AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

PUBLIC AND ENVIRONMENTAL HEALTH MICROBIOLOGY

Environmental Presence and Genetic Characteristics of Carbapenemase-Producing *Enterobacteriaceae* from Hospital Sewage and River Water in the Philippines

Yuki Suzuki,[a] Pearl Joy Nazareno,[b] Ryuichi Nakano,[a] Melisa Mondoy,[b] Akiyo Nakano,[a] Mark Philip Bugayong,[b] Josie Bilar,[b] Mauricio Perez V,[b] Emarld Julian Medina,[b] Mariko Saito-Obata,[c] Mayuko Saito,[c] Kazutoshi Nakashima,[d] Hitoshi Oshitani,[c] Hisakazu Yano[a]

[a]Department of Microbiology and Infectious Diseases, Nara Medical University, Kashihara, Japan
[b]Department of Microbiology, Research Institute for Tropical Medicine, Muntinlupa, Philippines
[c]Department of Virology, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan
[d]Faculty of Sports and Health Science, Daito Bunka University, Higashi-Matsuyama, Japan

Yuki Suzuki, Pearl Joy Nazareno, and Ryuichi Nakano contributed equally to this work.

ABSTRACT This study aimed to evaluate the prevalence and genetic characteristics of carbapenemase-producing *Enterobacteriaceae* (CPE) in hospital sewage and river water in the Philippines, which has a typical tropical maritime climate. We collected 83 water samples from 7 hospital sewage and 10 river water sites. CPE were identified using CHROMagar mSuperCARBA, and Gram-negative strains were identified using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) or 16S rRNA gene sequencing. Resistance genes in *Enterobacteriaceae* strains were identified using PCR and DNA sequencing, and transferability of carbapenemase genes from the CPE was investigated with conjugation experiments. Genotyping was performed using multilocus sequence typing (MLST) for *Escherichia coli* and *Klebsiella pneumoniae*. Out of 124 *Enterobacteriaceae* isolates, we identified 51 strains as CPE and divided these into 7 species, 11 *E. coli*, 14 *Klebsiella* spp., 15 *Enterobacter* spp., and 11 others, including 4 additional species. Conjugation experiments via broth mating and using *E. coli* J53 revealed that 24 isolates can transfer carbapenemase-encoding plasmids. MLST analysis showed that 6 of 11 *E. coli* isolates belonged to clonal complex 10 (CC10). Of 11 *K. pneumoniae* strains, 9 unique sequence types (STs) were identified, including ST147. Five types of carbapenemase genes were identified, with the most prevalent being NDM ($n = 39$), which is epidemic in clinical settings in the Philippines. *E. coli* CC10 and *K. pneumoniae* ST147, which are often detected in clinical settings, were the dominant strains. In summary, our results indicate that hospital sewage and river water are contaminated by CPE strains belonging to clinically important clonal groups.

IMPORTANCE Carbapenemase-producing *Enterobacteriaceae* (CPE) cause severe health care-associated infections, and their increasing prevalence is a serious concern. Recently, natural ecosystems have been recognized as important reservoirs of antibiotic resistance genes. We investigated the prevalence and genetic characteristics of CPE isolated from the environment (hospital sewage and river water) in the Philippines and found several CPE, including *Escherichia coli* and other species, with different carbapenemases. The most prevalent carbapenemase gene type was NDM, which is endemic in clinical settings. This study revealed that isolates belonging to carbapenemase-producing *E. coli* CC10 and *K. pneumoniae* sequence type 147 (ST147), which are often detected in clinical settings, were dominant in the natural environment. Our work here provides a report on the presence and characteristics of CPE in the environment in the Philippines and demonstrates that both hospital sew-

Citation Suzuki Y, Nazareno PJ, Nakano R, Mondoy M, Nakano A, Bugayong MP, Bilar J, Perez M, V, Medina EJ, Saito-Obata M, Saito M, Nakashima K, Oshitani H, Yano H. 2020. Environmental presence and genetic characteristics of carbapenemase-producing *Enterobacteriaceae* from hospital sewage and river water in the Philippines. Appl Environ Microbiol 86:e01906-19. https://doi.org/10.1128/AEM.01906-19.

