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Evaluation of a Double-Disk Synergy Test with a Common Metallo-β-Lactamase Inhibitor, Mercaptoacetate, for Detecting NDM-1-Producing *Enterobacteriaceae* and *Acinetobacter baumannii*

Jun-ichi Wachino[1,2], Mari Matsui[1], Hoang Huy Tran[3], Masato Suzuki[1], Satowa Suzuki[1], and Keigo Shibayama[1]*

[1]*Department of Bacteriology II, National Institute of Infectious Diseases, Tokyo 208-0011;*
[2]*Department of Bacteriology, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan; and*
[3]*National Institute of Hygiene and Epidemiology, Hanoi, Vietnam*

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New Delhi metallo-β-lactamase (NDM)-1 carbapenemase-producing bacteria are resistant to antibiotics of the carbapenem family, which are used as a last resort for the treatment of infectious diseases caused by drug-resistant bacteria. Therefore, the emergence of these bacteria presents a serious public health issue. This is particularly true given that NDM-1 carbapenemase has been detected in many clinical isolates worldwide since it was first identified in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* from a patient hospitalized in New Delhi (1). The NDM-1 carbapenemase gene has been predominantly identified in *Enterobacteriaceae*, but it can also occur in non-fermenters (2). Hence, it is necessary to monitor the emergence and spread of NDM-1 producers with a convenient and effective screening method.

NDM-1 carbapenemase is a class B zinc metallo-β-lactamase (MBL) (3). A variety of techniques have already been developed to detect MBL producers (4), including a disk-based synergy test, the sodium mercaptoacetate (SMA) test, which is conventionally used in Japanese clinical microbiology laboratories (5). This test uses a Kirby–Bauer (KB) disk containing a β-lactam antibiotic (ceftazidime [CAZ] recommended) and a disk containing SMA, an MBL inhibitor that can bind to the MBL active site through interactions with zinc ions (6). Although this test works well for the detection of IMP- and VIM-type MBL producers, which are the predominant MBL types in Japan (7), preliminary results have indicated that it may fail to detect NDM-1 producers when using the SMA test with the CAZ disk according to general recommendations (8). Hence, the present study aimed at improving the SMA test by replacing the CAZ disk for detecting NDM-1 producers among *Enterobacteriaceae* and *Acinetobacter baumannii*.

A collection of 15 NDM-1-positive bacterial isolates (5 *E. coli*, 4 *K. pneumoniae*, 1 *Enterobacter cloacae*, 1 *Citrobacter freundii*, and 4 *A. baumannii*), obtained from hospitals in Vietnam in 2010, and 1 NDM-1-positive *K. pneumoniae* strain (MRY10-722) isolated in a hospital in Japan in 2010, were used in this study. These isolates were identified using the API 20E and Vitek2 systems (bioMerieux, Marcy l'Etoile, France). Identification of *A. baumannii* isolates was further confirmed by *rpoB* gene sequencing (9). The NDM-1 gene was detected using PCR analysis with specific primers as described previously (10).

A total of 16 isolates were subjected to the SMA test. The inhibitory effect of CAZ was compared with that of the carbapenems, imipenem (IPM) and meropenem (MPM). Suspensions of the bacterial isolates were adjusted and spread on Mueller–Hinton agar plates according to the protocol recommended by the Clinical and Laboratory Standards Institute guidelines (11). The KB disks containing β-lactam antibiotics (CAZ [30 μg] or IPM [10 μg] or MPM [10 μg]) (Eiken Chemical Co., Ltd., Tokyo, Japan) were placed on the plates, and disks containing SMA (3 mg) (Eiken Chemical) were placed close to 1 β-lactam disk as shown in Fig. 1. The center-to-center diameter between the KB disk and the SMA disk was 16 mm. The plate was incubated at 35°C for 18 h and the growth-inhibitory zone around the KB disk close to the SMA disk was compared with that around the KB disk alone. An isolate was considered MBL-positive when an apparent expansion was observed, i.e., an enlargement of 5 mm or greater of the growth-inhibitory zone around the KB disk close to the SMA disk compared with that around the KB disk alone (Fig. 1, middle and lower panels). An isolate was considered MBL-negative if there was no expansion of the growth-inhibitory zone or if the expansion was less than 5 mm (Fig. 1, upper panel).

The results are summarized in Table 1. The highest sensitivity was obtained with a combination of the MPM and SMA disks. Of the 16 strains, 15 (93.8%) were confirmed as positive. The combination of the IPM and SMA disks resulted in the positive identification of 14 strains (87.5%). On the other hand, a combination of the CAZ and SMA disks was considerably less sensitive, as only 7 strains (43.8%) had positive results.

These results indicated that the carbapenems, IPM and MPM, are generally more suitable than CAZ for

*Corresponding author: Mailing address: Department of Bacteriology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan. Tel: +81-42-561-0771, ext. 3500, Fax: +81-42-561-7173, E-mail: keigo@niid.go.jp

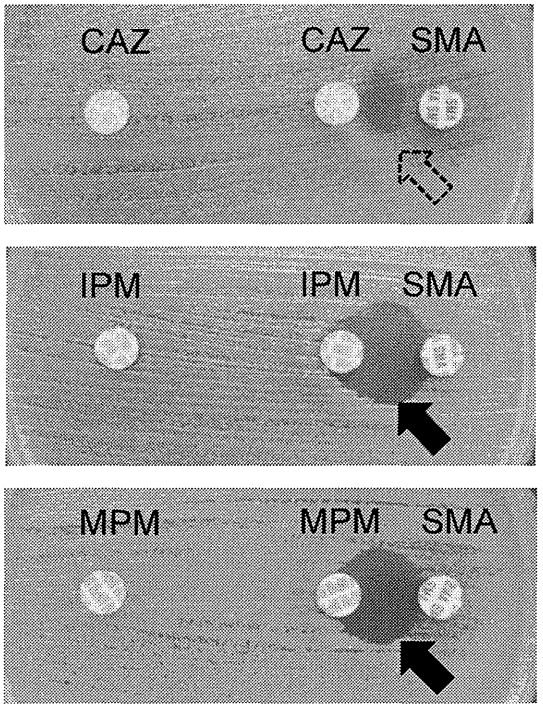

Fig. 1. Results of the disk-based synergy test, the sodium mercaptoacetate (SMA) test, for the New Delhi metallo-β-lactamase (NDM)-1-producing *Acinetobacter baumannii* isolate V-275. Apparent expansion of the growth-inhibitory zone (black arrows) between SMA and imipenem (IPM)/meropenem (MPM) disks were observed. On the other hand, a very slight inhibitory effect (arrow with dashed black line) was observed between the ceftazidime (CAZ) and SMA disks.

detecting NDM-1-producing bacteria. The low sensitivity of the CAZ disk could be ascribed to the coproduction of other β-lactamases such as extended-spectrum β-lactamases and plasmid-mediated or chromosomally encoded AmpC β-lactamases, which inactivate CAZ without being inhibited by SMA. In fact, it has been reported that NDM-1-producers often simaultaneously carry other β-lactamase genes, such as $bla_{\text{CTX-M}}$, bla_{CMY}, or bla_{DHA} (12,13). Nevertheless, 1 *E. cloacae* isolate (V-87) produced a clearly enlarged inhibitory zone between the SMA and CAZ disks but not between SMA and MPM or IPM, suggesting the importance of CAZ. We hypothesized that these findings may have been owing to the coproduction of OXA-48 carbapenemase, which is usually resistant to MPM but not to CAZ. The OXA-48 carbapenemase is a member of the serine-β-lactamases, whose activities are not inhibited by SMA. We used PCR to screen all isolates for the presence of $bla_{\text{OXA-48}}$. As expected, only the *E. cloacae* isolate V-87 carried the $bla_{\text{OXA-48}}$ gene; however, further examinations with additional isolates are required to confirm our hypothesis.

On the basis of these results, we conclude that the SMA test using both the MPM and CAZ disks is the most suitable method for screening carbapenem-resistant isolates for NDM-1-type MBL producers. As reported previously (8), this combination also allows for highly sensitive and specific detection of the IMP- and VIM-type MBL producers. Effective screening of MBL producers, including the NDM-1 type and other MBL types, can therefore be performed by the SMA test using MPM and CAZ disks in clinical laboratories followed by confirmation of the MBL genes by PCR analysis in specialized laboratories.

Table 1. Inhibitory activity of SMA disks for NDM-1-producing bacterial isolates

Bacterial isolate	Antibiotic disk		
	CAZ	IPM	MPM
E. coli V-22	+	+	+
E. coli V-48	+	+	+
E. coli V-91	−	+	+
E. coli V-102	−	+	+
E. coli V-134	−	+	+
K. pneumoniae MRY10-722	+	−	+
K. pneumoniae V-17	+	+	+
K. pneumoniae V-21	+	+	+
K. pneumoniae V-90	−	+	+
K. pneumoniae V-182	−	+	+
E. cloacae V-87	+	−	−
C. freundii V-868	−	+	+
A. baumannii V-275	−	+	+
A. baumannii V-303	+	+	+
A. baumannii V-320	−	+	+
A. baumannii V-357	−	+	+

SMA, sodium mercaptoacetate; NDM, New Delhi metallo-β-lactamase; CAZ, ceftazidime; IPM, imipenem; MPM, meropenem; +, positive; −, negative.

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Conflict of interest None to declare.

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Genetic Analysis of *Bordetella pertussis* Isolates from the 2008–2010 Pertussis Epidemic in Japan

Yusuke Miyaji[1,2], Nao Otsuka[1], Hiromi Toyoizumi-Ajisaka[1], Keigo Shibayama[1], Kazunari Kamachi[1]*

1 Department of Bacteriology II, National Institute of Infectious Diseases, Tokyo, Japan, **2** Department of Pediatrics, St Marianna University School of Medicine, Kawasaki, Japan

Abstract

A large pertussis epidemic occurred between 2008 and 2010 in Japan. To investigate epidemic strains, we analyzed 33 *Bordetella pertussis* isolates from the epidemic period by sequencing virulence-associated genes (*fim3*, *ptxP*, *ptxA*, and *prn*) and performing multilocus variable-number tandem repeat analysis (MLVA), and compared these results with those of 101 isolates from non-epidemic, earlier and later time periods. DNA sequencing of the *fim3* allele revealed that the frequency of *fim3B* was 4.3%, 12.8%, 30.3%, and 5.1% within isolates in 2002–2004, 2005–2007, 2008–2010, and 2011–2012, respectively. The isolation rate of the *fim3B* strain therefore temporarily increased during the epidemic period 2008–2010. In contrast, the frequencies of the virulence-associated allelic variants, *ptxP3*, *ptxA1*, and *prn2*, increased with time during overall study period, indicating that these variants were not directly involved in the occurrence of the 2008–2010 epidemic. MLVA genotyping in combination with analysis of allele types showed that the prevalence of an MT27d strain temporarily increased in the epidemic period, and that this strain carried virulence-associated allelic variants (*fim3B*, *ptxP3*, *ptxA1*, and *prn2*) also identified in recent epidemic strains of Australia, Europe, and the US. Phenotypic analyses revealed that the serotype Fim3 strain was predominant (≥87%) during all the periods studied, and that the frequency of adhesion pertactin (Prn) non-expressing *B. pertussis* decreased by half in the epidemic period. All MT27d strains expressed Prn and Fim3 proteins, suggesting that *B. pertussis* MT27d strains expressing Prn and Fim3B have the potential to cause large epidemics worldwide.

