J. Rakuno Gakuen Univ., **43** (2) : 85～96 (2019)

Comprehensive identification of bacteria in pig lung abscesses using the MALDI Biotyper system

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MALDI Biotyper を用いた豚の肺膿瘍における網羅的細菌解析

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(Accepted 3 December 2018)

ABSTRACT

Pig lung lesions typified by abscesses are one of the most serious and common problems facing the swine industry worldwide. The major primary causative agents of lung lesions are reported to be the bacteria *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae*, whereas *Streptococcus suis*, *Haemophilus parasuis* and *Pasteurella multocida* are considered secondary pathogens that may exacerbate the infection. To fully understand these multiple infections, comprehensive bacterial identification that includes minor bacterial species is necessary. However, recent studies have only focused on the major bacteria listed above. Here, we isolated bacteria from the lung abscesses (or healthy lung tissue) and tonsilla specimens of 135 pigs, and identified the species present using a MALDI Biotyper, a proteomic-based bacterial identification tool. Of 1,206 isolates, 981 were identified to the species level and another 126 isolates were identified to the genus level. *A. pleuropneumoniae* was isolated from 68% of the lung abscess samples, and coinfection with *Escherichia coli*, *P. multocida* or *Streptococcus* spp. was observed in 56% of samples. These four bacteria were isolated significantly more frequently from lung abscesses than from healthy lung tissue, but there was no difference in the prevalence among tonsillar microbiota. Principal component analysis revealed that the constitution of lung abscesses was related to the higher prevalence of *A. pleuropneumoniae*, *E. coli* and *P. multocida* in the lungs, and lower daily weight gain. Our results contribute towards a comprehensive understanding of the multiple bacterial infections in pig lung abscesses.

Key Words: Bruker MALDI Biotyper, lung abscess, matrix-assisted laser desorption ionization-time of flight mass spectrometry, pig, tonsilla microbiota.

INTRODUCTION

Lung lesions, which appear macroscopically as lung abscesses at slaughtering, are one of the most serious problems facing pig farming worldwide[21]. Economical losses associated with lung lesions and respiratory disease result from reduced weight gain and feed efficiency[11,21], in addition to the cost of antibiotics and other treatments[26]. Moreover, severe lung lesions, including pneumopleuritis, reduce the quality of the carcass. *Mycoplasma*

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hyopneumoniae and *Actinobacillus pleuropneumoniae* are considered the main primary bacterial pathogens causing lung lesions[7,27], and *Streptococcus suis*, *Haemophilus parasuis* and *Pasteurella multocida* are considered as secondary pathogens that can contribute to disease severity[4, 7]. To date, various studies have been conducted to elucidate the multiple infectious agents present in pig lung lesions; however, most of these studies have focused on the major bacterial species[4,7,27] rather than the contribution of minor bacterial species. Therefore, comprehensive evaluation of the causative agents of pig lung abscesses, including minor bacterial species, and risk analyses of the microbiota are required.

Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) and next-generation sequencing are effective techniques for the proteomic and genomic identification of bacterial species, respectively. Furthermore, the MALDI Biotyper (Bruker, Billerica, MA, USA) system couples MALDI-TOF MS with bacterial molecular fingerprinting and mass spectral databases to comprehensively identify bacterial species[18], offering the advantages of low running costs, ease of use and rapid diagnosis[20]. The MALDI Biotyper system is widely used in human clinical medicine and is becoming a routine bacterial identification method[3,9], but has not yet been extensively applied in veterinary medicine.

In recent years, the increased incidence of pig lung abscesses and the waste associated with lung lesions was observed in some pig herds brought to the Higashimokoto Meat Inspection Center at Hokkaido, Japan. This study aimed to clarify the causative agents of the lung abscesses in the herd and to assess the microbiota of healthy pigs compared with diseased pigs.

MATERIALS AND METHODS

Sample collection

Lung and tonsillar samples of pigs were collected between April, 2016 and January, 2017 at the Higashimokoto Meat Inspection Center in Hokkaido, Japan. In total, 135 lung and tonsillar samples were collected, 100 of which were derived from farm X where the incidence of lung abscesses was higher than on other farms. The other 35 samples were derived from farm Y where lung abscesses were rarely observed. Among the 100 samples from farm X, 50 samples (referred to below as "group A") were collected from diseased pigs in which an abscess was observed in their lungs at slaughtering. Another 50 samples from farm X (referred to below as "group B") and 35 samples from farm Y (referred to below as "group C") were collected from healthy pigs that did not have any abscesses in their lungs. The peripheral tissue between a lung abscess and the normal tissue was collected from group A, but only normal tissue was collected from groups B and C. After sample collection, the refrigerated organs were sent to Rakuno Gakuen University and experiments were performed within 36 h of slaughtering.

