in the Bac-Pre-LAMP.

Efficacies of three different DNA extraction methods

The detection limits of the Sz-LAMP in combination with the Loopamp PURE DNA Extraction Kit, InstaGene Matrix, and boiling method for pure culture were 1×10^5, 1×10^3, and 1×10^4 CFU/ml, respectively. These detection limits (CFU/ml) of the LAMP with the Loopamp PURE DNA Extraction Kit, InstaGene Matrix, and boiling method are equal to 200 CFU/reaction, 2 CFU/reaction, and 20 CFU/reaction, respectively.

Thirteen of 14 clinical samples with *S. zooepidemicus* concentrations greater than 1×10^4 CFU/ml were positive by the LAMP method plus the Loopamp PURE DNA

Extraction Kit; 11 of these 14 clinical samples were positive with LAMP plus InstaGene Matrix, and 7 were positive with LAMP plus boiling (Table 2-8). Thirty-four of 34 spiked samples were positive with LAMP plus the Loopamp PURE DNA Extraction Kit; 31 were positive with LAMP plus InstaGene Matrix and 27 with LAMP plus boiling. Among the 40 clinical samples in which *S. zooepidemicus* concentrations were either less than 20 CFU/ml or 20 to 1×10^4 CFU/ml, one clinical sample was positive with LAMP plus the Loopamp PURE DNA Extraction Kit, two with LAMP plus InstaGene Matrix, and one with LAMP plus boiling.

Detection limits

The detection limits of Sz-, Bac-Pre-, Sa-, and Pa-LAMPs in combination with the Loopamp PURE DNA Extraction Kit were 1×10^5 CFU/ml and those of Kp-, Sm-, and Ec-LAMPs were 1×10^4 CFU/ml. The detection limits of 1×10^4 CFU/ml and 1×10^5 CFU/ml were equal to 20 CFU/reaction and 200 CFU/reaction, respectively, in the LAMP assays.

Application for clinical samples

Of the 67 clinical samples, 36 gave positive results in at least one LAMP assay (Table 2-9): 19 samples were positive on Sz-LAMP, 19 on Bac-Pre-LAMP, four on Ec-LAMP, four on Kp-LAMP and two on Sm-LAMP (Table 2-10). Thirty-two of 36 clinical samples (88.9%) with $>1\times10^4$ CFU/ml of the target bacterium were positive in the relevant LAMP assays. The four samples (J13-15, J13-20, J13-31, and J13-35), with both $\geq1\times10^4$ CFU/ml of target bacterium and relevant LAMP negative, contained 2.4×10^4 CFU/ml to 8.6×10^4 CFU/ml of *S. zooepidemicus* or *S. aureus*—slightly lower than the detection limit of Sz- and Sa-LAMP (1×10^5 CFU/ml). Except for *S.*

zooepidemicus of which specific PCR for detection has not been reported available for clinical sample, all clinical samples that were LAMP positive—in some of which the bacterial concentrations were less than the detection limits of the LAMP assays—were also positive by each group- or species-specific PCR reaction. The overall accordance rates of the Sz-LAMP and Bac-Pre-LAMP results with the results of bacterial culture were both 91.0%, using a threshold bacterial concentration of 1×10^4 CFU/ml, and 85.1% and 88.1%, respectively, using a threshold of 20 CFU/ml (Table 2-10). The accordance rates of the other five LAMP assays were 94.0% to 100% (using 1×10^4 CFU/ml) and 95.5% to 100% (using 20 CFU/ml).

Discussion

Multiplex PCR [2] and multiplex real-time PCR [9] for detecting *S. zooepidemicus* and *S. equi,* have been described previously. These two published PCR-based methods use two genes: *sodA*, which encodes a manganese-dependent superoxide dismutase, for detecting both *S. zooepidemicus* and *S. equi*; and *seeI*, which encodes pyrogenic mitogen SePE-I, for detecting only *S. equi*. The two methods cannot judge the presence or absence of *S. zooepidemicus* when a sample contains *S. equi*, because they do not use a primer set that can detect only *S. zooepidemicus*. Therefore, the two methods are appropriate for identifying each species in pure culture, but are not suitable for detecting *S. zooepidemicus* in clinical samples. Carbohydrate (e.g. lactose, sorbitol, and trehalose) fermentation testing is commonly used to differentiate between *S. zooepidemicus* and *S. equi* [6]. All *S. equi* strains lack the *sorD* gene that encodes sorbitol-6-phosphate 2-dehydrogenase and therefore lack the ability to ferment sorbitol, whereas all *S. zooepidemicus* strains possess *sorD* [38]. This genetic basis indicates that *sorD* should be suitable for differentiating *S. zooepidemicus* from *S. equi* and, as a result, the Sz-LAMP targeting the *sorD* gene could specifically detect *S. zooepidemicus* in this study.

In the study described in CHAPTER 1, strains belonging to the *Bacteroides-Prevotella* cluster accounted for 67.2% (39 of 58 strains) of pathogenic obligate anaerobes in the equine respiratory tract infection; these bacteria were found in 93.5% (29 of 31 horses) of horses that were sick with lower respiratory tract infection involving with obligate anaerobes (data not shown). Although the author did not isolate *Porphyromonas* spp., which Bac-Pre-LAMP could detect, *Porphyromonas* is one of the pathogens causing equine pneumonia [65]. Bac-Pre LAMP, therefore, could broadly

detect pathogenic obligate anaerobes in lower respiratory tract specimens from horses. Collectively, the high specificities of the five new developed LAMP assays were confirmed.

In a previous study, 69 of 148 healthy Thoroughbred horses had bacteria in their tracheas at concentrations of 1 to 10^4 CFU/ml; the concentration of the bacteria was greater than 1×10^4 CFU/ml in only seven of these horses [18]. In another previous study, the average concentration of bacteria in tracheal washes from Standardbred horses with no clinical signs was 1.49×10^4 CFU/ml [43]. Moreover, the presence of bacteria at concentrations greater than 1×10^4 CFU/ml in human BAL samples is associated with clinical disease [8]. The author therefore considered a concentration of greater than 1×10^4 CFU/ml in clinical samples to be of clinical importance. Thus, the author divided the clinical samples into three groups by using two thresholds: 20 CFU/ml (the detection limit of my bacterial culture) and 1×10^4 CFU/ml.

The clinical efficacy of a genetic test can sometimes differ from the results of sensitivity testing in *in vitro* experiments using pure bacterial culture [53, 54], because each DNA extraction method has a different performance in terms of DNA purification or the yield of DNA extracted from clinical specimens. Therefore, the author used clinical samples and spiked samples to assess the clinical efficacies of three DNA extraction methods; Loopamp PURE DNA Extraction Kit, InstaGene Matrix, and boiling. Although the sensitivity of the LAMP method plus the Loopamp PURE DNA Extraction Kit was 10 to 100 times lower than those with InstaGene Matrix or boiling, the combination of LAMP plus the Loopamp PURE DNA Extraction Kit could detect *S. zooepidemicus* in both clinical and spiked samples more efficiently than the other methods. Because the bacterial concentrations in the spiked samples were sufficient to give positive results by the Sz-LAMP in pure culture, the LAMP method would

theoretically be expected to give positive results in all spiked samples. However, not all spiked samples were positive by LAMP plus InstaGene Matrix or the boiling method; 100% detection was obtained only with the Loopamp PURE DNA Extraction Kit. Many inhibitors are present in specimens from the respiratory tract, and they can cause false-negative results in PCR [42, 85]. These inhibitory substances either bind to polymerase or interact with the sample's DNA or polymerase or both, during primer extension [62, 85]. The inhibitors remaining after DNA extraction using InstaGene Matrix or boiling might inhibit the LAMP reaction; using the Loopamp PURE DNA Extraction Kit would be more likely to reduce the effect of these inhibitors than would the other extraction methods. My results suggest that the InstaGene Matrix and boiling methods are unsuitable for use on clinical samples because they can yield false negatives; the LAMP method plus the Loopamp PURE DNA Extraction Kit is the most appropriate for use on clinical samples. LAMP plus the Loopamp PURE DNA Extraction Kit gave a positive result in one clinical sample in which the *S. zooepidemicus* concentration was less than 20 CFU/ml, despite the fact that the detection limit of this combination in pure culture was 1×10^5 CFU/ml. This inconsistency may have been caused by the presence of dead *S. zooepidemicus* in the sample, because the samples were collected after the administration of antimicrobials.