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* E-mail: kamachi@nih.go.jp

Introduction

Bordetella pertussis, a highly communicable Gram-negative coccobacillus, is the cause of pertussis (whooping cough), a major acute respiratory infection resulting in severe childhood illness and infant death [1]. Although universal immunization programs have contributed to significant reductions in the morbidity and mortality rates associated with pertussis, the incidence of pertussis has increased in several countries despite high vaccination coverage [2-6]. In Japan, acellular pertussis vaccines (ACVs) were introduced in 1981 and are used to control pertussis with a schedule of 3 primary doses and single booster dose at ages 3, 4, 6, and 18−23 months, respectively. This vaccination schedule has been followed since 1994. The incidence of pertussis cases in adolescents and adults, however, has significantly increased since the early 2000s [7]. The waning of vaccine-acquired immunity and the decrease in opportunities of natural immune boosting owing to reduced levels of *B. pertussis* circulation have been proposed as explanations for the recent resurgences of pertussis [2,8,9]. Another possible underlying factor is the adaptation of the *B. pertussis* population to vaccine-induced immunity [2,10,11].

Antigenic and genetic shifts in *B. pertussis* circulating strains have been identified within virulence-associated genes encoding serotype 3 fimbriae (*fim3*), pertussis toxin S1 subunit (*ptxA*), pertactin (*prn*), and the pertussis toxin promoter (*ptxP*). Allele frequencies of the virulence-associated allelic variants, *fim3B*, *ptxA1*, *prn2*, and *ptxP3*, have increased within the *B. pertussis* population in several countries [10,12–17]. *B. pertussis* strains carrying *ptxP3* are more capable of producing pertussis toxin (PT) than are *ptxP1* strains, and the emergence of *ptxP3* strains was associated with pertussis resurgence in the Netherlands [18]. Similarly, a significant correlation was observed between an increase in *fim3B* strains and pertussis notifications in the US [13]. Strains with *fim3B* have a single amino-acid substitution (A87E) within the surface epitope of Fim3, which interacts with human serum [19,20]. Furthermore, multilocus variable-number tandem repeat analysis (MLVA) has revealed that the *B. pertussis* population has changed over the past 50 years worldwide. In Australia, the frequency of *B. pertussis* MLVA type 27 (MT27) and MT70 strains increased after the introduction of an ACV, and subsequently, the MT27 strain became predominant in 2008−2010 [21,22]. An increase in the frequency of *B. pertussis* MT27 strain was also observed in Europe and the US [13,16,23].

Pertussis epidemics still occur worldwide, and epidemic strains have been characterized by Fim serotyping and/or genotyping within some regions [24–26]. In a Dutch epidemic, significant changes in Fim serotypes and MTs were observed during a period when the pertussis vaccine dose was lowered [24]. Besides phenotypic variants of Fim, *B. pertussis* variants that do not express adhesion pertactin (Prn) have been recently identified in Japan as

well as in other countries [27–30]. Since Prn is a component of ACVs, it is reasonable to hypothesize that Prn-negative variants have increased fitness in humans immunized with ACV. To date, the relationship between the prevalence of Prn-negative variants and pertussis epidemics has not been evaluated.

In Japan, a large pertussis epidemic occurred in 2008−2010 despite high vaccination coverage with ACVs. To elucidate the causes of the epidemic, we determined temporal trends in the frequencies of virulence-associated genes (*fim3*, *ptxP*, *ptxA*, and *prn*) and genotypes in the *B. pertussis* population from 2002 to 2012. In addition, phenotypes of epidemic isolates were characterized by their expression of Fim and Prn proteins.

Materials and Methods

Pertussis surveillance data

National surveillance data were obtained from the Ministry of Health, Labor and Welfare of Japan Infectious Disease Surveillance data. Each week, the number of pertussis cases was reported from approximately 3,000 sentinel clinics and hospitals within Japan. Diagnosis was based on bacterial culture, clinical symptoms, and/or the results of a serologic test. The reporting criteria did not change during the study period, 2002−2012.

Bacterial strains

We examined 134 clinical *B. pertussis* isolates collected in Japan from 2002 to 2012 (Table S1). Thirty-three of those isolates were collected during the 2008–2010 pertussis epidemic, while 101 isolates were collected from non-epidemic periods: 23 in 2002–2004, 39 in 2005–2007, and 39 in 2011–2012 (Fig. 1). All isolates were epidemiologically unrelated cases of pertussis. The isolates were cultured on Bordet-Gengou agar (Difco) or cyclodextrin solid medium (CSM) agar [31], and incubated at 36°C for 2−3 days. DNA was extracted from *B. pertussis* isolates by boiling, and stored at −20°C.

Sequence analysis of *fim3*, *ptxP*, *ptxA*, and *prn*

Four virulence-associated genes, *fim3*, *ptxP*, *ptxA* and *prn*, were analyzed using PCR-based sequencing [14,18,32,33]. Sequence reactions were carried out with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and resultant products were sequenced using an Applied Biosystems 3130xl Genetic Analyzer or 3730 DNA Analyzer. Subsequent sequencing of the variable region 2 (R2) of *prn* was performed where necessary, to distinguish between *prn1* and *prn7* alleles. Primer sets used in this study are listed in Table S2.

MLVA

MLVA typing was performed as previously described [22,28]. MTs were assigned using the MLVA typing tool found at http://www.mlva.net. Novel MLVA types were assigned by the webmasters, Drs. H. van der Heide and F. Mooi, from the Centre for Infectious Disease Control Netherlands, within National Institute for Public Health and the Environment, in the Netherlands.

Immunoblotting and serotyping analysis

Prn expression in *B. pertussis* isolates was analyzed by immunoblotting [28]. Briefly, protein samples (1 μg) were first subjected to 10% SDS-PAGE, then transferred onto nitrocellulose membranes (Bio-Rad), and finally incubated with anti-Prn antiserum. Antigen-antibody complexes were visualized using a horseradish peroxidase-conjugated secondary antibody (Bio-Rad) and the Western Lightning ECL Pro (PerkinElmer, Inc.). Resultant blots were imaged using a LAS-3000 (Fujifilm, Tokyo, Japan).

Serotyping of *B. pertussis* isolates was performed with indirect whole-cell ELISA using anti-Fim2 and anti-Fim3 monoclonal antibodies as previously described [34,35], with some minor modifications. Briefly, bacterial cells cultured on Bordet-Gengou agar were resuspended in phosphate-buffered saline (PBS) to an optical density of 0.01 at 620 nm, and then inactivated at 56°C for 1 h. The wells of 96-well ELISA plate (Nunc Immuno Plate Maxisorp) were coated with 100 μl of this suspension to each well and allowing it to evaporate overnight at 36°C. Anti-Fim2 (NIBSC 04/154) and anti-Fim3 (NIBSC 04/156) antibodies were used at a 1:1,000 dilution in PBS. Antibody binding to bacterial cells was detected following the addition of a 1:4,000 dilution of alkaline phosphatase-labeled goat anti-mouse IgG (Southern Biotechnology Associates, Inc.) and with the use of *p*-nitrophenylphosphate as a substrate. The optical density was measured at 405 nm with 650 nm as a reference using a Multiskan FC microplate reader (Thermo Fisher Scientific Inc.). *B. pertussis* strain 18323 that expresses both Fim2 and Fim3 was used as a positive control.