Isolation of bacteria

Heart infusion agar plates (Nissui Pharmaceutical Co., Tokyo, Japan) containing 5% horse defibrinated blood and 0.002% β-nicotinamide adenine dinucleotide (NAD) (Wako Pure Chemical Industries, Osaka, Japan) were used for bacterial isolation as they allow for growth of a broad spectrum of bacteria. Lung and tonsillar samples were immediately washed with sterile 0.9% saline and were then dipped in 90% ethanol and exposed to a flame for a few seconds to burn the surface of the sample. The inside of the lung was exposed by excision using scissors and was then stamped onto the plate. For the tonsillar samples, 25 mg of tissue was punched out from inside the tissue, and homogenized in the saline to make a 10% tissue homogenate. Then, 25 μl of a 1% to 0.01% diluted homogenate was plated. All sample-inoculated plates were then incubated at 37°C under atmospheric conditions. After 24 to 36 h, six isolates were picked at random, and replated onto fresh plates. A sample of the bacterial growth was also collected and stored in 10% skimmed milk (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at −80°C for further experiments.

Bacteria identification using the MALDI Biotyper

Bacterial identification using the MALDI Biotyper was performed based on the ethanol—formic acid extraction procedure[1]. A loopful of bacteria were suspended in 150 μl of double-distilled water and mixed well by pipetting. Then 450 μl of

ethanol was added and the tube was centrifuged at 15,490×g for 2 min, and the supernatant was discarded. The bacterial pellet was resuspended in 10 to 20 μl of 70% formic acid, and the same volume of acetonitrile was added. The tube was centrifuged at 15,490×g for 2 min and 1 μl of the supernatant was transferred onto a MALDI target plate and dried at room temperature. Finally, 1 μl of HCCA matrix solution (Bruker) was applied on the sample. A bacterial test standard (Bruker) was used to calibrate the instrument. The acquisition of mass spectra was performed using an Autoflex speed (Bruker) accompanied by the software, Flex Control v3.4.105 for calibration and Biotyper Real Time Classification v2.0 for bacterial identification using the reference database (5,627 database entries).

Molecular characterization of Actinobacillus

Bacterial DNA was extracted from isolated colonies by the boiling method[17]. Polymerase chain reaction (PCR) was performed in 25 or 50 μl total reaction mixture containing *Ex Taq* buffer, 0.2 mM of each dNTP, 0.5 μM of forward and reverse primers, 0.025 units/μl of *Ex Taq* (TaKaRa Bio Inc. Shiga, Japan) and 2.5 or 5 μl of DNA template. *A. pleuropneumoniae* species-specific primers, LPF and LPR, were used to confirm the species[10]. Primers, C70 and B37, were used to amplify and then sequence the *16S rRNA* gene[12]. PCR was performed according to the PCR Thermal Cycler Dice® Gradient (TaKaRa Bio Inc.) and previously published thermal cycler conditions[10,12]. The PCR products were electrophoresed on a 1.5% agarose gel at 100 V for 30 to 40 min to examine the product sizes. Sequencing of the *16S rRNA* gene was performed by the FASMAC sequencing service (Fasmac Co., Ltd., Kanagawa, Japan).

Data analysis

Data on the carcass weights and the period of fattening were provided by the Higashimokoto Meat Inspection Center. A Student's t-test was used to compare the daily weight gain (DWG) among the groups. Fisher's exact test was used to compare the bacterial prevalence rate among the groups. Principal component analysis (PCA) was used to simplify several parameters, such as the existence of abscesses, farm and group differences and the isolated bacterial species. All statistical analyses were performed using the statistical analysis software R v3.4.4 or EZR v2.14 (Easy R)[14].

RESULTS

Identification of bacteria by MALDI Biotyper

From the 135 lung and 135 tonsillar samples, 1,206 bacteria were isolated (Table 1). Of the 1,206 isolates, 981 yielded identification scores of ≥2.0, which was considered acceptable for species level assignment[24]. Another 126 isolates showed identification scores of ≥1.7 but <2.0, which was considered acceptable for genus level assignment. The remaining 99 isolates showed identification scores of <1.7, which was considered unreliable. In this study, 46 species in 33 genera of bacteria were isolated. Among the isolates from lung samples, the predominant species was P. multocida (n=59), followed by *A. pleuropneumoniae* (n=53), *Actinobacillus lignieresii* (n=44), *Streptococcus porcinus* (n=39) and *Escherichia coli* (n=29). Among tonsillar samples, *Str. porcinus* (n=140) was predominant, followed by *P. multocida* (n=110), *E. coli* (n=107) and *Str. suis* (n=75).

