別紙３

厚生労働科学研究委託費（新興・再興感染症に対する革新的医薬品等開発推進研究事業）
委託業務成果報告（業務項目）

Molecular epidemiology of *Bordetella pertussis* in the Philippines in 2012–2014

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Objectives: The present study was designed to determine the genotypes of circulating *Bordetella pertussis* in the Philippines by direct molecular typing of clinical specimens.

Methods: Nasopharyngeal swabs (NPSs) were collected from 50 children hospitalized with pertussis in three hospitals during 2012–2014. Multilocus variable-number tandem repeat analysis (MLVA) was performed on the DNA extracts from NPSs. *B. pertussis* virulence-associated allelic genes (*ptxP*, *ptxA*, *prn*, and *fim3*) were also investigated by DNA sequence-based typing.

Results: Twenty-six DNA extracts yielded a complete MLVA profile, which were sorted into 10 MLVA types. MLVA type 34 (MT34), rare in other countries, was the predominant strain (50%). Seven MTs (MT29, MT32, MT33, and MT283–286, total 42%) were single-locus variants of MT34, while two (MT141 and MT287, total 8%) were double-locus variants of MT34. All MTs had the combination of virulence-associated allelic genes, *ptxP1-ptxA1-prn1-fim3A*.

Conclusions: The *B. pertussis* population in the Philippines comprises genetically related strains. These strains are markedly different from those found in patients from other countries where acellular pertussis vaccines are used. The differences in vaccine types between these other countries and the Philippines, where the whole-cell vaccine is still used, may select for distinct populations of *B. pertussis*.

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A. 研究目的

Bordetella pertussis, a highly communicable Gram-negative coccobacillus, is the etiological agent of whooping cough (pertussis), a major acute respiratory infection resulting in severe childhood illness and infant death. In the Philippines, pertussis is controlled by the administration of the whole-cell pertussis vaccine (WCV), given as 3 doses at ages of 6, 10, and 14 months. Despite these controls, pertussis infections have sporadically occurred, primarily in unvaccinated children (<6 months of age). The molecular epidemiology of *B. pertussis* populations has been studied in several countries, but no information of the specific strains present in the Philippines has been reported. Molecular typing of the organism can be performed on both bacterial isolates and clinical specimens. We therefore determined the genotypes of the circulating strains of *B. pertussis* in the Philippines by direct molecular typing of clinical specimens.

B. 研究方法

Between September 2012 and May 2014, 50 children hospitalized with severe respiratory distress (median age, 2 months; range, 0−53 months) were diagnosed with *B. pertussis* infections by PCR targeting IS*481*. Patients were hospitalized at the Philippine General Hospital (PGH; 26 patients), Ospital ng Palawan (ONP; 17 patients), and the Research Institute for Tropical Medicine (RITM; 7 patients) (Figure 1). Nasopharyngeal swabs (NPSs) were obtained from the patients, and DNA was extracted from the NPSs using the QIAamp DNA Mini kit (Qiagen).

Multilocus variable-number tandem repeat analysis (MLVA) typing was performed on DNA extracted from NPSs. MLVA types (MTs) were assigned using the MLVA typing tool found at http://www.mlva.net. Novel MTs were assigned by Dr. H. van der Heide, National Institute for Public Health and the Environment, the Netherlands. To characterize the phylogenic relationships between the MTs, minimum spanning trees were generated using the FPQuest Software (Bio-Rad). DNA sequence-based typing for *B. pertussis* virulence-associated allelic genes (*ptxP*, *ptxA*, *prn*, and *fim3*) was also performed on DNA extracts that yielded a complete sixe allele MLVA profile with some modifications. Briefly, PCR cycling conditions were 94°C for 2 min followed by 11 cycles of touchdown PCR (98°C for 10 s, followed by annealing, initially at 65°C for 30 s, and decreasing 1°C/cycle until 55°C, and elongation at 68°C for 45 s) and 25 cycles of standard PCR (98°C for 10 s, 55°C for 30 s, and 68°C for 45 s). For *ptxP* and variable region 2 (R2) of *prn*, some DNA extracts that failed to yield DNA sequences were analyzed by nested PCR. PCR primer sets used in this study are listed in Table 1.

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NPSs were obtained for diagnostic purposes, and were retrospectively and anonymously analyzed for *B. pertussis* genotyping. No patient data beyond classification by age and hospital were stored.

Ethical approval was thus not required.

C. 研究結果

DNA extracts from NPSs were collected from 50 patients and analyzed by MLVA typing. Twenty-six (52%) samples yielded a complete 6-allele MLVA profile, and the remainder yielded either a partial profile (38%) or a negative MLVA result (10%) (Table 2). The complete MLVA profiles were obtained primarily from patients aged between 1 and <3 months; however, distributions of patient age among the three MLVA groups (complete profiles, partial profiles, no alleles) were not significantly different (P = 0.87, Fisher's exact test). The MLVA results therefore did not depend on patient age.