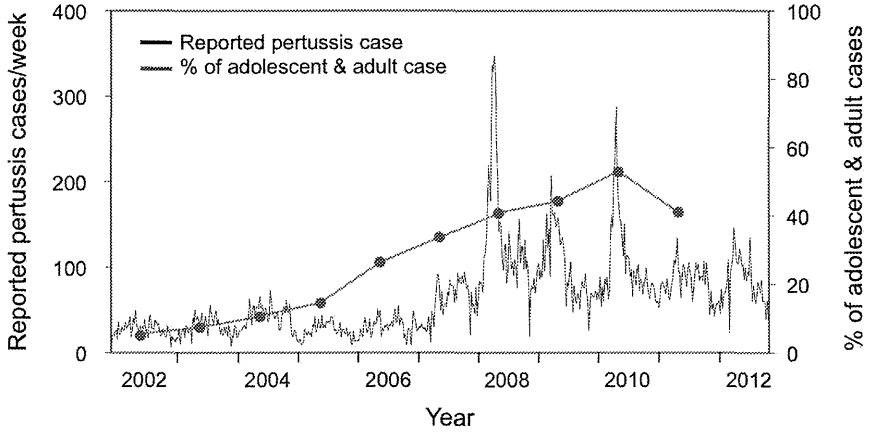

Figure 1. Number of reported pertussis cases per week in Japan from 2002 to 2012. Pertussis cases are shown by the black line, with each value representing a week of the year. The percentage of adolescent and adult cases (≥15 years old) per year is shown in red circles. The data were obtained from the Ministry of Health, Labor and Welfare of Japan Infectious Disease Surveillance data. Data regarding the number of adolescent and adult cases in 2012 were not available.
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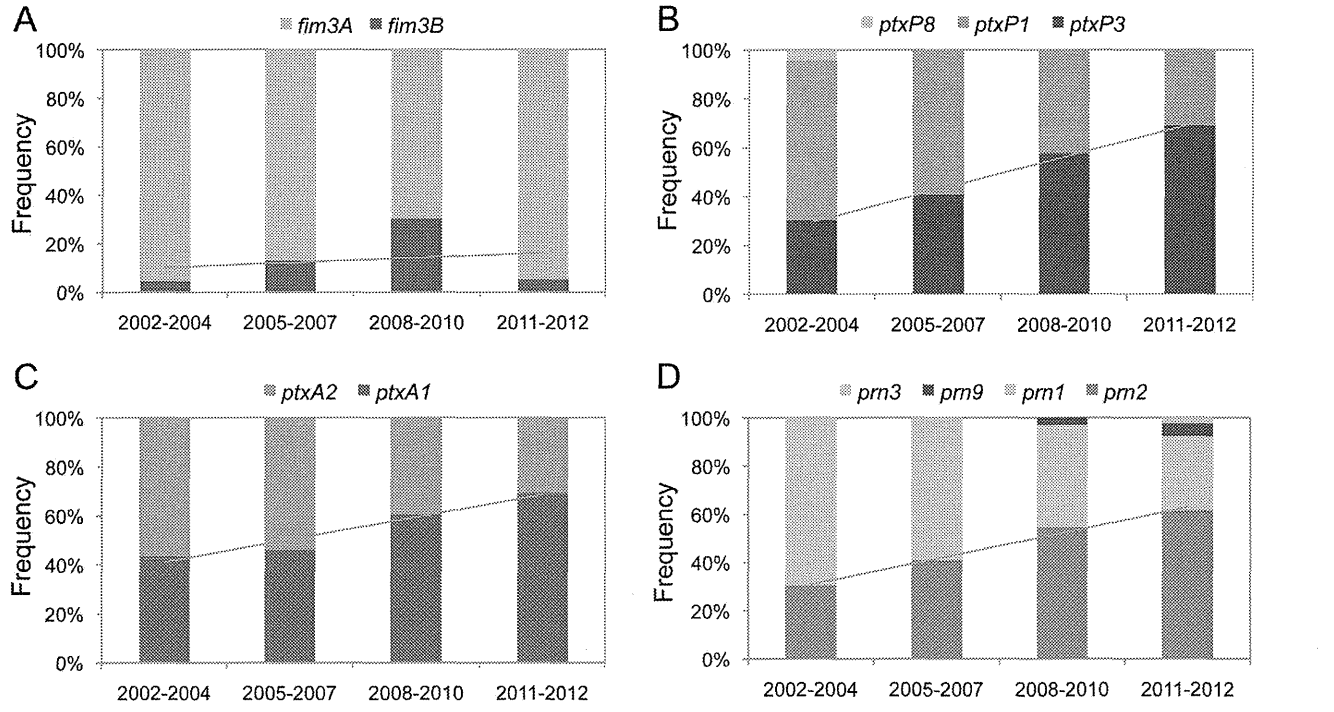

Figure 2. Temporal trends in the frequencies of *fim3*, *ptxP*, *ptxA*, and *prn* alleles within *Bordetella pertussis* isolates in Japan from 2002 to 2012. Four allelic genes, *fim3* (A), *ptxP* (B), *ptxA* (C), and *prn* (D), of 134 *B. pertussis* isolates were sequenced. Isolate allele frequencies are shown by time period: 2002–2004 (non-epidemic, n = 23), 2005–2007 (pre-epidemic, n = 39), 2008–2010 (epidemic, n = 33), and 2011–2012 (post-epidemic, n = 39). The regression line shows the relationship between the frequency of virulence-associated allelic variant (*fim3B*, *ptxP3*, *ptxA1*, or *prn2*) and the 4 time periods.
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Statistical analysis

Squared Pearson's correlation coefficient (R^2) was used to identify a linear dependence between allele frequency (*fim3B*, *ptxP3*, *ptxA1*, or *prn2*) and isolation periods. Fisher's exact test was performed to analyze the distribution of *B. pertussis* population. The Simpson's diversity index (DI) and 95% confidence interval (CI) of MTs was calculated as described by Hunter and Gaston [36] and Grundmann et al. [37], respectively, using the online tool available at http://www.comparingpartitions.info/.

Results

Characteristics of the 2008–2010 pertussis epidemic in Japan

There were 17,349 reported pertussis cases between January 2008 and December 2010 in Japan. Within this pertussis epidemic period, 3 sharp peaks representing increases in case frequency were observed: 1 in late May 2008 (347 cases at week 22), 1 in mid-May 2009 (207 cases at week 20), and 1 in mid-June 2010 (289 cases at week 24) (Fig. 1). The number of reported cases per year in 2008–2010 was ≥2.7 times higher than the previous 5-year average. Although the number of pertussis patients over 15 years of age steadily increased in the 2000s, alongside increases of adolescent and adult incidence rates (40.7%, 44.2%, and 52.9% of all reported cases in 2008, 2009, and 2010, respectively), in 2011, the number of those patients decreased and the incidence rate in adolescents and adults also decreased to 41%.

Temporal changes in the frequencies of *fim3*, *ptxP*, *ptxA*, and *prn* alleles

Among the 134 *B. pertussis* isolates tested, 2 *fim3* (*fim3A* and *fim3B*), 3 *ptxP* (*ptxP1*, *ptxP3* and *ptxP8*), 2 *ptxA* (*ptxA1* and *ptxA2*), and 4 *prn* (*prn1*, *prn2*, *prn3*, and *prn9*) alleles were identified. Figure 2 shows the temporal trends of the allele frequencies. The frequency of the allele *fim3B* temporarily increased during the epidemic period (2008–2010): it was 4.3% in 2002–2004, 12.8% in 2005–2007, 30.3% in 2008–2010, and 5.1% in 2011–2012 (Fig. 2A). In contrast, the frequencies of *ptxP3*, *ptxA1*, and *prn2* increased with time from 2002 to 2012 (Fig. 2B-D). High correlations ($R^2 \geq 0.95$) were observed between these latter allele frequencies and the isolation periods.

Temporal changes in the frequencies of Prn and Fim variants

The Prn and Fim expression phenotypes of the 134 *B. pertussis* study isolates were determined with immunoblotting and indirect whole-cell ELISA, respectively. Figure 3A shows the temporal trend of the frequencies of the 2 identified Prn variants, Prn-expressing and Prn-negative strains, with the frequency of Prn-negative strains at 43.5%, 41.0%, 21.2%, and 25.6% during 2002–2004, 2005–2007, 2008–2010, and 2011–2012, respectively. A decreased frequency of Prn-negative strains was observed during the epidemic period (2008–2010); however, this decrease was not statistically significant ($P > 0.05$). All the Prn-negative strains carried the *prn1* allele (Table S1). Prn-negative strains carrying *prn1* were previously found in Finland [30], and those carrying *prn2* were found in France and the US [27,29].

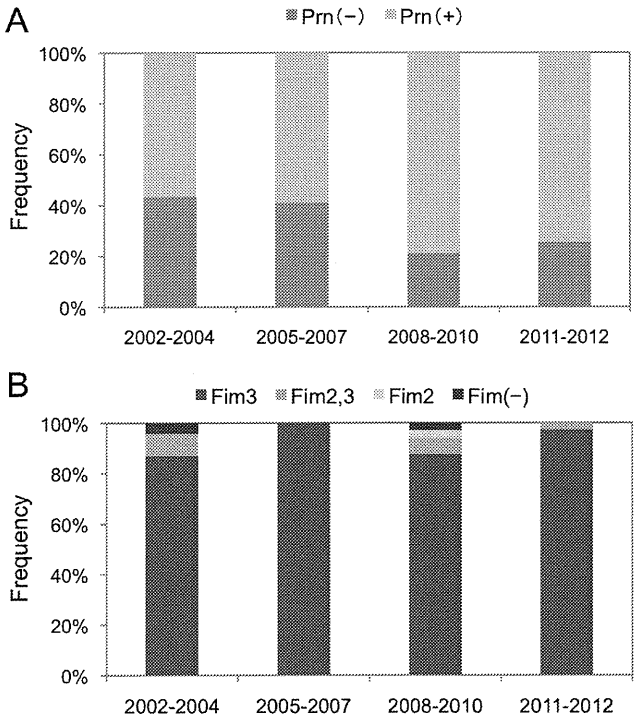

Figure 3. Temporal trends in the frequencies of Prn and Fim3 variants of *Bordetella pertussis* isolates in Japan from 2002 to 2012. Prn (A) and Fim (B) expression was analyzed within 134 *B. pertussis* isolates. Two Prn variants, Prn(+) and Prn(−), and 4 Fim variants, Fim2, Fim3, Fim2,3, and Fim(−) strains, were identified. Variant frequencies are shown by time period: 2002−2004 (non-epidemic, n = 23), 2005−2007 (pre-epidemic, n = 39), 2008−2010 (epidemic, n = 33), and 2011−2012 (post-epidemic, n = 39).
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On the other hand, 4 Fim variants, Fim2, Fim3, Fim2,3, and Fim(−), were identified among the *B. pertussis* isolates. The Fim2,3 strain was detected by both Fim2 and Fim3 antigens, while the Fim(−) strain was not detected by either. As shown in Figure 3B, the Fim3 strain was predominant during all the time periods studied: 87.0% in 2002−2004, 100% in 2005−2007, 87.9% in 2008−2010, and 97.4% in 2011−2012. Two Fim(−) strains were isolated in 2002−2004 and 2008−2010. Interestingly, one Fim(−) strain was previously identified in Ontario, Canada [12].

Temporal changes in MTs and genetic diversity

Among the 134 *B. pertussis* study isolates, 24 different MTs were identified, of which 2 were novel (MT251 and MT253). Figure 4 shows minimum spanning trees that revealed the genetic diversity of the *B. pertussis* population during the time periods of 2002−2004, 2005−2007, 2008−2010, and 2011−2012. Eighteen *B. pertussis* isolates carrying *fim3B* were identified during the 4 time periods, and these *fim3B* strains were divided into 4 MTs: MT26 (n = 1), MT27 (n = 14), MT32 (n = 1), and MT69 (n = 2). Although MT27 and MT186 were the predominant types during all the time periods, the *fim3B* strain did not belong to MT186. MT27 strains carrying *fim3B* were most frequent in MT27 during the epidemic period (2008−2010) at 56% (10/18), and their frequency decreased to 7% (2/27) in 2011−2012. The temporal increase of MT27 strains carrying *fim3B* was statistically significant ($P < 0.05$) when compared with non-MT27 strains carrying *fim3B*.