The seven LAMP methods in combination with the Loopamp PURE DNA Extraction Kit obtained high accordance rates with bacterial culture. These high accordance rates indicate that LAMP assays could be useful tools for detecting the bacterial pathogens causing lower respiratory tract infection in horses.

In conclusion, the author developed LAMP methods for detecting *S. zooepidemicus*, strains of the *Bacteroides-Prevotella* group, *K. pneumoniae*, *S. maltophilia* and *S. aureus*. These methods can yield results within 1 hr, and unlike

previous PCR-based methods, it can be applied to clinical samples without the need for special equipment. The seven LAMP assays, the five new LAMP assays and two previously published LAMP assays targeting *Escherichia coli* or *Pseudomonas aeruginosa*, could be conducted simultaneously at the same temperature (65 °C). The LAMP method in combination with the Loopamp PURE DNA Extraction Kit is efficient for clinical and spiked samples containing clinically significant concentrations, and it takes a total of only about 90 min from receiving the specimens to obtaining the LAMP results. In this way, the author can concurrently perform LAMP assays to detect both the primary and secondary causative pathogens of lower respiratory bacterial infections in adult horses within only 90 min with the naked eye; consequently, it will be possible to institute appropriate antimicrobial therapies more quickly in horses with bacterial lower respiratory tract infection.

Table 2-1. Primer sets used in this study

Target bacteria of LAMP assay	Target gene	Primer	Sequence (5'→3')	Reference
Streptococcus zooepidemicus	*sorD* gene	F3	ATGGCCTCTGAGGCAGG	This study
		B3	TCTGGTCAACGGTTTTTCCT	
		FIP [b]	GCCCAAGAGCGTGTATAGCTGTGAAGGCTCAGAAGGGCAAAG	
		BIP [c]	GGCAAGCATGGCGTTCGAGTCTTCATAAGCCAATGTCCGCA	
		Loop B	ACCAGGTATCATGGAGGCGAC	
Obligate anaerobes belonging to *Bacteroides-Prevotella* cluster [a]	16S rRNA gene	F3	GGCTACCTTGTTACGACTTAGC	This study
		B3	GTGAAGCTGGATTCGCTAGT	
		FIP [b]	CCGGGGGTACCTGAAGTACCCCAGTCACCAGTTTTACCCTAG	
		BIP [c]	GCTTGACGGGCGGTGTGTACAATCGCGCATCAGCCAC	
		Loop B	CGGG AACGTATTCACCGC	
Klebsiella pneumoniae	tyrosine aminotransferase (*tyrB*) gene	F3	GCCTCGCTGTATCTGCCAA	This study
		B3	CGATGTGGTTTTCCCAGGT	
		FIP [b]	CGGTATGCTCTGCGCCAAACAAGGGTTGAGCGGCTACC	
		BIP [c]	CTGAAGGTCGGGGCCGACTTGATCGCTGACCCAGACATGA	
		Loop B	CCTCAAACGTTACTTTCCTGAGTC	
Stenotrophomonas maltophilia	*smeT* gene	F3	CGCAAGACCAAAGAGGACAC	This study
		B3	GCACGCGCTCGACGAT	
		FIP [b]	GGCCACGCCGTGTTCATGGAAAGGCAACCCGGGAAGG	
		BIP [c]	ACCACGCTGGAGATGATCGGTGGCACCTCGCTCTTGTTCTT	
		Loop B	CGCGGTCTACTGGCACT	
		Loop F	TTCGGCGGCGTCAAGGA	
Staphylococcus aureus	nuclease (*nuc*) gene	F3	AACAGTATATAGTGCAACTTCAA	This study
		B3	CTTTGTCAAACTCGACTTCAA	
		FIP [b]	TGTCATTGGTTGACCTTTGTACATTAAAATTACATAAAGAACCTGCGA	
		BIP [c]	GTTGATACACCTGAAACAAAGCATCATTTTTTTCGTAAATGCACTTGC	
		Loop B	AAAGGTGTAGAGAAATATGGTCCTG	
		Loop F	ACCGTATCACCATCAATCGCTT	
Escherichia coli	*malB* gene	F3	GCCATCTCCTGATGACGC	(35)
		B3	ATTTACCGCAGCCAGACG	
		FIP [b]	CATTTTGCAGCTGTACGCTCGCAGCCCATCATGAATGTTGCT	
		BIP [c]	CTGGGGCGAGGTCGTGGTATTCCGACAAACACCACGAATT	
		Loop B	ATCAATCTCGATATCCATGAAGGTG	
		Loop F	CTTTGTAACAACCTGTCATCGACA	
Pseudomonas aeruginosa	outer membrane lipoprotein (*oprL*) gene	F3	GCGTTGCCGCCAACAATG	(32)
		B3	CATGCGGGCAACCTCTC	
		FIP [b]	GTTGTCACCCCACCTCCGGGCGGCAACGTTCCTCC	
		BIP [c]	CTCCGTGCAGGGCGAACTGCAGGCGAGCCAACTC	
		Loop B	GTTCATGCAGCTCCAGCAG	
		Loop F	ACCTGCCGTGCCATACC	

a) These bacteria are revealed to be dominant causative obligate anaerobes of lower respiratory tract infection in horses in Chapter 1.

b) FIP primer consists of F1c and F2 regions.

c) BIP primer consists of B1c and B2 regions.

Table 2-2. GenBank accession numbers used in this study

Target bacteria of LAMP assay	Gene name	GenBank accession numbers
Streptococcus zooepidemicus	*sorD* gene	NC011134, CP002904, and FM204884
Klebsiella pneumoniae	tyrosine aminotransferase (*tyrB*) gene	AF074934, AY016787, AY016789, AY016785, AY016783, AY016781, AY016770, and M12047
Stenotrophomonas maltophilia	*smeT* gene	AM743169, HE798556, CP002986, AJ316010, AY520544, AY520543, AY450956, and AY450955
Staphylococcus aureus	nuclease (*nuc*) gene	DQ507382, DQ507377, DQ399678, EF529607, and EF529589
Obligate anaerobes belonging to *Bacteroides-Prevotella* cluster	16S rRNA gene	obligate anaerobe X83935, NR119165, L16486, NR074515, AB547651, L16481, NR042843, NR040838, L16493, NR117287, EF025906, AB525414, NR043332, L04168, and AB075768 facultative anaerobe NR024570, AF130981, NR042887, AB008509, NR027552, AF015929, EF406034, and FJ468344 aerobe AF094713

Table 2-3. Bacteria used for Sz-LAMP

Strain	Numbers of examinated strain	Number of Sz-LAMP positive
Streptococcus equi subsp. zooepidemicus	51	51
Streptococcus equi subsp. *equi*	50	0
Streptococcus dysgalactiae subsp. *equisimilis*	23	0
Streptococcus bovis	4	0
Streptococcus pneumoniae	4	0
Actinobacillus equuli	3	0
Escherichia coli	3	0
Klebsiella pneumoniae	3	0
Pasteurella pneumotropica	3	0
Pseudomonas aeruginosa	3	0
Rhodococcus equi	3	0
Salmonella enterica subsp. enterica serovar Abortusequi	3	0
Salmonella enterica subsp. enterica serovar Typhimurium	3	0
Staphylococcus aureus	3	0
Staphylococcus hyicus	3	0
Streptococcus mitis	3	0
Streptococcus acidominimus	2	0
Streptococcus equinus	2	0
Streptococcus mutans	2	0
Streptococcus constellatus	1	0