Among the 26 DNA extracts that yielded a complete MLVA profile, 10 distinct MLVA types (MTs) were identified, of which 5 were novel (MT283, MT284, MT285, MT286, and MT287). Figure 2 shows a minimum spanning tree that revealed the genetic diversity of the *B. pertussis* population. MT34 was the most prevalent type (n = 13) and MT33 was the second most prevalent (n = 4). Eight MTs (MT29, MT32, MT141, MT283, and MT284–287) were minor subtypes, appearing only rarely (n = 1 or 2). All MTs had a combination of *ptxP1-ptxA1-prn1-fim3A* alleles as demonstrated by DNA sequence-based typing. The MT distribution was not statistically different among the three hospitals, PGH, ONP, and RITM (P = 0.23, Fisher's exact test).

D. 考察

In the present study, we demonstrated that the *B. pertussis* MT34 strain was the predominant (50%) subtype in the Philippines during 2012–2014. Seven MTs (MT29, MT32, MT33, and MT283–286, total 42%) were single-locus variants of MT34, and two others (MT141 and MT287, total 8%) were double-locus variants of MT34. All MT strains carried the same virulence-associated allelic genes, *ptxP1-ptxA1-prn1-fim3A*. These data suggest that the *B. pertussis* population in the Philippines comprises genetically related strains.

In Australia, Europe, and the US, the *B. pertussis* MT27 strain was the predominant type during the past decade. In Japan, both MT27 and MT186 were the predominant types during 2002–2012, with only one MT34 strain found out of 134 *B. pertussis* isolates tested. The Japanese MT34 strain carried the same virulence-associated genes, *ptxP1-ptxA1-prn1-fim3A*, as the MT34 strain from the Philippines described in this study. MT34 strains have been identified very rarely throughout the world. In Australia, the US, Japan, and most European countries, acellular pertussis vaccines (ACVs) have been used, whereas a WCV is still used in the Philippines. Thus, the different vaccine types may select for different *B. pertussis* populations.

This study provides a baseline for future studies on the *B. pertussis* population in the Philippines.

E. 結論

The *B. pertussis* population in the Philippines comprises genetically related strains. These strains are markedly different from those found in patients from other countries where acellular pertussis vaccines are used. The differences in vaccine types between these other countries and the Philippines, where the whole-cell vaccine is still used, may select for distinct populations of *B. pertussis*.

F. 研究発表

1. 論文発表

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2. 学会発表

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- 平松征洋, 大塚菜緒, 柴山恵吾, 鈴木英里, 渡邉峰雄, 蒲地一成. 百日咳類縁菌 *Bordetella holmesii* の自己凝集抑制因子 BipA に関する研究. 第 88 回日本細菌学会総会, 平成 27 年 3 月, 岐阜.

G. 知的財産権の出願･登録状況
(予定を含む。)

1. 特許取得:なし
2. 実用新案登録:なし
3. その他:なし

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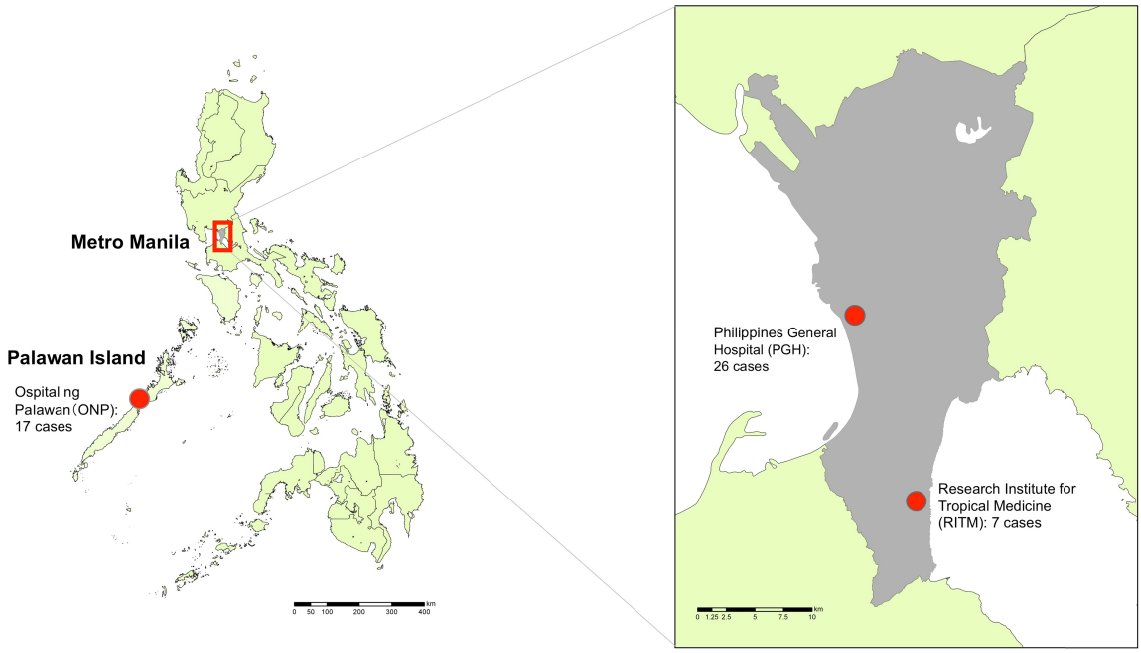