MT27 strains could be further classified into 5 subtypes (MT27a to MT27e) based on their allele types (Table 1). MT27a strains

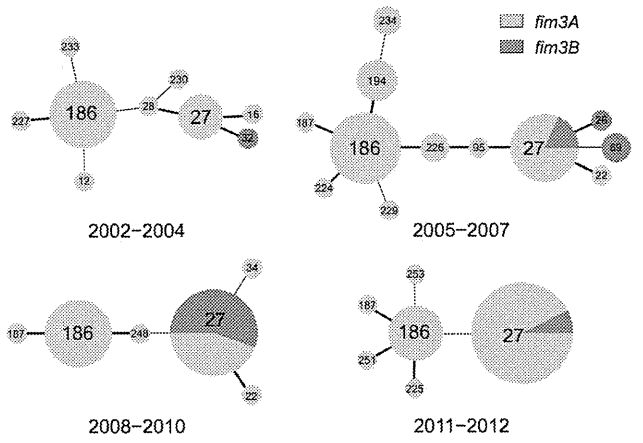

Figure 4. Minimum spanning trees revealing the genetic diversity of the *Bordetella pertussis* population in Japan from 2002 to 2012. MTs were determined for 134 *B. pertussis* isolates. The resultant phylogenetic trees based on MTs are shown by time period: 2002−2004 (non-epidemic, n = 23), 2005−2007 (pre-epidemic, n = 39), 2008−2010 (epidemic, n = 33), and 2011−2012 (post-epidemic, n = 39). Each circle within a tree represents a different MT, with the MT number noted. Thick lines separate single-locus variants, while thin lines separate double-locus variants, and dotted lines signify a more distant relationship. Pink and red colors indicate, respectively, the *fim3A* and *fim3B* allele frequencies within MTs.
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carried *fim3A*, *ptxP3*, *ptxA1*, and *prn2*, and were the predominant subtype. Notably, they were collected in both non-epidemic and epidemic periods. In contrast, MT27d strains carrying *fim3B*, *ptxP3*, *ptxA1*, and *prn2* were predominantly isolated during the epidemic period, with 10 of 12 isolates (83%) being of this subtype in 2008−2010. An MT27c strain carrying *fim3A*, *ptxP3*, *ptxA1*, and *prn9* was also isolated during the epidemic period. The other MT27 subtypes, MT27b and MT27e, were found only in 2011−2012.

The genetic diversity of MTs and the 5 MT27 subtypes was subsequently determined. As shown in Figure 5, Simpson's DI was 0.74 (95% CI, 0.58−0.90), 0.85 (0.78−0.92), 0.77 (0.70−0.84), and 0.59 (0.43−0.75) in 2002−2004, 2005−2007, 2008−2010, and 2011−2012, respectively. Therefore, the genetic diversity within *B. pertussis* population decreased after the 2008−2010 pertussis epidemic.

Relationship between MTs and phenotypes in 2008−2010

During the 2008−2010 pertussis epidemic, MT186 (33%), MT27a (21%), and MT27d (30%) were the predominant MTs, and most of them were serotype Fim3 strains (89%) (Table 2). Fim3 strains were also identified within other minor MTs (MT22, MT27c, MT187, and MT248), while a Fim2 strain belonged to MT34, the 2 Fim2,3 strains were typed as MT27d and MT186, and the Fim(−) strain belonged to MT27a. Of the 10 MT27d strains, 9 expressed Fim3 and 1 expressed both Fim2 and Fim3. On the other hand, Prn-negative strains were all observed within MT186. Seven (64%) of the 11 MT186 strains did not express Prn. All other MTs expressed Prn.

Discussion

In the present study, we demonstrated that the prevalence of *B. pertussis* strains carrying *fim3B*, which were classified as MT27d, temporarily increased during the 2008–2010 pertussis epidemic in

Table 1. Comparison of MTs and allele types between *Bordetella pertussis* isolates collected in non-epidemic and epidemic periods.

	Allele types				No. of isolates detected within the time period			
MT	*fim3*	*ptxP*	*ptxA*	*prn*	2002–2004	2005–2007	2008–2010*	2010–2012
12	A	8	2	1	1			
16	A	3	1	2	1			
22	A	3	1	2		1	1	
26	B	3	1	2		1		
27a	A	3	1	2	5	9	7	24
27b	A	3	1	3				1
27c	A	3	1	9			1	
27d	B	3	1	2		2	10	
27e	B	3	1	9				2
28	A	1	1	1	1			
32	B	3	1	2	1			
34	A	1	1	1			1	
69	B	3	1	2		2		
95	A	3	1	2		1		
186	A	1	2	1	11	12	11	8
187	A	1	2	1		1	1	1
194	A	1	2	1		4		
224	A	1	2	1		1		
225	A	1	2	1				1
226	A	1	2	1		2		
227	A	1	2	1	1			
229	A	1	2	1		1		
230	A	1	1	1	1			
233	A	1	1	1	1			
234	A	1	1	1		2		
248	A	1	2	1			1	
251	A	1	2	1				1
253	A	1	2	1				1

*Pertussis epidemic period.

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Japan. The MT27d strains of the epidemic period were collected in several areas (Table S1). All MT27d strains carried *fim3B*, *ptxP3*, *ptxA1*, and *prn2*, and expressed Prn and Fim3 proteins. Although Prn-negative strains have recently increased in their prevalence within Japan and in other countries [27–30], here, a lowered frequency of Prn-negative strains was observed during the epidemic period specifically, indicating that Prn-negative strain types were not involved in the epidemic. Similarly, we found that the prevalence of the virulence-associated allelic variants, *ptxP3*, *ptxA1*, and *prn2*, has increased with time from the early 2000s, indicating that the variants were also not directly associated with the epidemic. Taken together, our findings demonstrate that *B. pertussis* strains carrying *fim3B* (i.e., MT27d) were associated with the 2008–2010 pertussis epidemic.

We evaluated the regional effect on *B. pertussis* population because of the low number of samples of isolates. When the number of isolates was compared, the difference in regional population between epidemic (2008–2010) and post-epidemic (2011–2012) periods was statistically significant ($P < 0.01$), possibly because of the high number (18/39) of isolates collected in the Kinki district (including Osaka) during the post-epidemic period. A sampling bias cannot be excluded from the analysis of the trend in 2011–2012. However, no significant difference was observed between pre-epidemic (2005–2007) and epidemic periods ($P > 0.05$). The regional effect was therefore small or negligible for detection of the emergence of strain MT27d in the 2008–2010 epidemic.

In the past decade, the prevalence of *B. pertussis* strains carrying *fim3B* has increased worldwide [12–14,25]. In Ontario, Canada, 1 predominant *B. pertussis* clone was identified in the 2000s [12]. This strain carried the same virulence-associated allelic variants (*fim3B*, *ptxP3*, *ptxA1* and *prn2*) as our epidemic strains within MT27d and was identical to the strains involved in recent pertussis resurgences within Europe and Australia. Similarly, *fim3B* strains carrying *ptxP3*, *ptxA1*, and *prn2* were predominant in the US during the 2000s, and most were genotyped to MT27 [13]. Interestingly, the pertussis resurgence in the US was correlated with the emergence and predominance of the *fim3B* allele, but not with the *ptxP3* allele. On the other hand, in the Netherlands, the prevalence of *fim3B* strains temporarily increased in the early

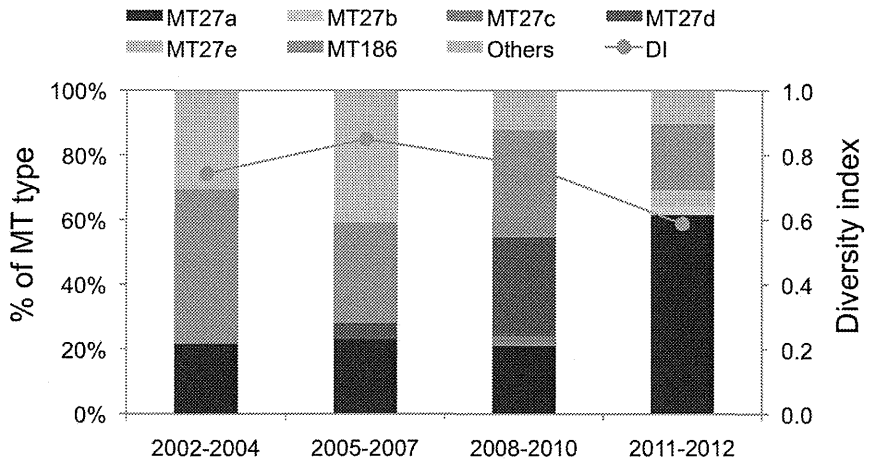

Figure 5. Frequency of MLVA types and genetic diversity of the *Bordetella pertussis* population in Japan from 2002 to 2012. The diversity index (DI) and 95% confidence interval (CI) were calculated from the MT frequencies within 4 time periods: 2002–2004 (non-epidemic), 2005–2007 (pre-epidemic), 2008–2010 (epidemic), and 2011–2012 (post-epidemic). For convenience, minor MTs (MT22, MT34, MT187, and MT248) are shown as "Others".
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2000s, although the strains disappeared in 2010 [14]. Likewise, MT27d strains (carrying *fim3B*) were not identified after the 2008–2010 epidemic period, and the reasons behind the disappearance of this strain type are unclear. Our findings along with those of previous studies, therefore, suggest that the MT27d strain is a recent epidemic strain that is found worldwide, and that this strain is not only associated with pertussis resurgence but can also be correlated with pertussis epidemics.

Fimbriae of *B. pertussis* are composed of Fim2 and/or Fim3 and FimD. The minor fimbrial subunit FimD forms the adhesive tip [38]. Fim2 and Fim3 are encoded by the single-copy genes *fim2* and *fim3*, respectively [39,40], and are serologically distinct [41]. Fim resulting from the *fim3B* strain is Fim3B, which differs from Fim3A by a single amino-acid substitution (A87E) at the surface epitope. To date, the biological differences between Fim3A and Fim3B are unknown. In an effort to address this issue, we recently observed *B. pertussis* clinical strains and found that most strains carrying *fim3B* had strong autoagglutination capability, unlike those carrying *fim3A*, following the suspension of CSM agar cultures into saline (Otsuka and Kamachi, unpublished data).

Table 2. Relationship between MTs and phenotypes in *Bordetella pertussis* isolates collected during the 2008–2010 pertussis epidemic period.

		Fim expression				Prn expression	
MT	No. of isolates	Fim2	Fim3	Fim2,3	Fim(−)	Prn(+)	Prn(−)
22	1		1			1	
27a	7		6		1	7	
27c	1		1			1	
27d	10		9	1		10	
34	1	1				1	
186	11		10	1		4	7
187	1		1			1	
248	1		1			1	

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Surprisingly, autoagglutination was not observed when the strains were cultured on Bordet-Gengou agar. Bacterial autoaggregation is a phenomenon associated with pathogen virulence in many Gram-negative bacteria [42–46], suggesting Fim3B strains are more virulent than Fim3A strains because of their ability to autoagglutinate. Further study is necessary to fully elucidate the relationship between autoagglutination and Fim3B. Attempts to identify the molecular mechanism that regulates autoagglutination within *fim3B* strains are currently underway.