Editor Christopher A. Elkins, Centers for Disease Control and Prevention

Address correspondence to Ryuichi Nakano, rnakano@naramed-u.ac.jp.

Received 20 August 2019
Accepted 1 November 2019
Accepted manuscript posted online 8 November 2019
Published 7 January 2020

age and river water are contaminated by CPE strains belonging to clinically important clonal groups.

KEYWORDS Carbapenemase-producing *Enterobacteriaceae*, hospital sewage, river water, the Philippines

Antibiotic resistance, in particular to carbapenems, is a threat to global health. Infections caused by carbapenemase-producing *Enterobacteriaceae* (CPE) have limited treatment options and are associated with high mortality rates (1). CPE cause severe health care-associated infections, and their increasing prevalence is a serious concern (2).

Carbapenemase genotypes originally differed geographically, as the U.S. epidemic was associated with KPC (class A in the Ambler classification) (3), the European epidemic with VIM (class B) and OXA-48-like (class D), and the Asian epidemic with NDM and IMP (class B). The NDM type was first detected in India in tap water and sewage and has now spread to clinical settings worldwide (4, 5). Carbapenemase genes are frequently located on plasmids and mobile genetic elements that can be transmitted between species and often coexist with other classes of antibiotic resistance genes, such as aminoglycosides and fluoroquinolones (6).

The "One Health" approach is a strategic framework for reducing the risk of infectious diseases at the animal-human-environment interface and was officially adopted by international organizations and professional bodies in 2008 (7). Antibiotic resistance has traditionally been viewed as a clinical problem, but within the last decade, natural ecosystems have been recognized as important reservoirs of antibiotic resistance genes (8). CPE have been reported in the natural environment (9), and previous studies have reported the presence of carbapenemase- and/or extended-spectrum β-lactamase-producing *Enterobacteriaceae* in rivers, effluent, and hospital sewage systems worldwide (10–12), highlighting the interrelationship between human infection and water sources (13). It can thus be inferred that the primary origin of these bacteria is humans, from clinical settings and community settings, and that these bacteria are disseminated to the environment.

The CPE epidemic gene type in clinical settings in the Philippines is NDM (NDM-1 and NDM-7) (14, 15); however, the prevalence of CPE in the natural environment and the molecular relatedness between CPE in human settings and CPE in the natural environment are unclear. Therefore, we investigated the prevalence and genetic characteristics of CPE isolated from the environment (hospital sewage and river water) in the Philippines.

RESULTS AND DISCUSSION

Identification and characterization of CPE isolates. We collected 83 water samples from 7 hospital and 10 river sites and then plated them on CHROMagar mSuperCARBA plates to select CPE. High-density colonies grew on all CHROMagar mSuperCARBA plates incubated with the hospital sewage and river water samples. This agar plate inhibits extended spectrum β-lactamase (ESBL)/AmpC producers, and CPE can be selected based on the morphology of red or blue colonies that is characteristic of *Enterobacteriaceae*. In total, 124 *Enterobacteriaceae* isolates were obtained from 83 samples (Table 1), of which 33 were *Escherichia coli*, 35 *Klebsiella* spp., 28 *Enterobacter* spp., 24 *Citrobacter* spp., 1 *Serratia marcescens*, 1 *Kluyvera ascorbata*, 1 *Raoultella ornithinolytica*, and 1 *Providencia stuartii*. Among the 124 isolates, 51 were identified as CPE using PCR, and of these, 11 were *E. coli*, 14 *Klebsiella* spp., 15 *Enterobacter* spp., 8 *Citrobacter* spp., 1 *S. marcescens*, 1 *K. ascorbata*, and 1 *R. ornithinolytica*. CPE were recovered from five hospital sewage sites (including pretreatment) and four river sites. Of the four hospitals with treatment systems, CPE were detected only from pretreatment samples. Of the 51 CPE, 27 isolates were found in hospital sewage and 24 in river water. For CPE analysis, we removed isolates that have the same antimicrobial susceptibility, resistance genes, and sequence type (ST) patterns to reduce the possibility of having duplicate strains and, finally, obtained 51 CPE. The other 73 isolates were