Table 2-4. Bacteria used for the Bac-Pre-LAMP

	Strain	Numbers of examinated strain	Number of Bac-Pre-LAMP positive
Strains of *Bacteroides-Prevotella-Porphyromonas* group	*Bacteroides fragilis* (ATCC 25285^T and clinical strains)	16	16
	Bacteroides pyogenes	4	4
	Bacteroides thetaiotaomicron (ATCC 29741 and clinical strain)	2	2
	Bacteroides uniformis (ATCC 8492^T)	1	1
	Bacteroides vulgatus (ATCC 8482^T)	1	1
	Bacteroides ovatus (ATCC 8483^T)	1	1
	Bacteroides helcogenes	1	1
	Bacteroides xylanisolvens	1	1
	Prevotella heparinolytica	9	9
	Prevotella loescheii (ATCC 15930^T)	1	1
	Prevotella melaninogenica (ATCC 25845^T)	1	1
	Prevotella dentasini	1	1
	Other *Prevotella* spp.	7	7
	Strain related to *Paraprevotella*	1	1
	Porphyromonas gingivalis (ATCC 33277^T)	1	1
	Porphyromonas levii (ATCC 29147^T)	1	1
Obligate anaerobe except for *Bacteroides-Prevotella-Porphyromonas* group	*Veillonella parvula* (ATCC 10790^T)	1	0
	Clostridium perfringens	2	0
	Clostridium orbiscindens	1	0
	Clostridium difficile	1	0
	Clostridium argentinense	1	0
	Fusobacterium nucleatum subsp. *nucleatum* (ATCC 25586^T)	1	0
	Fusobacterium spp.	3	0
	Peptostreptococcus anaerobius (ATCC 27337^T)	1	0
	Other anaerobic species reralated to *Clostridium* or *Eubacterium*	13	0
Aerobe and facultive anaerobe	*Streptococcus equi* subsp. *zooepidemicus*	9	0
	Rhodococcus equi	3	0
	Klebsiella pneumoniae	3	0
	Pseudomonas aeruginosa	3	0
	Escherichia coli	3	0
	Pasteurella pneumotropica	3	0
	Staphylococcus aureus	3	0
	Staphylococcus hyicus	3	0
	Actinobacillus equuli	3	0
	Streptococcus dysgalactiae subsp. *equisimilis*	3	0
	Salmonella Abortusequi	3	0
	Salmonella Typhimurium	3	0

Table 2-5. Bacteria used for Kp-LAMP

Strain	Numbers of examinated strain	Number of Kp-LAMP positive
Klebsiella pneumoniae (ATCC 13883^T and clinical strains)	100	100
Citrobacter freundii (ATCC 8090^T)	1	0
Hafnia alvei (ATCC 51815)	1	0
Morganella morganii subsp. morganii (ATCC 25830^T)	1	0
Plesiomonas shigelloides (ATCC 14029^T)	1	0
Raoultella terrigena (ATCC 33257^T)	1	0
Yersinia enterocolitica subsp. enterocolitica (ATCC 9610^T)	1	0
Escherichia coli	12	0
Salmonella Abortusequi	3	0
Salmonella Typhimurium	3	0
Klebsiella oxytoca	11	0
Enterobacter sp.	2	0
Enterobacter cloacae (ATCC 13047^T and clinical strains)	5	0
Pantoea sp.	3	0
Serratia marcescens (ATCC 13880^T and a clinical strain)	2	0
Streptococcus equi subsp. *zooepidemicus*	11	0
Pasteurella pneumotropica	3	0
Actinobacillus equuli	3	0
Streptococcus dysgalactiae subsp. *equisimilis*	3	0
Rhodococcus equi	3	0
Staphylococcus hyicus	3	0
Staphylococcus aureus	3	0
Pseudomonas aeruginosa	3	0
Bacteroides pyogenes	3	0
Prevotella heparinolytica	3	0

Table 2-6. Bacteria used for Sm-LAMP

Strain	Numbers of examinated strain	Number of Sm-LAMP positive
Stenotrophomonas maltophilia	26	26
Streptococcus equi subsp. *zooepidemicus*	11	0
Pasteurella pneumotropica	3	0
Actinobacillus equuli	3	0
Streptococcus dysgalactiae subsp. *equisimilis*	3	0
Rhodococcus equi	3	0
Salmonella Abortusequi	3	0
Salmonella Typhimurium	3	0
Staphylococcus hyicus	3	0
Staphylococcus aureus	3	0
Klebsiella pneumoniae	3	0
Pseudomonas aeruginosa	15	0
Escherichia coli	3	0

Table 2-7. Bacteria used for Sa-LAMP

Strain	Numbers of examinated strain	Number of Sa-LAMP positive
Staphylococcus aureus	88	88
Staphylococcus xylosus	13	0
Staphylococcus hyicus	8	0
Staphylococcus scuri	15	0
Staphylococcus epidermidis	5	0
Staphylococcus intermedius	5	0
Staphylococcus chromogens	1	0
Klebsiella pneumoniae	3	0
Pseudomonas aeruginosa	3	0
Escherichia coli	3	0
Streptococcus equi subsp. *zooepidemicus*	3	0
Pasteurella pneumotropica	3	0
Streptococcus dysgalactiae subsp. *equisimilis*	3	0
Rhodococcus equi	3	0
Salmonella Abortusequi	3	0
Salmonella Typhimurium	3	0
Stenotrophomonas maltophilia	3	0
Actinobacillus equuli	3	0

Table 2-8. Comparison of efficacies of the three methods of extracting DNA from clinical and spiked samples

Sample	*S. zooepidemicus* concentration (CFU/ml)	Number	Numbers (%) of LAMP-positive samples		
			Loopamp PURE DNA Extraction kit	InstaGene matrix	boiling method
Clinical sample	<20 [b)]	34	1 (2.9)	1 (2.9)	0
	20 to 1×10^4	6	0	1 (16.6)	1 (16.6)
	$>1\times10^4$	14	13 (92.9)	11 (78.6)	7 (50)
Spiked sample [a)]	1×10^6	34	34 (100)	31 (91.2)	27 (79.4)

a) Type strain of *S. zooepidemicus* (ATCC 43079^T) was added to clincal samples with *S. zooepidemicus* concentrations less than 20 CFU/ml.

b) Detection limit of bacterial culture was 20 CFU/ml in this study.

Table 2-9. Detailed data on all 67 clinical samples

Sample	Derived from	Isolate	Bacterial concentration (CFU/ml)	Positive LAMP assays [d]
J10-1	TW [a]	*Streptococcus zooepidemicus*	8.5×10^{4}	Sz-LAMP
J12-1	BALF [b]	*Alcaligenes faecalis*	8.0×10^{3}	Sm-LAMP
		Gram-positive facultative anaerobic rod	6.0×10^{3}	
		Streptococcus zooepidemicus	4.0×10^{3}	
		Stenotrophomonas maltophilia	4.0×10^{3}	
J12-2	BALF	*Bacteroides* sp. or *Prevotella* sp.	8.0×10^{6}	Bac-Pre-LAMP
		Staphylococcus sp.	6.0×10^{3}	
		Gram-positive facultative anaerobic rod	4.0×10^{3}	
		beta-hemolytic *Streptococcus* sp.	4.0×10^{3}	
J12-3	BALF	*Bacteroides* sp. or *Prevotella* sp.	1.0×10^{6}	Bac-Pre-LAMP
		beta-hemolytic *Streptococcus* sp.	3.3×10^{2}	
J12-4	pleural effusion	*Bacteroides* sp. or *Prevotella* sp.	4.8×10^{4}	Bac-Pre-LAMP
J12-5	TW	*Bacteroides* sp.	3.5×10^{7}	Bac-Pre-LAMP
		Prevotella sp.	2.0×10^{5}	
J12-6	BALF	*Klebsiella pneumoniae*	1.8×10^{6}	Kp-LAMP
J12-7	TW	*Klebsiella pneumoniae*	3.0×10^{7}	Kp- and Ec-LAMP
J12-8	BALF	N.D. [c]	-	
J12-9	TW	N.D.	-	Sz-LAMP
J12-10	guttural pouch lavage fluid	*Streptococcus zooepidemicus*	6.0×10^{5}	Sz- and Bac-Pre-LAMP
		Prevotella sp.	3.8×10^{6}	
J12-11	BALF	N.D.	-	
J12-12	BALF	N.D.	-	
J12-13	BALF	*Bacteroides fragilis*	5×10	Bac-Pre- and Ec-LAMP
J12-14	BALF	*Klebsiella pneumoniae*	1.5×10^{6}	Kp-LAMP
J12-15	BALF	*Klebsiella pneumoniae*	8.0×10^{3}	Kp-LAMP
J12-16	BALF	*Streptococcus zooepidemicus*	4.4×10^{6}	Sz- and Bac-Pre-LAMP
		Bacteroides sp. or *Prevotella* sp.	9.0×10^{6}	
J12-17	TW	*Streptococcus zooepidemicus*	1.9×10^{6}	Sz- and Bac-Pre-LAMP
J12-18	right pleural effusion	*Streptococcus zooepidemicus*	9.0×10^{5}	Sz-LAMP
J12-19	left pleural effusion	*Streptococcus zooepidemicus*	2.0×10^{6}	Sz-LAMP
J12-20	BALF	*Streptococcus zooepidemicus*	6.0×10^{5}	Sz- and Bac-Pre-LAMP
J12-21	TW	*Streptococcus zooepidemicus*	1.0×10	
J12-22	right pleural effusion	N.D.	-	
J12-23	left pleural effusion	N.D.	-	
J12-24	BALF	N.D.	-	
J12-25	BALF	N.D.	-	
J13-1	BALF	N.D.	-	
J13-2	BALF	obligate anaerobe	1.0×10^{3}	
J13-3	TW	*Streptococcus zooepidemicus*	3.0×10^{3}	
		unidentifiable	2.0×10^{3}	
		unidentifiable	2.0×10^{3}	
J13-4	BALF	N.D.	-	
J13-5	TW	N.D.	-	
J13-6	BALF	N.D.	-	
J13-7	TW	N.D.	-	
J13-8	BALF	N.D.	-	
J13-9	TW	N.D.	-	
J13-10	BALF	*Prevotella oralis/denticola*	2.0×10^{3}	
		Pseudomonas aeruginosa	2.0×10^{3}	
		Obligate anaerobic Gram-negative rod	2.0×10^{2}	
		Stenotrophomonas maltophilia	1.6×10^{2}	
		Prevotella buccae	4.0×10^{1}	
J13-11	BALF	*Streptococcus zooepidemicus*	3.2×10^{2}	
		Obligate anaerobic Gram-negative rod	3.5×10^{2}	
		Prevotella intermedia	1.7×10^{2}	
J13-12	BALF	N.D.	-	
J13-13	BALF	alpha-hemolytic streptococci	4.0×10^{1}	