Molecular characterization of Actinobacillus

To date, *A. lignieresii* has been reportedly isolated from cattle, horse, sheep and humans[15], but not pigs. To confirm the results obtained with the MALDI Biotyper, species-specific PCR and 16S rRNA sequencing analysis were performed on 29 randomly selected isolates identified by the MALDI Biotyper as *A. pleuropneumoniae* (n=19), *A. lignieresii* (n=8) and *Actinobacillus equuli* (n=2). *A. pleuropneumoniae* species-specific PCR products were observed for all *A. pleuropneumoniae* (19/19) isolates and the majority of *A. lignieresii* (7/8) isolates, indicating misidentification by the MALDI Biotyper. Two isolates of *A. equuli* did not show any amplification in the *A. pleuropneumoniae* species-specific PCR. Moreover, the *16S rRNA* sequences of four of the *A. pleuropneumoniae* isolates and two of the *A. lignieresii* isolates were obtained, but Basic Local Alignment Search Tool (BLAST) analysis could not distinguish these two species due to similarly high identity scores (99%) (Table 2). Finally, phylogenetic analysis clarified that *A.*

Table 1 MALDI Biotyper identification of bacteria isolated from lung and tonsillar samples

Bacteria isolated	Number of isolates						
	Group A		Group B		Group C		Total
	Lung (including abscess)	Tonsilla	Lung (Normal tissue)	Tonsilla	Lung (Normal tissue)	Tonsilla	
Acinetobacter spp.[a]	2		2		1		5
Actinobacillus equuli		2		1		4	7
Actinobacillus lignieresii[b]	44			2			46
Actinobacillus pleuropneumoniae	48		1	1	4	1	55
Actinobacillus rossii		1					1
Actinobacillus suis					1		1
Aeromonas spp.	3	2	1				6
Alcaligenes faecalis						1	1
Bacillus infantis		1					1
Bordetella sp.[c]	1						1
Brevundimonas diminuta					2		2
Burkholderia multivorans			1				1
Citrobacter sp.[a]	1	1					2
Corynebacterium spp.[a]			3	1			4
Cryseobacterium hominis			1				1
Enterobacter cloacae			1			1	2
Enterococcus faecium			1				1
Escherichia albertii				1			1
Escherichia coli	21	50	7	40	1	17	136
Escherichia fergusonii		1	1				2
Haemophilus haemolyticus				1			1
Haemophilus parasuis	4	7	4	6	3	4	28
Klebsiella pneumoniae		1	1				2
Klebsiella sp.[d]	1					2	3
Mannheimia sp.[a]					1		1
Microbacterium oxydans	1						1
Moraxella canis	1				3		4
Moraxella osloensis	3	1					4
Moraxella pluranimalium	3	4	7	1	8		23
Morganella morganii		1		1			2
Neisseria weaveri			1		1		2
Paenibacillus glucanolyticus					1		1
Pasteurella aerogenes	1	2		1	3		7
Pasteurella multocida	35	41	22	47	2	22	169
Proteus mirabilis				2			2
Proteus vulgaris		2		1			3
Providencia rettgeri						3	3
Providencia stuartii		1				1	2
Pseudomonas aeruginosa					2	1	3
Pseudomonas oryzihabitans			2				2
Pseudomonas putida			1				1
Pseudomonas sp.			1				1
Rothia nasimurium	4	4	3		4		15
Serratia sp.[e]			1				1
Staphylococcus aureus	10	25	13	19		4	71
Staphylococcus chromogenes	1	6	2	15		2	26
Staphylococcus epidermidis	1	2	1	4			8
Staphylococcus felis		2		1			3
Staphylococcus haemolyticus	1						1
Staphylococcus hyicus	2	16	4	17	1	10	50
Staphylococcus simulans	1	7		1		1	10
Stenotrophomonas maltophilia	2		4		1		7

Table 1のつづき

Bacteria isolated	Number of isolates						
	Group A		Group B		Group C		Total
	Lung (including abscess)	Tonsilla	Lung (Normal tissue)	Tonsilla	Lung (Normal tissue)	Tonsilla	
Streptococcus agalactiae		6		11			17
Streptococcus alactolyticus					2		2
Streptococcus gallinaceus		1				2	3
Streptococcus orisratti		1				2	3
Streptococcus parauberis	2						2
Streptococcus pluranimalium		1					1
Streptococcus porcinus	20	46		60	19	34	179
Streptococcus spp.[f]	4	30		25		5	64
Streptococcus suis	4	28		26	13	21	92
Streptococcus uberis		1					1
Trueperella pyogenes						1	1
Yersinia enterocolitica		2		7			9
Unidentified	12	20		12	22	33	99
Total isolates	233	316	86	304	95	172	1206