TABLE 1 Prevalence of Gram-negative bacteria and carbapenemase-producing *Enterobacteriaceae* (CPE) in the environment

Sampling site (area)	Sewage treatment plant	No. of samples (pretreatment)	No. of *Enterobacteriaceae* isolates grown on CHROMagar mSuperCARBA	No. of isolated CPE (isolates from pretreatment)
Hospital A (Metro Manila)	Yes	2 (12)	33	0 (10)
Hospital B (Metro Manila)	Yes	1 (4)	11	0 (6)
Hospital C (Metro Manila)	Yes	2 (5)	2	0 (2)
Hospital D (Metro Manila)	Yes	1 (3)	3	0 (3)
Hospital E (Metro Manila)	No	3	18	6
Hospital F (Biliran)	No	3	12	0
Hospital G (Leyte)	No	4	16	0
River Q (Metro Manila)	-	5	0	0
River R (Metro Manila)	-	1	2	1
River S (Metro Manila)	-	1	9	9
River T (Metro Manila)	-	3	0	0
River U (Metro Manila)	-	1	1	1
River V (Metro Manila)	-	8	17	13
River W (Rizal)	-	4	0	0
River X (South Cotabato)	-	4	0	0
River Y (Pampanga)	-	4	0	0
River Z (Benguet)	-	12	0	0
Total		83	124	51

identified as non-CPE as evidenced by negative PCR for carbapenemase; these isolates possibly include duplicate isolates, but further analysis was not performed. Furthermore, we also selected white colonies that were non-*Enterobacteriaceae* and identified them as *Acinetobacter* spp. and/or *Pseudomonas* spp. using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). PCR analysis indicated that they possessed some carbapenemases, such as NDM-1, OXA-58, and OXA-72 (16). We would like to further analyze this in detail in the future.

Table 2 lists the MICs for antimicrobial agents against the 51 CPE. A total of 44 isolates were nonsusceptible to carbapenems (imipenem or meropenem, $\geq$2 mg/liter), and 7 were susceptible (4 KPC-2, 2 OXA-48-like, and 1 GES-20 producer). Regarding non-β-lactams, 28 isolates were nonsusceptible to levofloxacin (MIC, $\geq$4 mg/liter) and 25 were nonsusceptible to gentamicin (MIC, $\geq$8 mg/liter). A total of 32 isolates with resistance to carbapenems were also resistant to levofloxacin and/or aminoglycosides, and 15 isolates were resistant to carbapenems, fluoroquinolones, and aminoglycosides. The distribution of carbapenemase genes is shown in Table 2. Among the 51 CPE, 39 isolates were positive for NDM (NDM-1, NDM-5, and NDM-7), 7 were positive for KPC (KPC-2), 2 were positive for OXA-48-like (OXA-48 and OXA-181), 2 were positive for GES (two GES-20), and 1 was positive for IMI (IMI-18). In addition, 24 isolates were positive for the CTX-M gene (CTX-M-1G), including CTX-M-15 (22 isolates) and CTX-M-3 (2 isolates). Moreover, NDM producers also carried the CTX-M gene (20/24 isolates). Seven isolates were positive for the 16S rRNA methylase gene RmtC and were highly resistant to aminoglycosides (gentamicin and amikacin MICs, $>$256 mg/liter); these isolates were all positive for NDM-1. Of the 15 isolates that were resistant to carbapenems, fluoroquinolones, and aminoglycosides, 14 were positive for NDM (4 *Enterobacter* spp., 4 *Citrobacter* spp., 3 *E. coli*, and 3 *Klebsiella* spp.).