Figure 1. Geographical locations of the three medical institutes (PGH, ONP, and RITM) in the Philippines

Table 1. Primers used for virulence-associated allelic genes

Target gene	Region	Primer name	PCR§	Sequence (5' to 3')	Coordinate*	Reference
ptxP		ptxP-PF	1st	AATCGTCCTGCTCAACCGCC	3987811	Mooi et al., Emerg Infect Dis, 2009
		ptxP-PR	1st	GGTATACGGTGGCGGGAGGA	3988365	
		ptxP-innerF	2nd	GCTTCATCGCAGACGAGATCCA	3987924	This study
		ptxP-innerR	2nd	TGTTCTTGCGGTTTGGCGAATT	3988275	
ptxA		ptxA-innerF		GACCACGACCACGGAGTATT	3988824	Nakamura et al., Clin Microbiol Infect, 2011
		ptxA-innerR		GTACACGAGAACCATCGCCT	3989021	
prn	R1	prn-innerF		GTCATTGCAGCCGGAAGACC	1098657	Nakamura et al., Clin Microbiol Infect, 2011
		prn-innerR		CCGGTCTCGATGACATTGCC	1099111	
	R2	prnR2-F	1st	GGTCAATACGCTGGCGGGTT	1099512	Miyaji et al., PLoS ONE, 2013
		prnR2-R	1st	CGTGTTGACCGCCGCGTT	1099921	
		prnR2-innerF	2nd	TGTTCCGCATGAATGTCTTCGC	1099538	This study
		prnR2-innerR	2nd	ACAACTCCCTGCCCGC	1099894	
fim3		fim3-innerF		CCAGCACCCTCAACCATATC	1647738	Nakamura et al., Clin Microbiol Infect, 2011
		fim3-innerR		GGCTTGCGTGGTTTTGTC	1648055	

*Coordinates in *Bordetella pertussis* Tohama genome sequence NC_002929.2
§ For nested PCR, the order of primer-pairs used is indicated.

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Table 2. Summary of MLVA results of 50 pertussis patients grouped by age

Patient age range	Number of clinical specimen	MLVA result[a]		
		Complete profile	Partial profile	No alleles generated
0 to <1 month	2	1	1	
1 to <2 months	21	11	8	2
2 to <3 months	14	8	5	1
3 to <12 months	8	4	3	1
1 to 4 years	4	2	2	
unknown	1[b]			1
Total	50	26	19	5

[a] Complete profile, six alleles; partial profile, from one to five alleles.
[b] Patient age was <2 months.

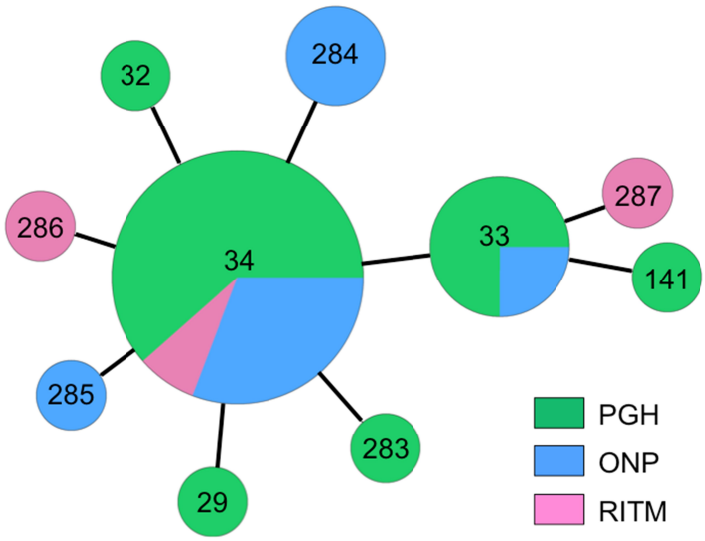

Figure 2. Minimum spanning tree revealing the genetic diversity of the *Bordetella pertussis* population in the Philippines during 2012–2014. MLVA types (MTs) were identified from DNA extracts of clinical specimens, collected from 26 patients. Each circle within a tree represents a unique MT, and the number denotes the MT. The sizes of circles are representative of the number of clinical specimens in each group. Lines connecting circles represent single-locus variants. All MTs carried *ptxP1*, *ptxA1*, *prn1*, and *fim3A* alleles. PGH, Philippine General Hospital; ONP, Ospital ng Palawan; RITM, Research Institute for Tropical Medicine.