[a] Identification at species level is unable by MALDI Biotyper
[b] *A. lignieresii* identified by MALDI Biotyper is considered as *A. pleuropneumoniae* based on the further molecular confirmation
[c] Distinction among *Bordetella bronchiseptica / pertussis / parapertussis* is unable by MALDI Biotyper
[d] Distinction among *Klebsiella oxytoca / ornithinolytica / planticola / terrigena* is unable by MALDI Biotyper
[e] Distinction among *Serratia liquefaciens / grimesii* is unable by MALDI Biotyper
[f] Distinction among *Streptococcus canis / dysgalactiae / equi / pyogenes* is unable by MALDI Biotyper

lignieresii isolated in this study belonged to the same sub-cluster as *A. pleuropneumoniae*, and that this group was distinguishable from other *A. lignieresii* [M75068.1] (Fig. 1). In conclusion, *A. lignieresii* identified by MALDI Biotyper in this study is subsequently considered to be *A. pleuropneumoniae*.

Causative agents of lung abscesses

A. pleuropneumoniae was isolated from 34 out of 50 lung abscess samples from group A, suggesting that *A. pleuropneumoniae* was the primary causative agent of lung abscesses in this group (Fig. 2). Among these 34 samples, 6 samples showed a single infection of *A. pleuropneumoniae* and 28 samples showed co-infection of *A. pleuropneumoniae* and other bacteria, such as *Streptococcus* spp., *P. multocida*, *Staphylococcus aureus*, *E. coli* and *H. parasuis* (Fig. 2). Next, Fisher's exact test was applied to each bacterial isolation rate for the three groups for all of the species (data not shown), and four prospective species were determined to have *P*-values of <0.05 (Table 3). As expected in the lung sample, the isolation rate of *A. pleuropneumoniae* from group A was significantly higher than for groups B and C. The isolation rates of *E. coli* and *Str. suis* from group A were significantly higher than for group C, but no significant differences were detected between groups A and B and groups B and C. Between groups A and C and B and C, the isolation rates of *P. multocida* differed significantly in the lung samples. As for the tonsillar samples, the isolation rates of the above four species did not differ. In summary, infection of *A. pleuropneumoniae* accompanied by other bacteria such as *E. coli*, *P. multocida* and *Streptococcus* seems to contribute to the composition of lung abscesses.

Principal component analysis (PCA) and the influence of lung abscesses on growth

To clarify the relationship between the constitution of lung abscesses and multiple bacterial infections, PCA based on a correlation coefficient matrix was performed. Four bacterial species, *A. pleuropneumoniae*, *E. coli*, *P. multocida* and *Str. suis*, were selected according to the results of Fisher's exact tests. As shown in Fig. 3A, factor loadings of the constitution of lung abscesses and the prevalence of *A. pleuropneumoniae* and *E. coli* located in a similar area, indicated that these parameters are positively related to each other. By contrast, the factor loadings of the farm and DWG

Table 2 Molecular characterization of *Actinobacillus*

Sample ID	Tissue	Biotyper		*A. pleuropneumoniae* species-spesific PCR	BLAST identification based on 16S rRNA sequence*	
		Identification	Identification score		Identification	Identification score
AL34-1	Lung	*A. pleuropneumoniae*	2.322	+	*A. pleuropneumoniae*	99%
					A. lignieresii	99%
					A. equuli subsp. *equuli*	98%
AL37-6	Lung	*A. pleuropneumoniae*	2.402	+	*A. pleuropneumoniae*	99%
					A. lignieresii	99%
					A. equuli subsp. *equuli*	98%
AL38-2	Lung	*A. pleuropneumoniae*	2.326	+	*A. pleuropneumoniae*	99%
					A. lignieresii	99%
					A. equuli subsp. *haemolyticus*	98%
AL39-6	Lung	*A. lignieresii*	2.395	+	*A. pleuropneumoniae*	99%
					A. lignieresii	99%
					A. equuli subsp. *haemolyticus*	98%
AL40-1	Lung	*A. pleuropneumoniae*	2.353	+	*A. pleuropneumoniae*	99%
					A. lignieresii	99%
					A. equuli subsp. *haemolyticus*	98%
AL40-4	Lung	*A. lignieresii*	2.399	+	*A. pleuropneumoniae*	99%
					A. lignieresii	99%
					A. equuli subsp. *haemolyticus*	98%
AT39-4	Tonsilla	*A. equuli*	2.043	−	*A. equuli* subsp. *equuli*	99%
					A. equuli subsp. *haemolyticus*	99%
					A. suis	99%
AT39-6	Tonsilla	*A. equuli*	2.061	−	*A. equuli* subsp. *haemolyticus*	99%
					A. equuli subsp. *equuli*	99%
					A. suis	99%
AL32-1	Lung	*A. lignieresii*	2.083	−	ND	
AL32-5	Lung	*A. lignieresii*	2.362	+	ND	
AL33-3	Lung	*A. pleuropneumoniae*	2.389	+	ND	
AL34-4	Lung	*A. pleuropneumoniae*	2.388	+	ND	
AL34-5	Lung	*A. pleuropneumoniae*	2.310	+	ND	
AL35-2	Lung	*A. lignieresii*	2.319	+	ND	
AL35-5	Lung	*A. pleuropneumoniae*	2.294	+	ND	
AL36-1	Lung	*A. pleuropneumoniae*	2.274	+	ND	
AL36-5	Lung	*A. pleuropneumoniae*	2.329	+	ND	
AL37-1	Lung	*A. pleuropneumoniae*	2.238	+	ND	
AL37-3	Lung	*A. pleuropneumoniae*	2.381	+	ND	
AL37-4	Lung	*A. pleuropneumoniae*	2.440	+	ND	
AL38-1	Lung	*A. pleuropneumoniae*	2.444	+	ND	
AL38-3	Lung	*A. lignieresii*	2.370	+	ND	
AL38-5	Lung	*A. pleuropneumoniae*	2.378	+	ND	
AL38-6	Lung	*A. pleuropneumoniae*	2.110	+	ND	
AL39-4	Lung	*A. lignieresii*	2.417	+	ND	
AL39-5	Lung	*A. lignieresii*	2.447	+	ND	
AL40-6	Lung	*A. pleuropneumoniae*	2.114	+	ND	
BL34-3	Lung	*A. pleuropneumoniae*	2.262	+	ND	
BT33-6	Tonsilla	*A. pleuropneumoniae*	2.177	+	ND	