Many CPE, including *E. coli* and other species, possessed different types of carbapenemase. The most prevalent carbapenemase gene type was NDM ($n = 39$; Table 2); NDM-1 and NDM-7 producers are endemic in clinical settings in the Philippines (14). We found that CPE isolates that are often found in clinical settings were also present in the environment. KPC, GES, and OXA-48 producers, which have not yet been detected in clinical settings in the Philippines (14, 15, 17), were detected in both hospital sewage and river water. Although it is unclear whether these CPE isolates originated from humans or the natural environment, it is possible that they may spread from the natural environment to clinical settings in the future.

Genotypes of CPE isolated from the environment (*E. coli* and *K. pneumoniae*). Various sequence types (STs) were identified from multilocus sequence typing (MLST)

TABLE 2 Distribution and characteristics of 51 carbapenemase-producing *Enterobacteriaceae*

Strain no.	Species	Sampling site	Carbapenemase	ESBL[a]	16S rRNA methylase	MIC (mg/liter) for[b]:					Plasmid incompatibility[c]	Transferability[d] (10^n)		ST (CC)[f]
						IPM	MEM	LVX	GEN	AMK		Carbapenemase	ESBL	
160	*E. coli*	Hospital A	NDM-7			2	8	32	32	64	FIA, FIB, X3	nt		448 (448)
222	*E. coli*	Hospital E	OXA-181	CTX-M-15		0.5	0.5	128	1	4	I1-Iɣ, F, X3	nt	nt	167 (10)
233	*E. coli*	Hospital E	NDM-7			4	4	16	64	4	FIA, FIB, X3, X4	nt		448 (448)
308	*E. coli*	River V	OXA-48	CTX-M-15		0.5	0.125	16	1	4	FIA, FIB, F	nt	−8	44 (10)
309	*E. coli*	River V	NDM-1			4	8	1	1	4	Y, I1-Iɣ, X3	−7		48 (10)
322	*E. coli*	River V	KPC-2			0.5	0.5	1	8	4	A/C, FIB, Y	nt		206 (206)
331	*E. coli*	River V	NDM-7			4	2	8	1	8	FIB, F, X3	nt		162 (469)
379	*E. coli*	River S	KPC-2			1	0.5	16	1	8	X4	−8		617 (10)
438	*E. coli*	River S	NDM-7	CTX-M-15		4	8	64	2	>256	X3	nt	nt	156 (156)
485	*E. coli*	River S	NDM-5	CTX-M-15		2	1	32	0.5	8	FIA, FIB, F, X4	nt	nt	167 (10)
536	*E. coli*	River U	NDM-7	CTX-M-15		8	2	16	0.5	4	FIA, FIB, F, X3	nt	nt	10 (10)
186	*K. pneumoniae*	Hospital A	KPC-2			2	1	2	128	8	A/C	−7		978
193	*K. pneumoniae*	Hospital A	KPC-2	CTX-M-15		1	1	2	64	8	A/C	−6	nt	978
270	*K. pneumoniae*	Hospital B	NDM-1	CTX-M-15	RmtC[e]	8	8	4	>256	>256	A/C	−6[e]	−6	Novel 1