In many countries, a shift from serotype Fim2 to Fim3 in *B. pertussis* circulating strains was observed after mass vaccination, and antigenic differences (in Fim, PT, and Prn) have been since noted between *B. pertussis* vaccine strains and circulating strains. In Japan, the *B. pertussis* strain Tohama carrying *ptxA2*, *prn1*, and *fim2* has been used as a vaccine strain to produce ACVs from 1981. Among the 4 currently used Japanese ACVs, 2 contain Fim2 and all do not contain Fim3 [47]. A recent study demonstrated that Fim2 and Fim3 are immunogenic antigens, and that individuals recently infected with pertussis had greater anti-Fim3 IgG concentrations than anti-Fim2 IgG concentrations, consistent with the current predominance of Fim3 strains [41]. Based on this finding, there is a clear need for the improvement of currently used ACVs. Specifically, the inclusion of Fim3 (Fim3A and/or Fim3B) in ACVs may be an effective way to reduce the number of current circulating *B. pertussis* strains, including Fim3B strains. Although Fim2 has been shown to be a protective antigen, the protective immunogenicity of Fim3 is still unknown [48]. Further study of this topic will be required to evaluate the effectiveness of Fim3 as a protective antigen.

In the present study, the genetic diversity of the *B. pertussis* population decreased after the 2008–2010 pertussis epidemic. This decrease reflects the expansion of the MT27a strain type and the disappearance of MT27d strains. The MT27a strains carried the same *ptxP3*, *ptxA1*, and *prn2* alleles as the MT27d strains, but additionally carried *fim3A*. Significant changes in the *B. pertussis* population were previously observed in a pertussis epidemic in the Netherlands, and this study suggested that strain surveillance may serve as an early detector of pertussis epidemics [24]. Here, we observed significant changes within the *B. pertussis* population during the 2008–2010 epidemic, lending further support for an early warning system of future pertussis epidemics.

In conclusion, the prevalence of *B. pertussis* MT27d strains temporarily increased during the 2008–2010 pertussis epidemic in Japan. The MT27d strains carried the same virulence-associated allelic variants (*fim3B*, *ptxP3*, *ptxA1*, and *prn2*) as recent epidemic strains observed in Australia, Europe, and the US. *B. pertussis* MT27d strains, therefore, likely have the potential to cause large epidemics in other countries, and, hence, further study and strain surveillance of the MT27d strain type is warranted.

Supporting Information

Table S1 Characteristics of *Bordetella pertussis* study isolates.
(XLSX)

Table S2 PCR primers in this study.
(XLSX)

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Author Contributions

Conceived and designed the experiments: YM NO KK . Performed the experiments: YM NO HT-A. Analyzed the data: YM NO KK. Contributed reagents/materials/analysis tools: NO KS KK. Wrote the paper: YM KK.

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Laboratory and Epidemiology Communications

First Report of OXA-48 Carbapenemase-Producing *Klebsiella pneumoniae* and *Escherichia coli* in Japan from a Patient Returned from Southeast Asia

Noriyuki Nagano[1,2,3], Yasunobu Endoh[4], Yukiko Nagano[3], Masami Toyama[1], Mari Matsui[3], Keigo Shibayama[3], and Yoshichika Arakawa[2,3]*

[1]*Medical Microbiology Laboratory, Funabashi Municipal Medical Center, Chiba 273-8588;*
[2]*Department of Bacteriology, Nagoya University Graduate School of Medicine, Nagoya 466-8550;*
[3]*Department of Bacteriology II, National Institute of Infectious Diseases, Tokyo 208-0011; and*
[4]*Clinical Laboratory, Japanese Red Cross Narita Hospital, Chiba 286-0041, Japan*

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Various carbapenemases such as NDM-types, KPC-types, and OXA-types have recently emerged and spread worldwide (1). Among these carbapenemases, OXA-types have been identified mainly in *Acinetobacter* spp., particularly in *Acinetobacter baumannii* (2). OXA-type carbapenemases in *A. baumannii* were shown to be OXA-51-like, OXA-23-like, OXA-24/40-like, and OXA-58-like. However, OXA-48 was first identified from *Klebsiella pneumoniae* clinically isolated in Turkey in 2001 (3). After 2009, OXA-48-producing *K. pneumoniae* spread rapidly among European countries and caused several outbreaks in hospital settings (4–6). The first cases in the United States were recently identified (7), although OXA-48-producing Gram-negative bacteria belonging to the family *Enterobacteriaceae* have been reported in several developing countries or regions (8,9).

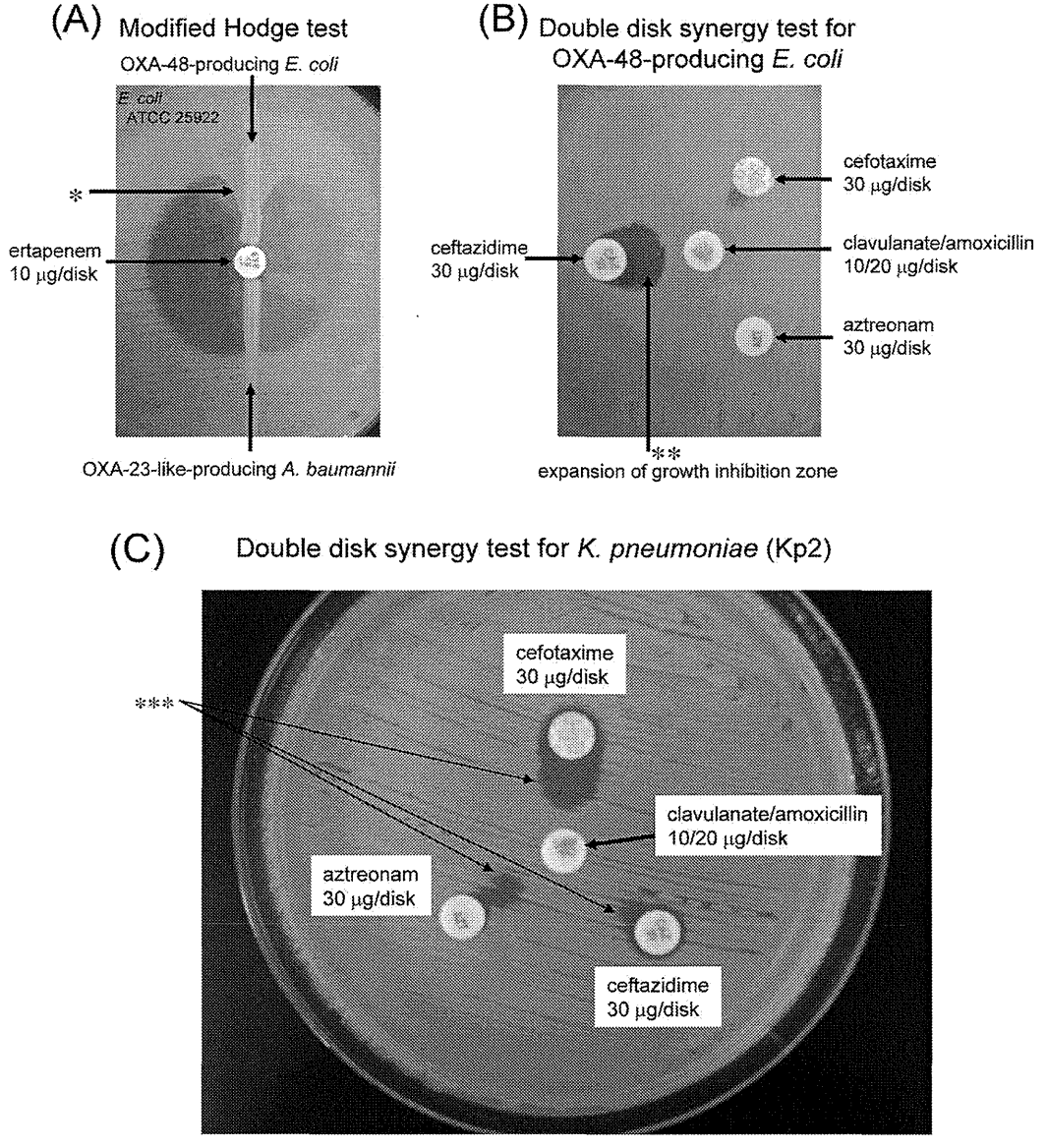
In November 2012, a man in his 60s was admitted to a general hospital in Kanto. This patient had a history of hospitalization in a Southeast Asian country for the treatment of cerebral infarction before admission to the hospital. Three types of antimicrobial-resistant microbes belonging to the family *Enterobacteriaceae*, namely, multidrug-resistant *K. pneumoniae* and *Escherichia coli*, as well as piperacillin-tazobactam-resistant *K. pneumoniae*, were isolated from the sputa and/or feces of this patient. The minimum inhibitory concentrations (MICs) of imipenem (IPM) and meropenem

Table 1. MICs of antimicrobials for OXA-48-producing *K. pneumoniae* and *E. coli*

Antimicrobial	MIC (μg/ml) for			Antimicrobial	MIC (μg/ml) for		
	Kp1[1)]	Kp2[2)]	Ec[2)]		Kp1	Kp2	Ec
ampicillin	>16	>16	>16	cefpirome	≤8	>16	>16
amoxicillin/clavulanate	>16	>16	>16	cefepime	≤1	>32	>32
piperacillin	>64	>64	>64	cefoxitin	4	4	>32
piperacillin/tazobactam	>64	>64	>64	cefmetazole	2	2	16
cefazolin	≤4	>16	>16	cefotetan	≤1	2	16
cefaclor	>16	>16	>16	imipenem	2	2	2
cefotiam	≤8	>16	>16	meropenem	2	2	2
cefotaxime	1	>128	>128	flomoxef	≤8	≤8	≤8
cefotaxime/clavulanate	0.25/4	>32/4	>32/4	gentamicin	≤1	≤1	>8
ceftazidime	≤0.5	>128	>128	amikacin	≤4	≤4	≤4
ceftazidime/clavulanate	≤0.12/4	4/4	16/4	tobramycin	≤1	4	>8
ceftriaxone	≤0.5	>64	>64	minocycline	2	8	>8
cefpodoxime	1	>64	>64	levofloxacin	≤0.5	1	>4
cefozopran	≤2	>16	>16	ciprofloxacin	≤0.25	2	>2
cefoperazone/sulbactam	≤16	>32	>32	fosfomycin	>16	16	≤4
aztreonam	≤0.5	>64	>64	sulfamethoxazole/trimethoprim	≤2	>2	>2

[1)]: OXA-48 (+), ESBL (−).
[2)]: OXA-48 (+), CTX-M-1-group ESBL (+).

*Corresponding author: Mailing address: Department of Bacteriology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Tel: +81-52-744-2106, Fax: +81-52-744-2107, E-mail: yarakawa@med.nagoya-u.ac.jp



*Typical expansion of growth area of *E. coli* ATCC25922 toward the ertapenem disk along the streak of OXA-48-producing *E. coli* was observed, suggesting production of carbapenemase.
**This phenomenon suggested probable production of class A enzymes such as ESBL.
***In each antimicrobial, atypical slight expansion of growth inhibition zone was observed toward the center disk containing clavulanate/amoxicillin.