* The top 3 highest score of BLAST identification is shown. ND: Not done.

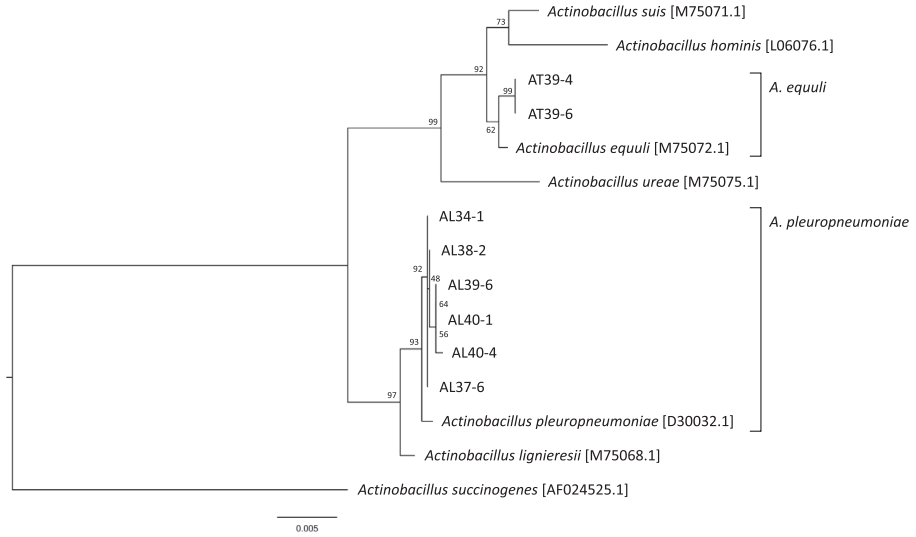

Fig. 1 Phylogenetic tree based on the 16S rRNA sequence of *Actinobacillus* spp.

A neighbor-joining phylogenetic tree based on 16S rRNA nucleotide sequences was constructed with ClustalW2 ver. 2.1[16] and 1,000 bootstrap replicates, and was drawn using the FigTree software ver. 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). The scale bar indicates 0.005 substitutions per nucleotide position. The numbers at the branches indicate bootstrap values. AL34–1, AL37–6, AL38–2, AL39–6, AL40–1, AL40–4, AT39–4 and AT39–6 are isolates identified in this study. GenBank accession numbers of the reference sequences are represented after the species name.