357	*K. pneumoniae*	Hospital C	NDM-1	CTX-M-15		4	2	2	16	4	NT	nt	nt	Novel 2
420	*K. pneumoniae*	Hospital D	NDM-7	CTX-M-15		4	8	4	0.5	2	FIIa, X3	nt	nt	147
221	*K. pneumoniae*	Hospital E	NDM-1			4	8	64	0.25	4	HI1, X3	nt		37
223	*K. pneumoniae*	Hospital E	NDM-1	CTX-M-15	RmtC[e]	4	4	256	>256	>256	N	−5[e]	nt	231
311	*K. pneumoniae*	River V	NDM-7	CTX-M-15		16	64	64	0.5	4	I1-Iɣ	nt	nt	16
327	*K. pneumoniae*	River V	NDM-1	CTX-M-15		8	8	32	0.5	8	NT	−6	nt	147
440	*K. pneumoniae*	River S	KPC-2	CTX-M-3		4	4	16	0.25	4	FIIa	−6	nt	11
484	*K. pneumoniae*	River S	KPC-2			4	4	2	0.5	2	FII, N, X3	nt		3026
220	*Klebsiella oxytoca*	Hospital B	NDM-1		RmtC	2	4	2	>256	>256	FIA	nt		
293	*K. oxytoca*	River V	NDM-7			8	8	64	128	8	A/C, X3	nt		
297	*K. oxytoca*	River V	GES-20			0.5	0.25	4	0.5	16	L/M	nt		
154	*Enterobacter cloacae*	Hospital A	NDM-1	CTX-M-15		4	2	1	>256	>256	A/C	nt	nt	
185	*E. cloacae*	Hospital A	NDM-1	CTX-M-15		4	2	1	128	8	NT	−6	nt	
191	*E. cloacae*	Hospital A	NDM-1	CTX-M-15		4	2	1	128	4	A/C	−6	nt	
259	*E. cloacae*	Hospital B	NDM-7			8	4	2	0.5	2	X3	−7		
260	*E. cloacae*	Hospital B	NDM-1	CTX-M-15	RmtC[e]	8	4	1	>256	>256	A/C	−6[e]	−4	
264	*E. cloacae*	Hospital B	NDM-7	CTX-M-3		16	4	1	0.25	2	X3	nt	nt	
267	*E. cloacae*	Hospital B	NDM-7			4	4	1	0.25	2	X3	−7		
310	*E. cloacae*	River V	NDM-1	CTX-M-15		4	8	128	64	4	A/C, X3	−7	−4	
460	*E. cloacae*	River R	IMI-18			32	16	≤0.06	0.25	1	FII	nt		
328	*Enterobacter kobei*	River V	GES-20			32	4	16	64	8	A/C	nt		
330	*Enterobacter tabaci*	River V	NDM-1	CTX-M-15	RmtC	64	32	32	>256	>256	A/C	nt	nt	
419	*Enterobacter xiangfangensis*	Hospital D	NDM-1			2	1	1	0.25	2	A/C	−6		
336	*E. xiangfangensis*	River V	NDM-1	CTX-M-15		8	16	64	128	4	A/C, X3	nt	nt	
377	*Enterobacter hormaechei*	River S	NDM-1			4	1	0.5	0.5	2	A/C	−6		
376	*Enterobacter* sp.	River S	NDM-1		RmtC	4	16	32	>256	>256	A/C, HI1	−5		
189	*Citrobacter freundii*	Hospital A	NDM-7	CTX-M-15		8	2	2	0.25	4	A/C, X3	−6	−4	
195	*C. freundii*	Hospital A	NDM-7	CTX-M-15		8	2	2	0.25	4	X3	−7	−4	
196	*C. freundii*	Hospital A	NDM-7	CTX-M-15		4	8	16	64	8	A/C, I1-Iɣ, F, X3	nt	nt	
231	*C. freundii*	Hospital E	KPC-2			1	1	256	0.25	2	A/C	nt		
413	*C. freundii*	Hospital D	NDM-7			8	4	0.5	0.5	2	HI1, X3	−7		

(Continued on next page)

TABLE 2 (Continued)