Fig. 1. (A) Results of modified Hodge test. Growth of *E. coli* ATCC25922, a carbapenem-susceptible reference strain, expands to the ertapenem disk along the streak of the *E. coli* strain Ec, shown in Table 1, suggesting the production of carbapenemase in the Ec. (B) and (C) Double disk synergy tests for *E. coli* strain Ec and *K. pneumoniae* strain Kp2. Since these strains produce both OXA-48 and CTX-M-type ESBL, the patterns of the growth inhibition zone around the disks containing ceftazidime (CAZ), cefotaxime, or aztreonam with clavulanic acid became atypical. In clinical isolates demonstrating atypical growth inhibition profiles with clavulanic acid and CAZ or cefotaxime increased, production of multiple and different types of β-lactamases should be considered.

(MEPM) for these isolates were below the breakpoint for "resistant," but two multidrug-resistant isolates, Kp2 and Ec (Table 1), consistently showed resistance to various broad-spectrum β-lactams, including cefotaxime, ceftazidime (CAZ), cefpirome, and cefepime, together with aminoglycosides and fluoroquinolones. In contrast, a piperacillin-tazobactam-resistant *K. pneumonia* isolate, Kp1 (Table 1), was found to be susceptible to many β-lactams. Inhibition tests for discriminating between β-lactamases produced in the isolates using sodium mercaptoacetate (SMA), EDTA, and 3-aminophenylboronic acid (APB) were all negative, but a modified Hodge test using the ertapenem disk indicated production of carbapenemases in these isolates (Fig. 1A). Polymerase chain reaction (PCR) analyses of carbapemase genes (10,11) such as bla_{NDM}, bla_{IMP}, bla_{VIM}, bla_{KPC}, and bla_{OXA} including $bla_{OXA\text{-}51\text{-}like}$, $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}24/40\text{-}like}$, $bla_{OXA\text{-}58\text{-}like}$, and $bla_{OXA\text{-}48\text{-}like}$ showed the presence of the $bla_{OXA\text{-}48\text{-}like}$ gene in these isolates. PCR analysis showed that two

multidrug-resistant isolates, Kp2 and Ec, as shown in Table 1, were also found to harbor a gene for the CTX-M-1-group ESBL. The MICs of CAZ for these isolates declined from $>128\,\mu g/ml$ to 4 or $16\,\mu g/ml$ in the presence of $4\,\mu g/ml$ clavulanate, suggesting the role of the ESBL production in CAZ resistance. Since these isolates co-produced the CTX-M-1-group ESBL and OXA-48, a double disk synergy test using a clavulanate/ampicillin disk with disks containing CAZ, cefotaxime, or aztreonam showed atypical inhibition profiles (Figs. 1B and 1C). Interestingly, the *E. coli* isolate was not agglutinated with anti-sera specific for *E. coli* serotype O25, although ESBL-producing and fluoroquinolone resistant *E. coli* clinical isolates are usually O25:H4-ST131, a global epidemic lineage. Moreover, an *A. baumannii* that harbors genes for acquired OXA-23-like carbapenemase and ArmA 16S rRNA methyltransferase was co-isolated from this patient.

It has been reported that some clinical isolates were "susceptible" to carbapenems despite harboring carbapenemase genes such as bla_{NDM}, bla_{KPC}, and bla_{OXA}. Therefore, clinical isolates suspected to be ESBL producers due to demonstrating consistent resistance to various cephalosporins and reduced susceptibility (MIC, 2–4 $\mu g/ml$) to carbapenems would worth being subjected to PCR analyses using PCR primer sets for detecting known carbapenemase genes after screening using modified Hodge test (12). Furthermore, even in cases in which the clinical isolates show susceptibility to broad-spectrum β-lactams as found in the piperacillin-tazobactam-resistant *K. pneumoniae* (Kp1 in Table 1), the resistance profile to piperacillin-tazobactam may be a good marker for detecting KPC- or OXA-type-carbapenemase producers in addition to the modified Hodge test. Two types of new carbapenemases, the NDM-1 and KPC-2, have already been identified in Gram-negative microbes in Japan, and OXA-48-like carbapenemase was newly identified in *K. pneumoniae* and *E. coli* in this study. Hence, early detection of invasion of carbapenemase-producing Gram-negative microbes and their containment in clinical settings is currently very important in Japan, where NDM, KPC, and OXA producers remain very rare. Furthermore, intensive surveillance of OXA-type carbapenemase-producing Gram-negative microbes should be conducted in Asian countries, as well as enhanced surveillance of NDM and/or KPC producers because OXA-181, a variant of OXA-48, has already spread in India (13). Positive results in the modified Hodge test using an ertapenem disk (14), as well as multidrug-resistance profiles, including piperacillin-tazobactam, are useful in the early detection of OXA-48-like carbapenemase-producing Gram-negative microbes.

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Conflict of interest None to declare.

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Detection of Tripoli metallo-β-lactamase 2 (TMB-2), a variant of *bla*$_{TMB-1}$, in clinical isolates of *Acinetobacter* spp. in Japan

Satowa Suzuki[1]*, Mari Matsui[1], Masato Suzuki[1], Akira Sugita[2], Yoko Kosuge[2], Nobuhiro Kodama[3], Yasuko Ichise[3] and Keigo Shibayama[1]

[1]*Department of Bacteriology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama Tokyo, Japan 208-0011;* [2]*Yokohama Municipal Hospital, 56 Okazawa-cho, Hodogaya Ward, Yokohama, Japan;* [3]*Fukuoka Tokushukai Medical Center, 4-5 Sugukita, Kasuga Fukuoka, Japan*

*Corresponding author. Tel: +81-42-561-0771; Fax: +81-42-561-7173; E-mail: suzukiss@nih.go.jp

Keywords: MBLs, TMB-1, carbapenemases

Sir,

Metallo-β-lactamase is an important resistance determinant among Gram-negative bacteria, and some metallo-β-lactamase genes are encoded on mobile gene elements that can spread among various clinically important bacterial species.[1] TMB-1 (Tripoli metallo-β-lactamase 1) was first identified in 2012 in an *Achromobacter xylosoxidans* strain isolated from a hospital environment sample in Tripoli, Libya.[2] Here, we report two cases in which *bla*$_{TMB}$-positive non-*baumannii Acinetobacter* spp. were isolated from patients with no history of international travel.

The first case, a carbapenem-resistant *Acinetobacter* sp. (MRY12-142) was isolated from a urine sample in December 2011. This case had no recent history of international travel. For the second case, *Acinetobacter* sp. (MRY12-226) was isolated from necrotic tissue in July 2012. This patient also had no international travel history. These two cases were identified in two hospitals that are separated by more than 1000 km, and there was no epidemiological link between the two cases.

Acinetobacter spp. were identified by sequencing the partial *rpoB* gene and the 16S–23S rRNA gene spacer region,[3,4] which revealed *Acinetobacter pittii* in the first case and *Acinetobacter* genomospecies 14BJ in the second case. Both isolates were resistant to penicillins, cephalosporins, imipenem, meropenem and trimethoprim/sulfamethoxazole, but susceptible to fluoroquinolones, amikacin, and minocycline according to MICs determined by the VITEK2 system (bioMérieux, Lyon, France) and the recommended breakpoints of CLSI 2012.[5] Metallo-β-lactamase production was screened using a disc containing sodium mercaptoacetic acid (SMA) (Eiken, Tokyo, Japan).[6] For both isolates, the growth inhibitory zone around the imipenem and ceftazidime discs expanded upon the addition of the SMA disc, which is strongly indicative of metallo-β-lactamase production.

Based on PCR analyses, both isolates were negative for *bla*$_{NDM-1}$, *bla*$_{KPC}$, *bla*$_{IMP}$ *bla*$_{VIM-1}$, *bla*$_{VIM-2}$, *bla*$_{OXA-23-like}$, *bla*$_{OXA-24-like}$, *bla*$_{OXA-51-like}$ and *bla*$_{OXA-58-like}$. However, PCR analyses for class 1 integron gene cassettes, in which primers targeted the 5′-conserved region (CS) and 3′-CS, revealed two bands of ~1.2 kbp and 1.8 kbp in both isolates. Sequence analysis of the 1.2 kbp PCR products of both isolates showed that the class 1 integron gene cassette contained only one gene that had 99% amino acid identity with TMB-1, and was thus designated TMB-2. The 738 bp sequence of *bla*$_{TMB-2}$ was identical to that of *bla*$_{TMB-1}$, except for one substitution at nucleotide position 544, which caused an amino acid change from serine to proline at position 228 according to the class B standard numbering[7] (GenBank accession numbers AB758277 and AB758278). Sequence analysis for the 1.8 kbp PCR product of MRY12-142 was conducted and showed that the class 1 integron gene cassette contained *aac(6′)-Ib* and a hypothetical open reading frame.

The PCR product of the class 1 integron gene cassette containing *bla*$_{TMB-2}$ was ligated into pGEM-T (Promega, WI, USA) and transformed into *Escherichia coli* strain DH5α. In addition, pGEM-T harbouring *bla*$_{TMB-1}$ was obtained by site-directed mutagenesis and transformed into *E. coli* DH5α to evaluate the role of this single amino acid substitution on antimicrobial susceptibility, the MICs being determined by Etest (bioMérieux). As shown in Table 1, the TMB-2-producing transformant was resistant to ceftazidime and susceptible to aztreonam, similar to the TMB-1-producing transformant. However, the TMB-2-producing transformant showed >256-fold and 16-fold lower MICs for doripenem and meropenem, respectively, compared with the TMB-1-producing transformant. The MICs of imipenem were not

Table 1. Antimicrobial susceptibility of isolates and strains determined by Etest

	MICs (mg/L) for isolates and strains						
Antimicrobial agents	*A. pittii* MRY12-142	*A.* genomospecies 14BJ MRY12-226	*E. coli* DH5α (pGEM-T-TMB-2)	*E. coli* DH5α (pGEM-T-TMB-1)	*E. coli* DH5α (pGEM-T)	*E. coli* DH5α	*A. pittii* ATCC 19004
Aztreonam	32	32	0.064	0.094	0.047	0.047	16
Ceftazidime	>256	>256	>256	>256	0.25	0.38	6
Imipenem	16	>32	2	1	0.38	0.38	0.25
Meropenem	>32	>32	2	32	0.064	0.064	0.75
Doripenem	>32	>32	0.064	32	0.032	0.032	0.19

different for the two strains. Both transformants also showed an apparent expansion of the growth inhibitory zone around the ceftazidime disc upon addition of the SMA disc.