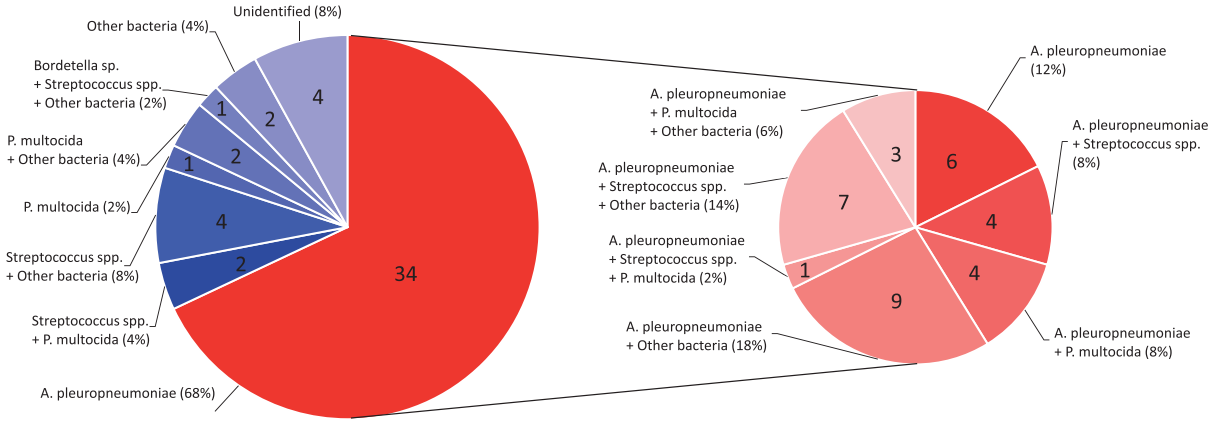

Fig. 2 Bacterial species isolated from lung abscesses in group A

Left cycle shows the total isolated bacterial species from lung abscesses in group A (n=50). Right cycle shows the breakdown of the isolation rate of *A. pleuropneumoniae* in the left cycle. Numbers in the cycles indicate the number of lung samples for which bacteria were isolated.

Table 3 Comparison of bacterial prevalence rate among 3 groups

Bacteria isolated	*P*-value of Fisher's exact test*					
	Lung			Tonsilla		
	Group A vs B	Group A vs C	Group B vs C	Group A vs B	Group A vs C	Group B vs C
A. pleuropneumoniae	<0.001*	<0.001*		0.242	0.412	0.640
E. coli	0.125	0.006*	0.231	0.541	0.065	0.237
P. multocida	0.483	<0.001*	0.004*	0.548	0.369	0.119
Str. suis	0.269	0.006	0.148		0.817	

*The *P*-values in the blanks are 1.0. *P*-values marked with an asterisk (*) indicate significant differences determined by a Fisher's exact test ($P<0.01$).

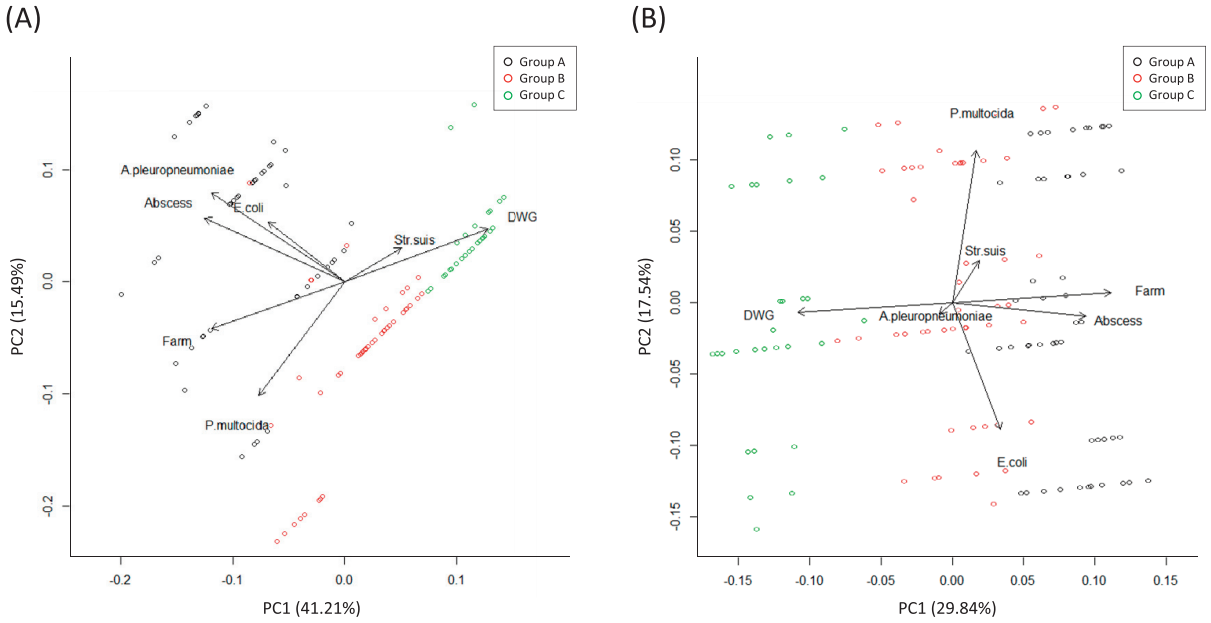

Fig. 3 Principal component analysis (PCA) of the multiple factors related to lung abscesses