Strain no.	Species	Sampling site	Carbapenemase	ESBL[a]	16S rRNA methylase	MIC (mg/liter) for[b]:					Plasmid incompatibility[c]	Transferability[d] (10^n)		ST (CC)[f]
						IPM	MEM	LVX	GEN	AMK		Carbapenemase	ESBL	
441	*C. freundii*	River S	NDM-1			4	2	16	16	4	N	−6		
472	*Citrobacter amalonaticus*	Hospital A	NDM-1		RmtC[e]	8	8	4	>256	>256	N	−6[e]		
447	*C. amalonaticus*	River S	NDM-7			8	4	4	128	2	X3	−7		
395	*Serratia marcescens*	Hospital C	NDM-1			4	1	1	64	8	A/C	−7		
227	*Kluyvera ascorbata*	Hospital E	NDM-1			1	2	2	0.25	4	X3	nt		
298	*Raoultella ornithinolytica*	River V	NDM-7			8	16	1	32	4	N, X3	nt		

[a]ESBL, extended-spectrum β-lactamase.
[b]Antibiotics: IPM, imipenem; MEM, meropenem; LVX, levofloxacin; GEN, gentamicin; AMK, amikacin.
[c]NT, nontypeable; underline indicates plasmid incompatibility type of transconjugant.
[d]nt, not transferred.
[e]RmtC was also transferred to transconjugants.
[f]ST, sequence type; CC, clonal complex.

analysis of *E. coli* and *Klebsiella pneumoniae* (Table 2). Six *E. coli* isolates belonged to clonal complex 10 (CC10; ST10, ST44, ST48, ST167, and ST617). Moreover, 9 STs were identified, including 2 novel STs, in 11 *K. pneumoniae* isolates (2 ST147, 2 ST978, 1 ST11, 1 ST16, 1 ST37, 1 ST231, and 1 ST3026).

Carbapenemase-producing *E. coli* isolates were divided into nine unique MLST types based on MLST analysis. *E. coli* belonging to ST131 is a major ST of ESBLs and/or carbapenemase-producing isolates in humans (18, 19); however, it was not detected in this study. Six isolates belonging to CC10 contained the carbapenemase gene types NDM (NDM-1, NDM-5, and NDM-7), KPC-2, and OXA-48-like (OXA-48 and OXA-181). A study conducted in the Netherlands found that ESBL-producing *E. coli* belonging to CC10 is present in a broad range of hosts, including humans, animals, and environmental surface water, with almost the same frequency (10%) (20). Moreover, CC10 has also been reported in the Danube River (Europe) (21), the Yamato River (Japan) (22), and sewage in Pakistan (23). A possible explanation is that CC10 circulates easily among different hosts and contains different types of carbapenemase genes. Therefore, the presence of carbapenemase-producing *E. coli* belonging to CC10 in the environment is a concern, because it can spread among humans, animals, and the environment.

Of the 11 *K. pneumoniae* strains identified, 2 isolates belonged to ST147 (1 NDM-1 producer and 1 NDM-7 producer) and 1 belonged to ST11 (a KPC-2 producer). In clinical settings worldwide, *K. pneumoniae* strains that belong to ST11, ST14, ST101, ST147, and ST258 are major carbapenemase-producing clones (24). *K. pneumoniae* strains that produce NDM-1 belonging to ST147 and KPC-2 belonging to ST11 are predominant in Germany and China in clinical settings, respectively (25, 26). We found that CPE isolated from clinical settings were also present in the natural environment, suggesting that CPE isolated from humans and the environment may be connected.

We found carbapenemase-producing *E. coli* belonging to CC10 and *K. pneumoniae* belonging to ST147 and ST11 in hospital sewage as well as in river water. It is probable that some hospital and domestic sewage is directly discharged into municipal sewage, and thus CPE were detected in river water. The Global Water Intelligence report states that the sewage system coverage rate is 31.2% in the Philippines, which is similar to that in other Southeast Asian countries (30 to 40%) (27); meanwhile, the coverage in Europe and North America is 70 to 80% (27). A previous study reported that CPE are spread throughout a community area by direct discharge (11); therefore, a broad sewage system coverage rate may prevent human contact with CPE-contaminated water. Increasing the sewage system coverage rate is expected to reduce the presence of CPE in the environment; however, a more in-depth understanding is needed because there are various factors at play, such as climate and regionality. Given the observed prevalence of CPE in river water and hospital sewage, transmission to humans through water contact is a realistic possibility. The probability of transmission depends upon the function and frequency of contact between humans and contaminated water bodies, e.g., whether for recreational activity or irrigation. In this study, CPE were detected only in pretreatment sewage, and not in posttreatment sewage, of four hospitals (hospitals A to D in Table 1). These hospitals treat sewage via aeration, chlorine treatment, and filtration. We theorized that CPE were not detected in posttreatment sewage due to the treatment process, which reduces the number of sewage bacteria. These findings highlight the need for constant monitoring of hospital sewage for antibiotic-resistant bacteria and for efficient sewage treatment plants in health care settings as part of biosecurity programs. Moreover, the findings support the importance and urgency of action needed to reduce environmental contamination by CPE.