Table 2-9. Detailed data on all 67 clinical samples (*continued*)

Sample	Derived from	Isolate	Bacterial concentration (CFU/ml)	Positive LAMP assays
J13-14	BALF	*S. aureus*	1.6×10^{2}	
J13-15	TW	*S. aureus*	8.6×10^{4}	
J13-16	BALF	*Streptococcus zooepidemicus*	4.2×10^{8}	Sz- and Bac-Pre-LAMP
		Pasteurella pneumotropica	6.0×10^{5}	
J13-17	BALF	*Streptococcus zooepidemicus*	1.2×10^{7}	Sz-LAMP
		Pasteurella pneumotropica	9.0×10^{3}	
J13-18	right pleural effusion	*Streptococcus zooepidemicus*	9.5×10^{4}	Sz-LAMP
		Pasteurella pneumotropica	$3..0\times10^{3}$	
J13-19	left pleural effusion	*Streptococcus zooepidemicus*	2.6×10^{5}	Sz-LAMP
		Pasteurella pneumotropica	5.0×10^{3}	
J13-20	BALF	Gram-negative facultative anaerobic rod	5.0×10^{4}	
		Streptococcus zooepidemicus	3.2×10^{4}	
J13-21	BALF	N.D.	-	
J13-22	pleural effusion	N.D.	-	
J13-23	BALF	*Bacteroides fragilis*	2.4×10^{6}	Sz- and Bac-Pre-LAMP
		Peptostreptococcus anaerobius	6.2×10^{6}	
		Streptococcus zooepidemicus	1.6×10^{4}	
J13-24	BALF	*Pseudomonas aeruginosa*	6.0×10	
J13-25	BALF	*Pantoea* sp.	1.4×10^{3}	
J13-26	TW	*Pantoea* sp.	3.0×10	Ec-LAMP
J13-27	BALF	*E. coli*	1.4×10^{3}	Ec-LAMP
		Enterobacteriaceae	4.6×10^{2}	
J13-28	BALF	*Streptococcus zooepidemicus*	1.8×10^{4}	Sz-LAMP
J13-29	BALF	*Bacillus* sp.	5.4×10^{2}	Bac-Pre-LAMP
J13-30	BALF	*Streptococcus zooepidemicus*	4.2×10^{4}	Sz- and Sm-LAMP
		Gram-positive facultative anaerobic rod	7.2×10^{2}	
J13-31	BALF	Obligate anaerobe	3.9×10^{8}	Bac-Pre-LAMP
		Gram-positive facultative anaerobic rod	5.8×10^{6}	
		Gram-negative facultative anaerobic cocci	4.0×10^{5}	
		Gram-negative obligate aerobic rod	1.2×10^{5}	
		Gram-negative facultative anaerobic rod	5.0×10^{4}	
		Streptococcus zooepidemicus	3.4×10^{4}	
J13-32	BALF	Gram-negative obligate anaerobic rod 1	1.0×10^{8}	Sz- and Bac-Pre-LAMP
		Gram-negative obligate anaerobic rod 2	2.0×10^{7}	
		Gram-positive facultative anaerobic rod	1.0×10^{5}	
		Streptococcus zooepidemicus	5.0×10^{3}	
J13-33	BALF	Gram-negative obligate anaerobic rod	2.8×10^{6}	Sz- and Bac-Pre-LAMP
		Streptococcus zooepidemicus	1.0×10^{6}	
J13-34	TW	Gram-negative obligate anaerobic rod 1	1.0×10^{6}	Sz- and Bac-Pre-LAMP
		Gram-negative obligate anaerobic rod 2	4.0×10^{5}	
		Streptococcus zooepidemicus	1.0×10^{4}	
J13-35	BALF	Gram-negative obligate anaerobic rod 1	3.2×10^{6}	Bac-Pre-LAMP
		Gram-negative obligate anaerobic rod 2	5.0×10^{5}	
		Streptococcus zooepidemicus	2.4×10^{4}	
J13-36	TW	alpha-hemolytic streptococci	2.8×10^{2}	
J14-1	right pleural effusion	*Clostridium* spp.	8.0×10^{4}	
		Streptococcus zooepidemicus	1.6×10^{3}	
J14-2	left pleural effusion	*Streptococcus zooepidemicus*	1.7×10^{3}	Sz-LAMP
J14-3	BALF	alpha-hemolytic Gram-positive facultative anaerobic cocci	3.2×10^{4}	
		Gram-positive facultative anaerobic coccobacillus	1.6×10^{3}	
J14-4	BALF	N.D.	-	Bac-Pre-LAMP
J14-5	pleural effusion	*Bacteroides* sp. or *Prevotella* sp.	2.0×10^{4}	Bac-Pre-LAMP
		Prevotella sp.	2.0×10^{3}	
		Streptococcus zooepidemicus	1.6×10^{2}	

a) TW: Tracheal wash
b) BALF: Bronchoalveolar lavage fluid
c) N.D.: Not detected
d) Blank columns indicate negative results.

Table 2-10. Comparison between LAMP assays and bacterial culture of respiratory specimens from horses.

LAMP assays	Results of LAMP	Bacterial cultivation (CFU/ml)			Accordance rate 1 [a)] (%)	Accordance rate 2 [a)] (%)
		$\geq 1 \times 10^4$	≥ 20 to $< 1 \times 10^4$	< 20		
Sz-LAMP	Positive	16	2	1	91.0	85.1
	Negative	3	6	39		
Bac-Pre-LAMP	Positive	13	1	5	91.0	88.1
	Negative	0	3	45		
Ec-LAMP	Positive	0	1	3	94.0	95.5
	Negative	0	0	63		
Kp-LAMP	Positive	3	1	0	98.5	100.0
	Negative	0	0	63		
Sm-LAMP	Positive	0	1	1	97.0	97.0
	Negative	0	1	64		
Pa-LAMP	Positive	0	0	0	100	97.0
	Negative	0	2	65		
Sa-LAMP	Positive	0	0	0	98.5	97.0
	Negative	1	1	65		

a) Accordance rate 1 was calculated on the basis of the bacterial culture value considered clinically important (1×10^4 CFU/ml). Accordance rate 2 was calculated by using the detection limit of bacterial culture (20 CFU/ml).