Seven factors including the presence or absence of lung abscesses, the farm, daily weight gain (DWG) and the prevalence of four bacteria isolated from the lung (A) and tonsilla (B) were assessed by PCA based on a correlation coefficient matrix. Daily weight gains (DWG) were calculated by dividing the carcass weight by the period of fattening. The first principal component (PC1) and second principal component (PC2) were represented on the x and y axes with each proportion of the variance. Arrows represent the factor loadings of each of the parameters on the two axes. Black, red and green open circles represent the principal component scores of groups A, B and C, respectively.

were located on opposite sides, indicating a negative correlation. Among the tonsillar bacterial species, similar patterns of factor loadings were observed to the lung bacterial species (Fig. 3B); however, the prevalence of *A. pleuropneumoniae*, *E. coli* and *P. multocida* among the tonsilla species did not seem to be strongly related to the constitution of lung abscesses.

Finally, to clarify the relationship between lung abscesses and pig growth, DWG was compared among the three groups. Fig. 4 presents the statistically significant differences in DWG among all three groups. The results suggested that lung abscesses have a negative effect both directly and indirectly on pig growth.

DISCUSSION

In this study, we applied the MALDI TOF MS system, MALDI Biotyper, to evaluate the multiple infections present in pig lung abscesses compared with the tonsillar microbiota. Together with the major pathogens present in lung lesions, such as *A. pleuropneumoniae*, *P. multocida* and *Streptococcus*

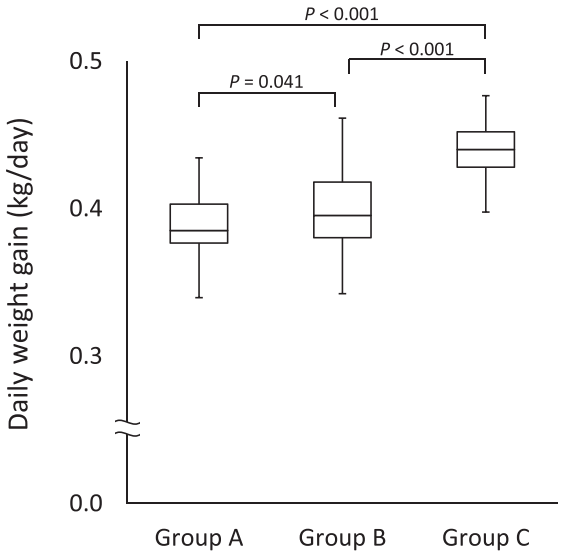

Fig. 4 Comparison of the daily weight gains (DWG) among the three groups

Box-whisker plot represents the minimum, lower quartile, median, upper quartile and maximum values of each DWG. The median of the DWG was compared by the Student's t-test and the *P*-values are shown above the boxes.

spp., significantly higher levels of *E. coli*, a minor causative agent of lung abscesses, were detected among the lung abscess samples. By contrast, among the tonsillar microbiota, the prevalence of these bacteria among the three groups was similar. PCA clarified that the high prevalence of *E. coli* in the lungs was related to the high prevalence of *A. pleuropneumoniae* and the constitution of lung abscesses. Moreover, our results suggested that lung abscesses have a negative effect on pig growth.

It should be noted that the MALDI Biotyper system employed in this study can identify a broad spectrum of bacteria without relying on species-specific diagnoses such as biochemical examination, serological testing and PCR. In this study, proportion of species identified was 91.8% (1,107/1,206 isolates), including genus level identification. In human clinical tests, the reported proportions of species identified were 96.8%[9] and 97.3%[3], similar to our data. However, a lack of identification and misidentification occurred in some cases, such as for *A. lignieresii*. Moreover, some isolates such as *Bordetella* and *Klebsiella* were limited to genus level identification by the MALDI Biotyper system. MALDI Biotyper screening is useful for gaining an overall understanding of the proportion of bacterial species present in an infection or in the microbiota, but additional biochemical and molecular tests are required for detailed identification.

It still remains to be determined whether the minor co-infecting bacteria present in lung lesions, such as *E. coli*, are the "cause" or "result" of lung abscesses. The isolation of *E. coli* from lung tissue during respiratory disease has previously been reported but reports are limited in pigs[6,8], and little is known about the relationship between *E. coli* infection and lung lesions. In humans, *E. coli* is one of the most serious concerns in cases of ventilator-associated pneumonia (VAP) in nosocomial infections[22]. An epidemiological study revealed that the B2 phylogenetic group was predominant in VAP patients and the isolates had a tendency to possess three virulence factor genes, *iroN*, *sfa* and *cnf1*[19]. It is possible that some unique *E. coli* subgroups can be the primary pathogens causing infections in lung abscesses in pig. Alternatively, *E. coli* may cause a secondary infection followed by immunological deterioration in the lung microenvironment. Apx toxins and lipopolysaccharides are major virulence factors produced by *A. pleuropneumoniae* that contribute to the formation of lung lesions showing hemolytic, cytotoxic and necrosis effects[5]. Further studies focused on the detailed characterization of *E. coli* and bacterial cross-talk will reveal the pathogenesis of *E. coli*-related pig lung abscesses.