Characteristics of carbapenemase-encoding plasmids and their transferability. Replicon typing revealed variability in the types of carbapenemase-encoding plasmids encountered in terms of incompatibility groups. Of the 51 isolates, 23 and 19 possessed X3 (circulating among *Enterobacteriaceae*) and A/C (broad-host-range) replicon regions, respectively, including *E. coli*, *Enterobacter* spp., and *Klebsiella* spp., whereas 3 isolates possessed nontypeable plasmids. Of the 23 IncX3-possessing isolates, 21 were NDM producers. All CPE isolates were tested for transferability of carbapenemase gene

determinants by conjugation with *E. coli* J53, and transconjugants containing carbapenemase-encoding plasmids were obtained from 24 (47.0%) isolates, with an average transfer frequency of 3.1×10^{-6} (range, 10^{-5} to 10^{-8}). Of the 24 transconjugants, 20 were positive for NDM and 4 were positive for KPC. There was no significant difference in transfer frequency between NDM and KPC producers.

Among the 20 transferable NDM producers, the most prevalent plasmid incompatibility types were IncA/C ($n = 9$) and IncX3 ($n = 8$), which have a broad host range (28). Of the NDM-positive transconjugants, IncX3 and A/C plasmids were obtained from 7 and 1 isolate, respectively, while the remaining 12 transconjugants had nontypeable plasmids (Table 2). Although the transfer frequency was not high (average transfer frequency, 10^{-6}), it is possible that the NDM-encoded plasmids are transferred to other species and strains. Indeed, IncX3 plasmids carrying various carbapenemases, such as NDM and KPC, were isolated from both clinical settings and sewage in the United Arab Emirates (UAE) (29), Myanmar (30), and China (31). Therefore, monitoring the prevalence of IncX3 plasmids in clinical settings and in sewage is necessary. Regarding the ESBL genes, conjugation experiments revealed that 6 of 24 CTX-M-1G-positive isolates were able to transfer their CTX-M-encoded plasmids to *E. coli* J53 (Table 2). It is possible that the carbapenemase and CTX-M genes are located on nontransferable plasmids or chromosomes of isolates that were not able to transfer both resistance genes. A previous study reported the presence of CTX-M and carbapenemase genes on chromosomes, raising concerns that these strains may disseminate worldwide (32–34). Nevertheless, it is not clear whether resistant genes that are not transferred are located on plasmids or chromosomes, and thus further analysis of the genome via next-generation sequencing is warranted.

In addition, we identified species rarely reported in clinical settings, such as *K. ascorbata* and *R. ornithinolytica*, which produce NDM. This suggests that carbapenemase-encoding genes are spreading to nonconventional organisms and may be more widespread in the environment than previously thought.

Conclusion. Our study reports the presence and genetic characteristics of CPE in the environment (hospital sewage and river water) in the Philippines. Various species produced different carbapenemases, with the most prevalent gene type being NDM, which is epidemic in clinical settings. We found that isolates belonging to carbapenemase-producing *E. coli* CC10 and *K. pneumoniae* ST147, which are also often detected in clinical settings, were dominant in the natural environment. Further studies are warranted for investigating the epidemiological links between isolates from the natural environment and humans.