It has been reported that carbapenem resistance among non-*baumannii Acinetobacter* spp. is usually due to the production of metallo-β-lactamase.[8–10] To our knowledge, this study is the first to report an *Acinetobacter* spp. positive for bla_{TMB} and to identify a new variant, bla_{TMB-2}. It is also the first report to identify bla_{TMB} in clinical isolates. The low MICs of carbapenems for transformant cells suggests that additional resistance mechanisms, such as the production of other classes of β-lactamase, a reduction in outer membrane protein expressions and an acceleration of efflux pump activities, may be involved in the carbapenem resistance of parental clinical isolates of *Acinetobacter* spp. Although the same phenomenon was reported in the IMP-type,[11] it is notable that one amino acid substitution from serine to proline in TMB-2 has drastically decreased the MICs of meropenem and doripenem. As neither patient had a history of international travel nor any epidemiological link, it is possible that bla_{TMB-2} had been endemic in Japan but unrecognized because of its reduced ability to hydrolyse carbapenems. The unrecognized spread of bla_{TMB-2} could be a concern as it can turn to bla_{TMB-1} by only one nucleotide substitution. Although this report discusses only two cases, it may be important to evaluate the spread of this emerging metallo-β-lactamase gene among non-*baumannii Acinetobacter* spp.

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Transparency declarations

None to declare.

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Ability of the VITEK® 2 system to detect group B streptococci with reduced penicillin susceptibility (PRGBS)

Kouji Kimura[1,2]*, Noriyuki Nagano[2,3], Yukiko Nagano[2], Jun-ichi Wachino[1,2], Keigo Shibayama[2] and Yoshichika Arakawa[1,2]

[1]Department of Bacteriology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan; [2]Department of Bacteriology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan; [3]Medical Microbiology Laboratory, Funabashi Municipal Medical Center, Chiba 273-8588, Japan

*Corresponding author. Tel: +81-52-744-2106; Fax: +81-52-744-2107; E-mail: koujikim@med.nagoya-u.ac.jp

Keywords: group B *Streptococcus*, GBS, penicillin G

Sir,

Group B *Streptococcus* (*Streptococcus agalactiae*, GBS) is the leading cause of neonatal sepsis and meningitis and an important pathogen among elderly people and those suffering from underlying medical disorders.[1,2] The highest GBS mortality and morbidity result from invasive infections in neonates.[1,2]

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ENVIRONMENTAL MICROBIOLOGY

Vibrio parahaemolyticus and Its Specific Bacteriophages as an Indicator in Cockles (*Anadara granosa*) for the Risk of *V. parahaemolyticus* Infection in Southern Thailand

Mingkwan Yingkajorn · Natthawan Sermwitayawong · Prasit Palitapongarnpimp · Mitsuaki Nishibuchi · William P. Robins · John J. Mekalanos · Varaporn Vuddhakul

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Abstract Correlation between the numbers of *Vibrio parahaemolyticus* and its specific bacteriophages in cockles was investigated from June 2009 to May 2010 in Hat Yai, Songkhla, Thailand. Cockles obtained monthly from a local market were sampled to determine the numbers of *V. parahaemolyticus* and bacteriophages that could form plaques on ten strains of pandemic and nonpandemic *V. parahaemolyticus*. In addition, *V. parahaemolyticus* isolates from clinical samples from Hat Yai hospital over the same period were investigated. All 139 cockles sampled were positive for *V. parahaemolyticus*. However, only 76 of them were positive for bacteriophages. During the testing period, the number of bacteriophages was not significantly correlated with the incidence of *V. parahaemolyticus*-infected patients, but the numbers of *V. parahaemolyticus* isolates from the cockle samples were closely related to the number of infected patients. The bacteriophages isolated from *V. parahaemolyticus* also infected *Vibrio alginolyticus* and *Vibrio mimicus*, suggesting that the broad host range of phages may be a factor of providing the possibility of their participation in the processes of genetic exchange between *V. parahaemolyticus* and closely related *Vibrio* spp. In conclusion, this study indicated that the number of *V. parahaemolyticus* in cockles may be a useful tool for predicting the relative risk of infection by *V. parahaemolyticus* in this area of Thailand.

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M. Yingkajorn · N. Sermwitayawong · V. Vuddhakul (✉)
Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand
e-mail: varaporn.v@psu.ac.th

M. Yingkajorn
Department of Biomedical Sciences, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand

P. Palitapongarnpimp
Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand

M. Nishibuchi
Center for Southeast Asian Studies, Kyoto, Japan

W. P. Robins · J. J. Mekalanos
Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA, USA

Introduction

Vibrio parahaemolyticus is a halophilic bacterium found worldwide in marine ecosystems and is an important causative agent of gastroenteritis after consumption of improperly cooked seafood. It requires 0.5–8 % sodium chloride for growth with the optimal temperature ranging from 30 to 35 °C. When grown with favorable conditions, the generation time is around 12 min [28]. Not all strains of *V. parahaemolyticus* are pathogenic. Most clinical strains of *V. parahaemolyticus* possess a major virulence factors, a thermostable direct hemolysin (TDH). Another virulence factor, the TDH-related hemolysin (TRH) has also been involved in some food-poisoning outbreaks [12]. Both TDH and TRH increased intracellular Ca^{2+} and Cl^- secretion from colonic cells [25, 26]. TDH and TRH are encoded by the *tdh* and *trh* genes, respectively [13, 29]. ToxR, a transmembrane regulatory protein encoded by the *toxR* gene, plays an important role in stimulation *tdh* [17]. Around 90 % of clinical isolates possess either the *tdh*, the *trh* gene, or both, whereas those genes are rarely detected in the environmental isolates [30, 32].

Marine bacterial viruses (bacteriophages) are ubiquitous in the environment, and their high host specificity for bacterial species makes them effective agents for the control of

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microbial communities [11]. In vitro study of bacteriophages and population dynamics of marine bacteria (*Pseudoaltermonas* sp, *Photobacteria* sp., and *Vibrio* sp.) indicated that bacteriophages influenced population dynamics, the species composition, and the clonal composition of the bacterial community in marine system [16]. In Dhaka, Bangladesh, a decrease in the bacteriophages that preyed on *Vibrio cholerae* in local waters was correlated with an increase in cholera patient admissions as well as an increase in the rate of isolation of pathogenic *V. cholerae* from the same water samples [6]. In addition, an increase in the environmental vibriophages was correlated with a decrease in *V. cholerae* and tended to coincide with the collapse of a concurrent cholera epidemic [7]. Data corroborating these conclusions have been reported by Nelson and colleagues [19]. In the case of *V. cholerae*, lytic phages adversely affect the infectivity of this organism [20, 34], and thus the transmission of *V. cholerae* in endemics settings where phages can amplify on *V. cholerae* in either the environment or within cholera victims [7, 13, 21].

The first isolation of *V. parahaemolyticus* bacteriophages was reported in 1966 [17]. The three bacteriophages described were isolated from seawater, human feces, and a lysogenic strain of *V. parahaemolyticus* and were found to be distinctly different from each other based on plaque morphology, host range, and serological specificity. In 2000, a filamentous bacteriophage f237 was isolated from a culture supernatant of tdh^+ *V. parahaemolyticus* O3/K6 pandemic strain [18]. It was speculative that epidemic potency of the pandemic *V. parahaemolyticus* was due to acquisition of the open reading frame 8 of f237. However, relationship between vibriophages and *V. parahaemolyticus* in environment has not been extensively investigated. In general, *Vibrio* spp. are more abundant in shellfish ($\sim 10^4$ cfu/g) than in seawater ($\sim 10^2$ cfu/ml) and vibriophages are more prevalent in mollusk extracts than in seawater [1, 2]. After studying the seasonal variations in *V. parahaemolyticus*, bacteriophages and *Vibrio* spp. in oysters and the water column, Comeau and colleagues [4] concluded that phages from *Vibrio* species in oysters were the major sources of viruses that infect *V. parahaemolyticus*. In Thailand, consumption of molluscan shellfish, especially the cockle, *Andara granosa*, contaminated with *V. parahaemolyticus*, is a major cause of acute gastroenteritis because semicooking is the popular way for consumption of this kind of mollusk. In a previous study, we reported that the DNA profiles of *V. parahaemolyticus* isolates from mollusks and patients were identical [30]. In this study, we focused on the possibility that cockle was a potential reservoir for both *V. parahaemolyticus* and their infecting phages and performed a year-long study on the numbers of bacteriophage and *V. parahaemolyticus* isolated from cockles and their relationship to the incidence of *V. parahaemolyticus* infections in Hat Yai city, southern Thailand.

Materials and Methods

Collection of Clinical Samples of *V. parahaemolyticus*

Clinical isolates of *V. parahaemolyticus* were obtained from patients in a Hat Yai hospital, Songkhla, Thailand. A rectal swab from each patient was cultured on thiosulfate citrate bile salt sucrose agar [5]. The sucrose nonfermenting colonies were selected after overnight incubation at 37 °C and confirmed as *V. parahaemolyticus* by a polymerase chain reaction (PCR) targeted to the *V. parahaemolyticus tox*R gene (Vp-*toxR*).

Collection of Environmental *V. parahaemolyticus*

After they were harvested from nearby cultivation farms, cockles were obtained from a local market in Hat Yai city. From each animal, 25 g of flesh was homogenized in 225 ml of alkaline peptone water (APW) containing 1 % NaCl, pH 8.6. Serial dilutions of these extracts were inoculated into a series of three APW tubes and incubated overnight at 37 °C. The highest dilution tubes that exhibited turbidity were plated on CHROMagar™ Vibrio (CHROMagar, France) and after incubation; one to five mauve-colored colonies were selected and used as a source of genomic DNA template to detect the Vp-*tox*R gene by PCR. If at least one colony was PCR positive, the corresponding tube and sample was considered positive for *V. parahaemolyticus* and used to calculate the most probable number (MPN) for the concentration of viable *V. parahaemolyticus* per gram in the corresponding cockle

Table 1 Numbers of bacteriophage positive cockle samples and susceptible hosts

Time	No. of samples collected	No. of samples positive for phage	~~Number~~ of susceptible *V. parahaemolyticus* hosts[a]
Jun 2009	14	12	9
Jul 2009	14	7	10
Aug 2009	22	8	9
Sept 2009	14	2	4
Oct 2009	7	2	3
Nov 2009	9	4	6
Dec 2009	10	4	7
Jan 2010	10	9	9
Feb 2010	10	7	10
Mar 2010	10	8	9
Apr 2010	10	9	10
May 2010	9	4	8
Total	139	76	–[b]

[a] The same ten host strains of *V. parahaemolyticus* were determined per month

[b] In each month, three to ten of *V. parahaemolyiticus* host strains were susceptible to phages