Fig. 2－1

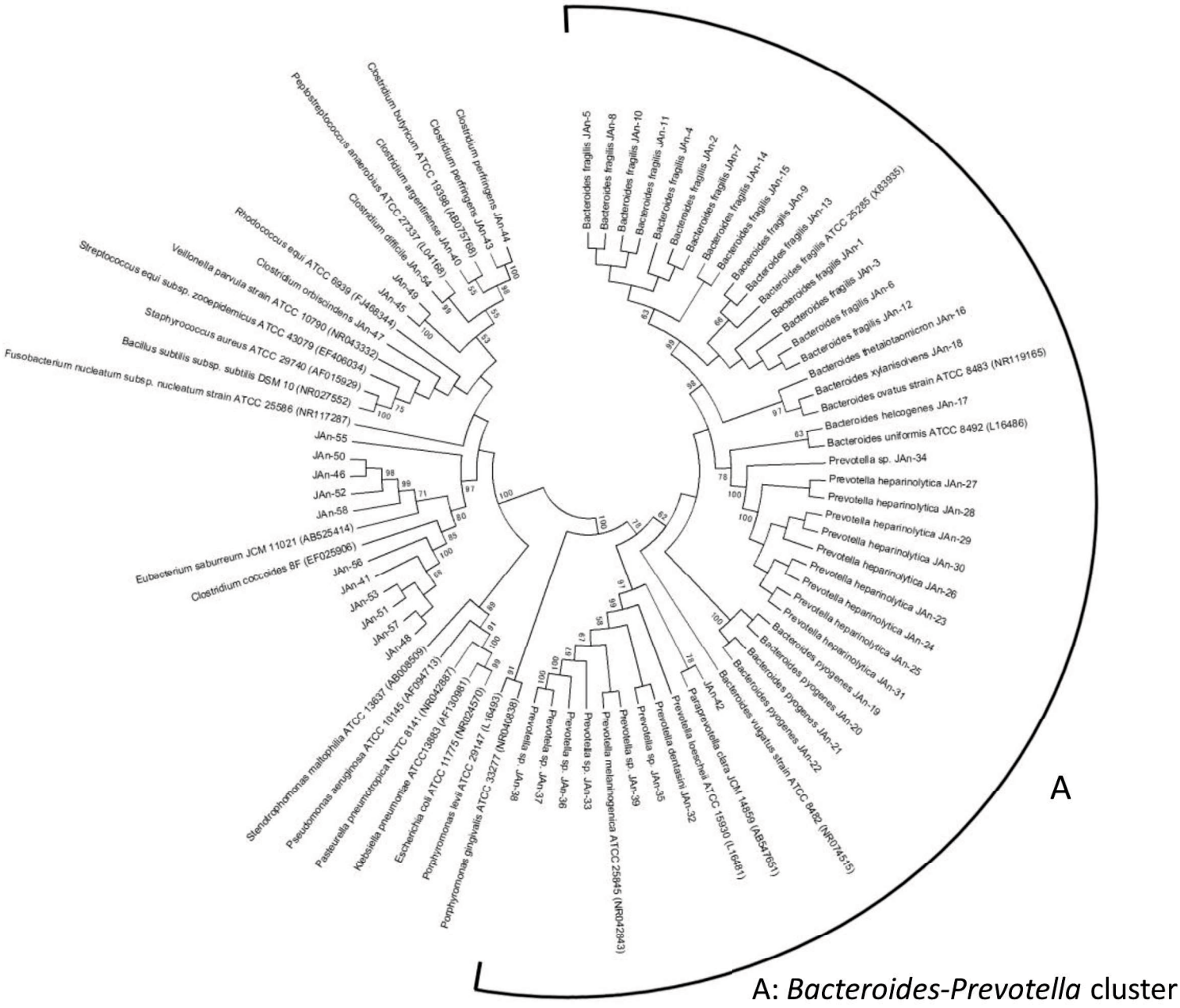

Fig. 2－1. Neighbor-joining phylogenetic tree derived from 16S rRNA gene sequences, showing the separation of *Bacteroides* and *Prevotella* from other obligate anaerobes, as well as from aerobes and facultative anaerobes. Accession numbers of published sequences are shown in parentheses. Bootstrap values (>50%) based on 1,000 replications are shown at branch nodes.

CHAPTER 3

Antimicrobial susceptibility patterns of dominant pathogenic bacteria from equine respiratory tract of ill horses

Summary

For appropriate antimicrobial use, veterinary practitioners should select antimicrobials on the basis of evidence, namely isolation frequencies of each pathogenic bacterium and antimicrobial susceptibility patterns. In this study, the author revealed antimicrobial susceptibility patterns of representative causative bacteria of respiratory tract in adult horses; *Streptococcus equi* subsp. *zooepidemicus*, obligate anaebores, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia,* and *Staphylococcus aureus*. 22 antimicrobials were examined for their susceptibilities against facultative anaerobic and aerobic bacteria, and 11 antimicrobials were examined against obligate anaerobes. The results showed all of *S. zooepidemicus* isolates were susceptible to all β-lactams, indicating β-lactams are first-line drug for bacterial infections in lower respiratory tract in adult horses. On the other hands, *S. zooepidemicus* and *E. coli* showed an increase in percent of isolates resistant to tetracycline and minocycline, in particular, minocycline-resistant *S. zooepidemicus* has significantly increased in 2010-2014. As for obligate anaerobes, all *Bacteroides* and *Prevotella* isolates were susceptible to imipenem, clindamycin, and metronidazole. These antimicrobial susceptibility patterns could be efficient evidence; veterinary practitioners could select appropriate antimicrobials for bacterial infections in lower respiratory tract in adult horses immediately after causative bacteria are revealed by using the LAMP methods described in CHAPTER 2.

Introduction

Antimicrobial use is one of the essential therapies to treat with bacterial infection in lower respiratory tract in horses, and veterinary practitioners have used various antimicrobials for those horses [28, 68]. Inappropriate antimicrobial therapies, e.g., usage against antimicrobial resistant pathogens, or improper dosage or interval, are associated with increased morbidity and fatality [26]. Therefore, veterinary practitioners should select appropriate antimicrobials on the basis of evidence, namely isolation frequencies of each pathogenic bacterium and its antimicrobial susceptibility patterns. Antimicrobial susceptibility tests of each bacterium should be ideally conducted in each clinical case. However, most veterinary practitioners are faced with difficulties to reveal antimicrobial susceptibility of the pathogens in each clinical case, because there are many limitations; e.g., no facilities for conducting susceptibility tests, short-handed, and costs. Moreover, even if veterinary practitioners proceed with an order of susceptibility tests, they should select antimicrobials empirically until results of susceptibility tests are revealed. Thus, many veterinary practitioners rely on antimicrobial susceptibility patterns of past strains. Antimicrobial susceptibility patterns of bacteria vary depending on the species or genus [70], geographic location, and hospital-specific factors [40, 78]. Therefore, veterinary practitioners should acquire antimicrobial susceptibility patterns which are constructed by accumulating the patterns of each regional bacterium.

In this CHAPTER, the author revealed antimicrobial susceptibility patterns of the pathogenic bacteria which cause bacterial infection in lower respiratory tract in horses and were also targeted of the LAMP methods in CHAPTER 2; i.e., *S. zooepidemicus*, obligate anaerobes, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *S. maltophilia,* and *S. aureus*. Combination of the results of the LAMP assays described in

CHAPTER 2 and the susceptibility patterns revealed in this chapter could enable veterinary practitioners to select appropriate antimicrobials rationally on the basis of evidence.

Materials and Methods

Bacteria used in this study

All bacterial isolates were collected from ill horses with respiratory tract infections in this study. Primary isolated strain was used if same bacterial species were isolated from several specimens of a single horse. Detailed information of bacterial isolates used in this study is shown in Table 3-1.