To date there have been several studies on the influence of *A. pleuropneumoniae* infection on pig fattening or growth, but no consensus has been reached[2,13,23,25]. In this study, *A. pleuropneumoniae* infection was found to have a significantly negative effect on pig growth, although there remains a possibility of confounding bias. Regarding the relationship with other factors, PCA revealed a positive correlation between *A. pleuropneumoniae* and *E. coli* infection in the lungs and the constitution of lung abscesses. In Fig. 3A, principal component (PC) 1 represents 41.21% of the variation, and it can be interpreted as a marker of hygiene or the health conditions. According to the results, worse sanitary conditions is correlate with a higher prevalence of *A. pleuropneumoniae*, *E. coli* and *P. multocida* in the lungs, although this does not appear to apply to *Str. suis*. In Fig. 3B, PC1 can also be considered a marker of hygiene, although the presence of four bacterial species in the tonsilla tissue does not seem to be related to the PC1. Interestingly, the amount of *A. pleuropneumoniae* in the tonsilla tissue may not be important to the constitution of lung abscesses because the factor loading in the Fig. is small. By contrast, factor loading of *E. coli* and *P. multocida* revealed an inverse relationship indicating mutual interference, although the influence on lung abscesses was unclear.

To date, the MALDI Biotyper system has not been widely applied in veterinary medicine, but it has great potential for the rapid and low-cost diagnoses and comprehensive analyses of microbiota. In this study, we revealed bacterial species present in pig lung abscesses and isolated unexpected bacteria as the cause of lung lesions. The methodology employed here would be applicable to other bacterial diseases of animals, such as infective endocarditis, arthritis and mastitis, to obtain an overview of the causative agents in multiple

bacterial infections. Our findings will contribute to the understanding of multiple bacterial infections in pig lung abscesses.

ACKNOWLEDGMENTS

This study was partially supported by a Grant-in-Aid for Cooperative Research from Rakuno Gakuen University in 2016. We thank Ms. Yurina Sato (Laboratory of Zoonotic Diseases), Dr. Jun Minaguchi (Laboratory of Veterinary Micro-anatomy) and Dr. Kohei Makita (Laboratory of Veterinary Epidemiology) in the School of Veterinary Medicine, Rakuno Gakuen University, for their technical support. We also thank Kate Fox, DPhil, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

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要　　約

豚の肺膿瘍はと殺解体時，膿瘍が破壊されることで可食部の汚染につながるため，食品衛生の観点から注意を要する病変である。肺膿瘍の原因は多岐に渡り，複合感染の状況を正しく把握することが衛生対策において重要と考えられる。本研究では，胸膜肺炎および肺膿瘍の発生割合が高い養豚場において，肺膿瘍を構成する細菌の網羅的同定と複合感染のリスクを評価した。2016 年 4 月～2017 年 1 月，北海道東藻琴食肉衛生検査所に，肺膿瘍の発生が多い農場より搬入される，肺膿瘍形成豚 50 頭（以下，A 群），肺膿瘍非形成豚 50 頭（以下，B 群），また

肺膿瘍の発生が少ない農場より搬入される肺膿瘍非形成豚 35 頭（以下，C 群）から肺（A 群については病変部），扁桃を採集し，NAD 添加馬血液寒天培地により菌分離を行った。分離された菌株は MALDI Biotyper を用い菌種同定を行い，群間での比較を行った。肺および扁桃から 30 属菌，計 1,206 株が分離された。A 群の肺のうち 68％から *Actinobacillus pleuropneumoniae*（App）が分離され，B，C 群との比較において，App，*Escherichia coli*，*Pasteurella multocida* が有意に多く分離された。一方，上記 3 種の扁桃における各群の保有率に差は見られなかった。主成分分析では，肺での App，*P. multocida*，*Streptococcus dysgalactiaegae* が肺膿瘍形成と関連することが示唆された。以上の結果は，病原体から見た豚の肺膿瘍形成機序の理解および，衛生対策を講じる上での一助になると期待される。今後は，分離された *Streptococcus suis* について，人への感染性，病原性を評価するべく，Multilocus Sequence Typing（MLST）法による遺伝子型別および血清型別を実施していく。