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t2.1 **Table 2** *V. parahaemolyticus* strains susceptible to bacteriophages isolated from cockles between June 2009 and May 2010

t2.2 Time	Positive sample(s) for bacteriophage									
t2.3	Vp pandemic strains					Vp nonpandemic strains				
t2.4 t2.5	PSU 2598 O4/K68	PSU 3916 O1/KUT	PSU 4099 O1/KUT	PSU 4118 O1/K25	PSU 4211 O3/K6	PSU 3622 O3/K29	PSU 3862 O5/K17	PSU 3909 O3/K5	PSU 3922 O3/K45	PSU 4117 O1/K56
t2.6 Jun 2009	3	2	3	4	10	8	0	4	4	4
t2.7 Jul 2009	4	3	1	3	1	2	3	2	2	3
t2.8 Aug 2009	1	1	0	1	3	2	2	4	1	1
t2.9 Sept 2009	1	0	0	2	0	1	0	0	0	2
t2.10 Oct 2009	0	0	1	0	0	0	0	0	1	1
t2.11 Nov 2009	1	1	0	1	2	3	0	2	0	0
t2.12 Dec 2009	1	2	0	0	2	0	1	1	1	3
t2.13 Jan 2010	1	0	1	2	5	4	1	3	5	2
t2.14 Feb 2010	2	1	3	3	3	3	4	2	1	2
t2.15 Mar 2010	1	0	3	4	4	4	6	2	1	2
t2.16 Apr 2010	3	9	1	3	7	5	3	3	5	7
t2.17 May 2010	1	4	1	0	1	0	1	1	1	1
t2.18 Total	19	23	14	23	38	32	21	24	22	28

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extract (Mike Curiale, http://i2workout.com/mcuriale/mpn/index.html). In addition, both the *tdh* and *trh* virulence genes of *V. parahaemolyticus* were examined by PCR as described below.

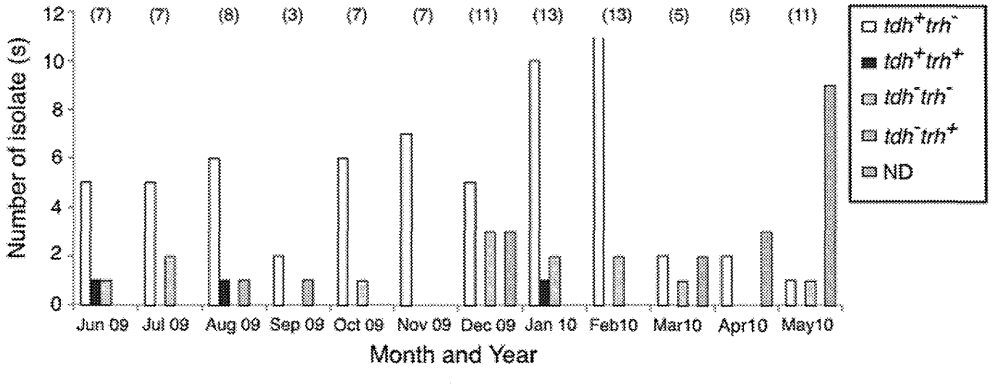
V. parahaemolyticus Confirmation and Detection of Virulence Genes

The test strains of *V. parahaemolyticus* were inoculated into 1 ml of Luria Bertani broth supplemented with 1 % NaCl and grown overnight with shaking at 150 rpm at 37 °C. Template DNA was obtained by boiling for 10 min; the supernatant was diluted 1:10 in sterile deionized water and used for the PCR assay. The *toxR* gene was amplified using primers T4 and T7 as previously described [14]. Testing for the *tdh* and *trh* genes by PCR was carried out using the previously reported primers D3–D5 and R2–R6, respectively [24]. The PCR conditions were as follows: the reaction mixture consisted of 1.5 µl template DNA, 2 µl 10× buffer, 1.6 µl 25 mM $MgCl_2$, 0.1 µl *Taq* DNA polymerase, 1.6 µl 2.5 mM dNTPs, 5 µl 2 µM primer mix, and 8.2 µl sterile MilliQ water. The amplification conditions were 35 cycles of amplification including a denaturation step at 94 °C for 1 min, annealing at 63 °C (Vp-*tox*R) for 1.5 min or 55 °C (*tdh* and *trh*) for 1 min, and extension at 72 °C for 1.5 min for Vp-*tox*R or for 1 min for *tdh* and *trh*.

Isolation and Enumeration of *V. parahaemolyticus* Bacteriophages

All other bacteria used in this study were from the culture collection of the Department of Microbiology, Prince of Songkla University, Thailand. Different O and K serotype strains of *V. parahaemolyticus* including five clinical pandemic strain isolates (PSU2598-O4/K68, PSU3916-O1/KUT, PSU4099-O1/KUT, PSU4118-O1/K25, and PSU4211-O3/K6) and five nonpandemic clinical isolates (PSU3622-O3/K29, PSU3862-O5/K17, PSU3909-O3/K5, PSU3922-O3/

Q5 **Fig. 1** Clinical isolation of *V. parahaemolyticus* detected in patients between June 2009 and May 2010. *Number in parenthesis* is total number of isolates/month; *ND* no PCR result



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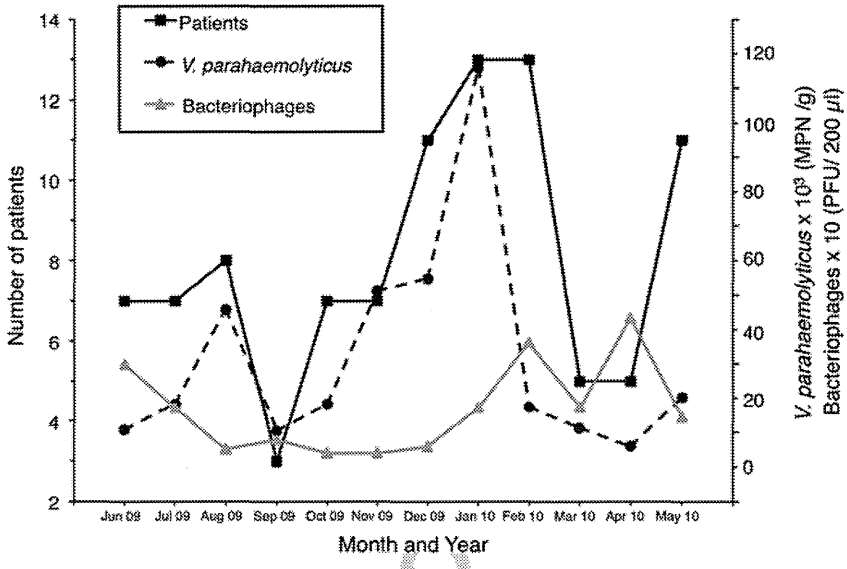

Fig. 2 Correlation between the number of patients, *V. parahaemolyticus* and bacteriophages obtained in 1 year (June 2009–May 2010)

K45, and PSU4117-O2/K56) were used as host strains for screening for *V. parahaemolyticus* bacteriophages in the cockle filtrates.

In each experiment, 25 g of cockle was squeezed out and the liquid extract obtained was centrifuged three times at 1,100×*g* for 3 min. The supernatant was collected and subjected to Millipore filtration (0.45 µm; Corning, Germany) to produce the starting filtrates. Enumeration of vibriophages was performed by plaque assay using the agar-overlay method [3, 6]. Briefly, 200 µl of *V. parahaemolyticus* culture (~10^8 cells/ml; optical density, 0.2–0.3 at 600 nm) was mixed with 200 µl of cockle filtrate and 3 ml of soft agar, which was then overlain on nutrient agar containing 17 mM NaCl. The plate was incubated overnight at 37 °C. Plaques observed on the bacterial lawn indicated a positive *V. parahaemolyticus* bacteriophages and were counted to estimate the concentration of phage particles in the cockle filtrates. Single plaque isolates of the detected phages were amplified on a susceptible strain of *V. parahaemolyticus* by harvesting the plate lysates and stored at 4 °C over chloroform.

Susceptibility of Clinical and Environmental *V. parahaemolyticus* Isolates to Bacteriophages

Each of the eight bacteriophages that were propagated on the most three common pandemic serotypes of *V. parahaemolyticus* strains isolated in this area (PSU2598-O4/K68, PSU4118-PSUO1/K25, and PSU 4211-O3/K6) [32], and the one nonpandemic *V. parahaemolyticus* strain (PSU 3622-O3/K29) were randomly selected from different plaque morphologies and amplified by a plate-lysate method. The high-titer bacteriophages (10^{10} PFU/ml) were subsequently examined for their host range specificity against 18 clinical and 18 environmental isolates of *V. parahaemolyticus*. Bacterial strains were first grown to an optical density of ~0.2–0.3 at 600 nm and were then added to nutrient soft agar; the mixture was overlain onto nutrient agar supplemented with 17 mM NaCl to make the bacterial lawns. Ten microliters of high-titer phage stocks were spotted into agar-overlay lawns of bacteria. The plates were incubated overnight at 37 °C and inspected on the next day for the single plaques or bacterial growth inhibition zones.

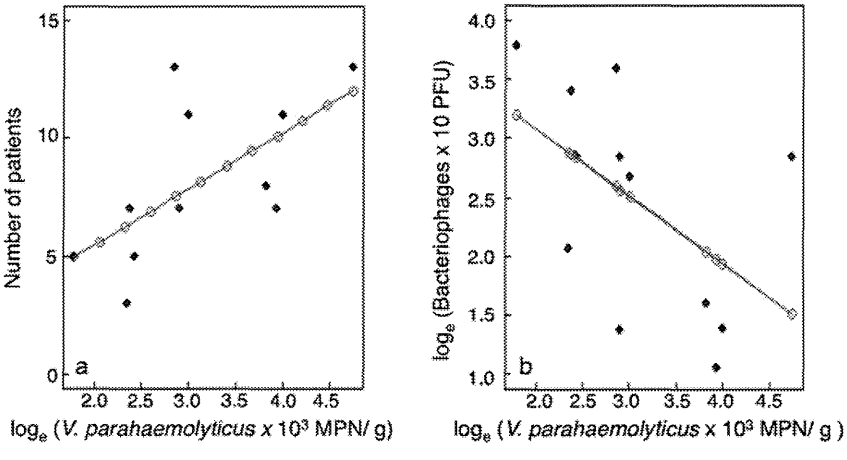

Fig. 3 **a** Pearson's product–moment correlation showing correlations between *V. parahaemolyticus* level in cockle extracts and the number of patients; p=0.02 (95 % confidence interval). **b** No correlation between *V. parahaemolyticus* level and bacteriophages level in cockle extracts and filtrates, respectively; p=0.07 (95 % confidence interval)

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