Antimicrobial susceptibility test

The author measured the minimum inhibitory concentrations (MICs) of antimicrobials by using customized commercial panels (Eiken Chemical Co., Ltd., Tokyo, Japan). The MIC_{50} was the concentration of drug required to inhibit the growth of 50% of the isolates tested, and the MIC_{90} was the concentration required to inhibit 90% of them. The panels for all strains except for obligate anaerobes include 22 antimicrobials, namely, penicillin, ampicillin, cephalothin, ceftiofur, ceftriaxone, imipenem, enrofloxacin, marbofloxacin, levofloxacin, garenoxacin, vancomycin, sulfamethoxazole/trimetoprim, tetracycline, minocycline, tobramycin, amikacin, gentamicin, kanamycin, rifampicin, fosfomycin, chloramphenicol, and azithromycin. The panel for obligate anaerobes includes nine antimicrobials; penicillin, ampicillin, cephalothin, imipenem, clindamycin, metronidazole, minocycline, doxycycline, and moxifloxacin. The results were interpreted according to CLSI guidelines [21, 22, 24] and the author used *Bacteroides fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29741, and *Staphylococcus aureus* ATCC 29213 as quality control strains. MICs of the antimicrobial agents for these three strains were within the quality control limits described in CLSI standard M100-S22 [24].

Statistics

Fisher's exact test was conducted for statistical analysis of comparisons between years for each resistant rate. A *P*-value ≤ 0.05 was considered to indicate a significant difference in this study.

Results

Antimicrobial susceptibility patterns of the five facultative anaerobic and one aerobic bacteria are shown in Table 3-2. All isolates of *S. zooepidemicus* were susceptible to β-lactams, levofloxacin, and sulfamethoxazole/trimethoprim in this study. On the other hands, all of *S. zooepidemicus* were resistant to aminoglycosides such as amikacin, gentamicin, and kanamycin. *S. zooepidemicus* showed an increase in percent of isolates resistant to tetracycline and minocycline, in particular, minocycline-resistant *S. zooepidemicus* significantly increased in 2010-2014 (Table 3-3). Similarly, *E. coli* showed an increase in percent of isolates resistant to tetracycline and minocycline. However, all of *E. coli* were susceptible to ceftriaxone, imipenem, and fosfomycin, and 91.7% to 95.8% of the isolates were susceptible to aminoglycosides and levofloxacin. As for *P. aeruginosa*, all of the isolates were susceptible to imipenem, levofloxacin, and amikacin. Except for amikacin, 24.1% to 96.6% of *P. aeruginosa* were resistant to aminoglycosides. Unlike *S. zooepidemicus*, 12 of 13 *S. maltophilia* isolates were resistant to sulfamethoxazole/trimethoprim in this study. All of the isolates were susceptible to levofloxacin and minocycline, and 76.9% to 100% of *S. maltophilia* were resistant to aminoglycosides. No isolates of *K. pneumonia* and *S. aureus* were resistant to the cephalosporins, and all of both species were susceptible to levofloxacin, sulfamethoxazole/trimethoprim, minocycline, and amikacin. Five of six *S. aureus* isolates were resistant to gentamicin and kanamycin.

As for obligate anaerobes, *Bacteroides* isolates tended to be resistant to most of the β-lactam antimicrobials, including penicillin, ampicillin, cephalothin (Table 3-4). In contrast, approximately 80% of *Prevotella* isolates were susceptible to these antimicrobials. All *Bacteroides* and *Prevotella* isolates were susceptible to imipenem,

clindamycin and metronidazole.

Discussion

In Japan, clinically ill adult horses with bacterial infection in lower respiratory tract have been generally administered penicillins or cephalosporins as first-line drug, e.g., penicillin, cephalothin, cefazolin, because *S. zooepidemicus* which is primary causative pathogen of bacterial pneumonia tends to be susceptible to β-lactams [68]. Although a few *S. zooepidemicus* isolates which were resistant to β-lactams were reported in equine medicine in USA and UK [29, 40], all *S. zooepidemicus* isolates used in this study were susceptible to β-lactams. Therefore, penicillins and cephalosporins are still useful as first-line drug for bacterial infection in lower respiratory tract in adult horses in Japan. Combination of β-lactams and aminoglycosides are usually used for bacterial infection in lower respiratory tract in equine medicine because the combination can cover broad range of bacteria; Gram positive bacteria tend to be susceptible to β-lactams and Gram negative bacteria tend to be susceptible to aminoglycoside [28, 68]. Moreover, the combination was reported to have synergy effects to some bacteria in human medicine [47, 83]. Although 55% to 87.5% of *S. zooepidemicus* and 89% to 100% of *S. aureus* isolates were reported to be gentamicin-susceptible in some previous papers [20, 29, 73], all *S. zooepidemicus* and five of six *S. aureus* isolates were resistant to gentamicin and kanamycin in this study. Clinical efficacy of the combination of β-lactams and aminoglycosides for the resistant strains is unclear, even though synergy effects might exist. Levofloxacin showed good efficacies in *in vitro* experiments for facultative anaerobic and aerobic bacteria in this study. Moreover, new fluoroquinolones, unlike aminoglycosides, retain high activity in acid environment and in abscess cavity [13, 82]. Therefore, the combinations of β-lactams and new fluoroquinolones might be more effective than those of β-lactams and aminoglycosides to treat the mixed

infections caused by Gram-positive and Gram-negative bacteria.

Although Sulfamethoxazole/trimethoprim is used for bacterial infection in lower respiratory tract in foreign equine medicine [17, 51, 81], only 30.9% to 63% of *S. zooepidemicus* isolates were susceptible to sulfamethoxazole/trimethoprim in some country [20, 29, 73]. However, because all *S. zooepidemicus* isolates were susceptible to sulfamethoxazole/trimethoprim in this study, sulfamethoxazole/trimethoprim might be effective to treat with a bacterial infection in lower respiratory tract in Japan. Sulfamethoxazole/trimethoprim is also used for some pathogens such as pneumocystis pneumonia [71] or *S. maltophilia* infection in human medicine [19]. Unlike *S. zooepidemicus*, 12 of 13 *S. maltophilia* isolates were resistant to sulfamethoxazole/trimethoprim in this study and thus, sulfamethoxazole/trimethoprim could not be recommended to treat with the lower respiratory tract infections caused by *S. maltophilis* in equine medicine in Japan.

In addition to β-lactams, minocycline is frequently added for severe bacterial pneumonia or pleuropneumonia in equine medicine in Japan. However, because *S. zooepidemicus* and *E. coli* showed an increase in percent of isolates resistant to minocycline, veterinary practitioners should conduct antimicrobial susceptibility test and confirm the bacterial susceptibility to minocycline before the agent would be used.

In agreement with previous studies in human medicine [49, 69], most of my *Bacteroides* isolates were resistant to most β-lactams. In contrast, approximately 80% of *Prevotella* isolates were susceptible to β-lactams, although previous studies have reported that many *Prevotella* isolates in human medicine are resistant to penicillin [12, 49, 69]. 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 [15, 48, 76],

metronidazole-resistant *Bacteroides* and *Prevotella* strains have recently been reported in humans [70]. All of my *Bacteroides* and *Prevotella* isolates were susceptible to metronidazole, suggesting that this drug may still be useful against obligate anaerobes in horses.

Administration of antimicrobials is one of the standard therapies for bacterial pneumonia or pleuropneumonia in horses [68]. Veterinarians should empirically select antimicrobials on the basis of evidence; e.g., isolation frequencies of each causative bacterium and antimicrobial susceptibility patterns. Antimicrobial susceptibility patterns of bacteria vary depending on the species or genus [70], geographic location, and hospital-specific factors [40, 78]. Therefore, antimicrobial susceptibilities revealed in this chapter could be very useful information for bacterial infection in lower respiratory tract in horses in Japan. Furthermore, veterinarians can promptly decide reasonable selection of antimicrobials by using both LAMP methods described in CHAPTER 2 and the antimicrobial susceptibility patterns. The author considers that these quick identifications of causative pathogens and appropriate selection of antimicrobials lead to improvement of therapeutic efficacy for adult horses with lower respiratory bacterial infection.

Table 3-1. Detailed information of bacteria in this study

Bacteria	Numbers	Year of isolation	Specimens[a)]						
			BALF	TW	Pleural effusion	Lung	Guttural pouch	Nasal swab	Other[b)]
Streptococcus zooepidemicus	66	2000-2014	44	9	5	2	1	1	4
Esherichia coli	24	1985-2013	12	4	1	1			6
Pseudomonas aeruginosa	29	1993-2013	15	4	1		5		4
Stenotrophomonas maltophilia	13	1995-2013	9	3			1		
Klebsiella pneumoniae	7	1995-2013	5			1	1		
Staphylococcus aureus	6	1996-2013	2	3		1			
Obligate anaerobes	75	2001-2014	51		14	6			4

a) BALF, bronchoalveolar lavage fluid; TW, trachial wash

b) Other include unplaceable

Table 3-2. Antimicrobial susceptibilities of facultative anaerobic and aerobic bacteria to 22 antimicrobial agents

Bacteria	Antimicrobials	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	Suscestible (%)	Intermediate (%)	Resistant (%)
Streptococcus zooepidemicus (n=66)	Ampicillin	≤0.12	≤0.12	100	0	0
	Penicillin	≤0.06	≤0.06	100	0	0
	Cephalothin	0.12	0.12	100	0	0
	Ceftriaxone	≤0.25	≤0.25	100	0	0
	Ceftiofur	0.25	0.25	100	0	0
	Imipenem	≤0.12	≤0.12	100	0	0
	Enrofloxacin	1	1	-[a)]	-	-
	Marbofloxacin	1	2	-	-	-
	Levofloxacin	1	1	100	0	0
	Garenoxacin	≤0.06	≤0.06	-	-	-
	Vancomycin	0.5	0.5	100	0	0
	Sulfamethoxazole/Trimethoprim	≤2.38/0.12	4.75/0.25	100	0	0
	Tetracycline	4	>8	39.4	30.3	30.3
	Minocycline	≤0.25	8	74.2	9.1	16.7
	Tobramycin	16	>16	-	-	-
	Amikacin	>64	>64	0	0	100
	Gentamicin	>8	>8	0	0	100
	Kanamycin	64	>64	0	0	100
	Rifampicin	≤1	≤1	-	-	-
	Fosfomycin	32	64	-	-	-
	Chloramphenicol	≤2	4	100	0	0
	Azithromycin	≤0.25	≤0.25	98.5	0	1.5
Escherichia coli (n=24)	Ampicillin	8	>32	83.3	0	16.7
	Penicillin	>0.5	>0.5	-	-	-
	Cephalothin	8	32	54.2	29.2	16.7
	Ceftriaxone	≤0.5	≤0.5	100	0	0
	Ceftiofur	0.5	0.5	-	-	-
	Imipenem	≤1	≤1	100	0	0
	Enrofloxacin	≤0.5	≤0.5	-	-	-
	Marbofloxacin	≤0.5	≤0.5	-	-	-
	Levofloxacin	≤1	≤1	95.8	0	4.2
	Garenoxacin	≤0.12	≤0.12	-	-	-
	Vancomycin	>32	>32	0	0	100
	Sulfamethoxazole/Trimethoprim	≤19/1	>76/4	75	0	25
	Tetracycline	≤4	>16	62.5	0	37.5
	Minocycline	≤2	16	66.7	12.5	20.8
	Tobramycin	≤4	≤4	95.8	4.2	0
	Amikacin	≤16	≤16	95.8	4.2	0
	Gentamicin	≤2	≤2	91.7	4.2	4.2
	Kanamycin	≤16	>64	83.3	0	16.7
	Rifampicin	>4	>4	-	-	-
	Fosfomycin	≤64	≤64	100	0	0
	Chloramphenicol	8	16	87.5	4.2	8.3
	Azithromycin	8	>8	-	-	-

a) Median line indicates that the CLSI guidelines do not contain criteria.

Table 3-2. Antimicrobial susceptibilities of facultative anaerobic and aerobic bacteria to 22 antimicrobial agents (*continued*)

Bacteria	Antimicrobials	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	Suscestible (%)	Intermediate (%)	Resistant (%)
Pseudomonas aeruginosa (n=29)	Ampicillin	>32	>32	-	-	-
	Penicillin	>0.5	>0.5	-	-	-
	Cephalothin	>32	>32	0	0	100
	Ceftriaxone	16	32	-	-	-
	Ceftiofur	>8	>8	-	-	-
	Imipenem	≤1	2	100	0	0
	Enrofloxacin	1	2	-	-	-
	Marbofloxacin	≤0.5	1	-	-	-
	Levofloxacin	≤1	≤1	100	0	0
	Garenoxacin	2	2	-	-	-
	Vancomycin	>32	>32	-	-	-
	Sulfamethoxazole/Trimethoprim	>76/4	>76/4	-	-	-
	Tetracycline	>16	>16	0	0	100
	Minocycline	16	>16	0	3.4	96.6
	Tobramycin	≤4	>16	75.9	0.0	24.1
	Amikacin	≤16	≤16	100	0	0
	Gentamicin	≤2	>8	58.6	10.3	31.0
	Kanamycin	>64	>64	3.4	0	96.6
	Rifampicin	>4	>4	-	-	-
	Fosfomycin	≤64	128	-	-	-
	Chloramphenicol	>32	>32	0	0	100
	Azithromycin	>8	>8	-	-	-
Stenotrophomonas maltophilia (n=13)	Ampicillin	>32	>32	-	-	-
	Penicillin	>0.5	>0.5	-	-	-
	Cephalothin	>32	>32	0	0	100
	Ceftriaxone	>64	>64	-	-	-
	Ceftiofur	>8	>8	-	-	-
	Imipenem	>16	>16	0	0	100
	Enrofloxacin	1	1	-	-	-
	Marbofloxacin	1	1	-	-	-
	Levofloxacin	2	2	100	0	0
	Garenoxacin	4	4	-	-	-
	Vancomycin	>32	>32	-	-	-
	Sulfamethoxazole/Trimethoprim	>76/4	>76/4	7.7	-	92.3
	Tetracycline	>16	>16	0	7.7	92.3
	Minocycline	≤2	≤2	100	0	0
	Tobramycin	>16	>16	-	-	-
	Amikacin	>64	>64	23.1	0	76.9
	Gentamicin	>8	>8	-	-	-
	Kanamycin	>64	>64	0	0	100
	Rifampicin	>4	>4	-	-	-
	Fosfomycin	≤64	128	-	-	-
	Chloramphenicol	>32	>32	0	23.1	76.9
	Azithromycin	>8	>8	-	-	-

Table 3-2. Antimicrobial susceptibilities of facultative anaerobic and aerobic bacteria to 22 antimicrobial agents (*continued*)

Bacteria	Antimicrobials	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	Suscestible (%)	Intermediate (%)	Resistant (%)
Klebsiella pneumoniae (n=7)	Ampicillin	32	32	0	0	100
	Penicillin	>0.5	>0.5	-	-	-
	Cephalothin	2	2	100	0	0
	Ceftriaxone	≤0.5	≤0.5	100	0	0
	Ceftiofur	0.5	≤0.25	-	-	-
	Imipenem	≤1	≤1	100	0	0
	Enrofloxacin	≤0.5	≤0.5	-	-	-
	Marbofloxacin	≤0.5	≤0.5	-	-	-
	Levofloxacin	≤1	≤1	100	0	0
	Garenoxacin	≤0.12	≤0.12	-	-	-
	Vancomycin	>32	>32	0	0	100
	Sulfamethoxazole/Trimethoprim	≤19/1	≤19/1	100	0	0
	Tetracycline	≤4	≤4	100	0	0
	Minocycline	≤2	≤2	100	0	0
	Tobramycin	≤4	≤4	100	0	0
	Amikacin	≤16	≤16	100	0	0
	Gentamicin	≤2	≤2	100	0	0
	Kanamycin	≤16	>64	85.7	0	14.3
	Rifampicin	>4	>4	-	-	-
	Fosfomycin	≤64	≤64	100	0	0
	Chloramphenicol	≤4	≤4	100	0	0
	Azithromycin	>8	>8	-	-	-
Staphylococcus aureus (n=6)	Ampicillin	>32	>32	0	-	100
	Penicillin	>0.5	>0.5	0	-	100
	Cephalothin	≤0.5	1	100	0	0
	Ceftriaxone	4	16	83.3	16.7	0
	Ceftiofur	1	4	-	-	-
	Imipenem	<1	<1	100	0	0
	Enrofloxacin	≤0.5	≤0.5	-	-	-
	Marbofloxacin	≤0.5	1	-	-	-
	Levofloxacin	≤1	≤1	100	0	0
	Garenoxacin	≤0.12	≤0.12	-	-	-
	Vancomycin	≤1	≤1	100	0	0
	Sulfamethoxazole/Trimethoprim	≤19/1	≤19/1	100	0	0
	Tetracycline	16	>16	16.7	16.7	66.7
	Minocycline	≤2	≤2	100	0	0
	Tobramycin	>16	>16	16.7	0	83.3
	Amikacin	≤16	≤16	100	0	0
	Gentamicin	>8	>8	16.7	0	83.3
	Kanamycin	>64	>64	16.7	0	83.3
	Rifampicin	≤1	≤1	100	0	0
	Fosfomycin	≤64	≤64	-	-	-
	Chloramphenicol	8	>32	50.0	16.7	33.3
	Azithromycin	1	>8	83.3	0	16.7

Table 3-3. Proportion of *Streptococcus zooepidemicus* and *Escherichia coli* isolates resistant to tetracycline or minocycline

Bacteria	Antimicrobials	Numbers of resistant bacteria (%)		
		-2004	2005-2009	2010-2014
Streptococcus zooepidemicus	tetracycline	7/30 (23.3)	4/18 (22.2)	9/18 (50)
	minocycline	3/30 (10.0)†	1/18 (5.6)†	7/18 (38.9)
Escherichia coli	tetracycline	1/9 (11.1)	4/8 (50)	4/7 (57.1)
	minocycline	1/9 (11.1)	1/8 (12.5)	3/7 (42.9)

†, Dagger indicates significant lower number of resistant isolates than those which were isolated in 2010-2014.

Table 3-4. Antimicrobial susceptibilities of 75 obligate anaerobes to 9 antimicrobial agents

Bacteria	Antimicrobials	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	Suscestible (%)	Intermediate (%)	Resistant (%)
Total anaerobes (n = 75)	Penicillin	0.5	>4	52	5.3	42.7
	Ampicillin	≦ 0.5	>16	56	2.7	41.3
	Cephalothin	4	>32	61.3	1.3	37.3
	Imipenem	≦ 0.5	1	98.7	0	1.3
	Clindamycin	≦ 0.25	1	94.7	0	5.3
	Metronidazole	≦ 1	4	94.7	0	5.3
	Minocycline	1	4	93.3	6.7	0
	Doxycycline	4	8	86.7	12	1.3
	Moxifloxacin	0.5	4	82.7	16	1.3
Bacteroides spp. (n = 25)	Penicillin	>4	>4	8	0	92
	Ampicillin	16	>16	8	0	92
	Cephalothin	>32	>32	12	0	88
	Imipenem	≦ 0.5	≤1	100	0	0
	Clindamycin	≦ 0.25	0.5	100	0	0
	Metronidazole	2	4	100	0	0
	Minocycline	2	4	100	0	0
	Doxycycline	4	8	88	12	0
	Moxifloxacin	0.5	2	100	0	0
Prevotella spp. and related strains (n = 27)	Penicillin	≤0.25	4	74.1	7.4	18.5
	Ampicillin	≦ 0.5	2	81.5	0	18.5
	Cephalothin	≦ 1	32	81.5	3.7	14.8
	Imipenem	≤1	1	100	0	0
	Clindamycin	≦ 0.25	0.5	100	0	0
	Metronidazole	≦ 1	2	100	0	0
	Minocycline	≦ 0.5	8	85.2	14.8	0
	Doxycycline	8	≤4	81.5	14.8	3.7
	Moxifloxacin	0.5	2	96.3	3.7	0

CONCLUSION

In this thesis, the author have described studies relating to bacterial infection of the lower respiratory tract in adult horses with the aim of enabling veterinary practitioners to diagnose the causative bacteria promptly and to select appropriate antimicrobials.

In CHAPTER 1, the author described the use of 16S rRNA gene sequencing for precise species-level identification of obligate anaerobes isolated from the lower respiratory tract of ill horses, with the aim of revealing the causative obligate anaerobes. The obligate anaerobes that the author isolated most commonly from horses with signs of lower respiratory tract infection were *Bacteroides* spp. (22 of 58 isolates; 37.9%)—in particular *B. fragilis* (15 isolates). The second most commonly isolated obligate anaerobes were *Prevotella* spp. (17 of 58 isolates; 29.3%)—in particular *P. heparinolytica* (nine isolates). Isolates belonging to either *Bacteroides* or *Prevotella* were found in 93.8% (30 of 32 horses) of horses that were sick with lower respiratory tract infection involving obligate anaerobes. The results indicated that strains of *Bacteroides* and *Prevotella* were the most important obligate anaerobes in lower respiratory tract infection in adult horses.

In CHAPTER 2, the author described my development of five LAMP methods to detect bacteria causing lower respiratory tract infection in horses, namely *S. equi* subsp. *zooepidemicus*, strains of the *Bacteroides-Prevotella* group, *K. pneumoniae*, *S. maltophilia*, and *S. aureus*. Four of the newly developed LAMPs (Sz-, Kp-, Sm-, and Sa-LAMPs) were confirmed to be species-specific, whereas Bac-Pre-LAMP was verified to broadly detect bacteria of the *Bacteroides-Prevotella-Porphyromonas* group, which were dominant obligate anaerobes in lower respiratory tract specimens from

horses. The author then compared the clinical efficacies of three DNA extraction methods, namely the Loopamp PURE DNA Extraction Kit, InstaGene Matrix, and boiling. Thirteen of 14 clinical samples and 34 of 34 samples spiked with *S. zooepidemicus* at concentrations greater than 1×10^4 CFU/ml were positive by Sz-LAMP plus the Loopamp PURE DNA Extraction Kit; 11 of these 14 clinical samples and 31 of 34 spiked samples were positive with LAMP plus InstaGene Matrix, and 7 and 27 were positive with LAMP plus boiling. The inhibitors remaining after DNA extraction using InstaGene Matrix or boiling might have inhibited the LAMP reaction; the Loopamp PURE DNA Extraction Kit would have been more likely than the other extraction methods to reduce the effect of these inhibitors, indicating that this kit was the most suitable DNA extraction method for use on clinical samples from the equine respiratory tract. The use of seven LAMP methods—the five new developed ones and two previously described ones targeting *E. coli* and *P. aeruginosa*—in combination with the Loopamp PURE DNA Extraction Kit obtained high accordance rates with bacterial culture: 91.0% to 100% (using a threshold of 1×10^4 CFU/ml) and 85.1% to 100% (using 20 CFU/ml). These high accordance rates indicated that LAMP assays could be useful tools for detecting the bacterial pathogens causing lower respiratory tract infection in horses.

In CHAPTER 3, the author revealed the antimicrobial susceptibility patterns of those pathogenic bacteria for which the LAMP detection methods in CHAPTER 2 had been developed. Beta-lactams such as penicillin and cephalothin are useful as first-line drugs for bacterial infection of the adult equine lower respiratory tract because the dominant causative bacterium, *S. zooepidemicus*, was consistently susceptible to these agents in this study. The percentage of minocycline-resistant *S. zooepidemicus* and *E. coli* isolates increased between 2010 and 2014. The dominant causative obligate

anaerobes, *Bacteroides* and *Prevotella*, were all susceptible to metronidazole, although metronidazole-resistant *Bacteroides* and *Prevotella* strains have recently been reported in humans. This result suggests that metronidazole may still be useful against obligate anaerobes in horses. The information revealed here on antimicrobial susceptibility patterns could be very useful for Japanese veterinarians to select appropriate antimicrobials for horses with bacterial infections of the lower respiratory tract.

Using the LAMP methods plus the Loopamp PURE DNA Extraction Kit will enable veterinary practitioners to detect seven dominant bacterial pathogen groups in the lower respiratory tract of adult horses. These methods can be applied in the clinic with the naked eye and take no more than 90 min. Veterinary practitioners can select appropriate antimicrobials as the initial step in treatment through the quick identification of causative bacteria and antimicrobial susceptibility patterns. These swift and appropriate therapies will improve therapeutic efficacy in adult horses with lower respiratory bacterial infection.

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