MATERIALS AND METHODS

Collection of environmental samples. We collected water samples from 7 hospital sewage and 10 river sites between August 2016 and August 2018. The study area included Metropolitan Manila, Benguet, South Cotabato, Pampanga, Rizal, Leyte, and Biliran, Philippines, which have a typical tropical maritime climate (Table 1, Fig. 1). For hospital sewage, pretreatment samples were collected from seven hospitals. Moreover, four of the seven hospitals had sewage treatment systems, and thus posttreatment samples were also collected.

CPE selection and species identification. To select for CPE, 100 μl of each water sample was plated on CHROMagar mSuperCARBA (Kanto Chemical, Tokyo, Japan) and incubated at 35°C for 24 to 48 h. We selected 20 to 50 colonies that differed in form and color and gave priority to red and blue colonies with morphology characteristic of *Enterobacteriaceae*, according to the manufacturer's instructions. Primary identification was conducted with MALDI-TOF MS using a Vitek MS system (bioMérieux, Marcy-l'Étoile, France). Isolates that could not be identified using MALDI-TOF MS were identified using 16S rRNA gene sequencing (35).

Antimicrobial susceptibility testing. The antimicrobial susceptibility of various antimicrobial agents was determined using the agar dilution method (36), and quality control was performed using *E. coli* ATCC 25922. MICs were interpreted according to the breakpoints defined by the Clinical and Laboratory Standards Institute (37).

Detection of antimicrobial resistance genes. PCR was performed for all isolates from the environmental samples using AmpliTaq Gold 360 master mix (Thermo Fisher Scientific, Waltham, MA) to detect the carbapenemase genes bla_{IMP}, bla_{VIM}, bla_{KPC}, $bla_{OXA\text{-}48\text{-}like}$, bla_{NDM}, bla_{GES}, bla_{IMI}, and bla_{SME} (38–44). Carbapenemase-positive isolates were also tested for other resistance genes using PCR, including CTX-M-type ESBL ($bla_{CTX\text{-}M\text{-}1Group}$, $bla_{CTX\text{-}M\text{-}2G}$, $bla_{CTX\text{-}M\text{-}9G}$, and $bla_{CTX\text{-}M\text{-}8/25G}$) and 16S rRNA methylase genes (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, and *npmA*) (45–47).

FIG 1 Map of the study area. I. Red dots indicate hospital sewage sites, and blue dots indicate river water sites. Map templates were taken from http://www.freemap.jp/ (left) and https://www.openstreetmap.org/ (right). II. Sampling sites. (a and b) Hospital sewage; (c and d) river.

DNA sequencing was conducted using BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA) and an ABI3730xl analyzer (Applied Biosystems). BLAST version 1.12 (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to process the sequencing data and to identify genes.

Plasmid characterization and conjugation experiments. Plasmid incompatibility groups were identified using the PCR-based replicon-typing method (48, 49). Conjugation experiments were conducted with the broth mating method using CPE isolates as the donor and sodium azide-resistant *E. coli* J53 as the recipient as previously described (50). Exponential-phase Luria-Bertani broth cultures of donor strains and recipient *E. coli* J53 were mixed at a ratio of 1:1 (by volume); these mating mixtures were incubated overnight at 35°C. Transconjugants were selected on Luria-Bertani agar plates containing cefpodoxime (8 μg/ml) and sodium azide (100 μg/ml).

MLST. MLST was performed using Achtman's (51) and Institut Pasteur's (52) schemes for *E. coli* and *K. pneumoniae* isolates, respectively. Housekeeping genes in *E. coli* (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) and *K. pneumoniae* (*rpoB*, *phoE*, *infB*, *gapA*, *mdh*, *pgi*, and *tonB*) were sequenced. DNA sequence variations were analyzed using an MLST database for *E. coli* and *K. pneumoniae* (https://pubmlst.org/bigsdb?db=pubmlst_mlst_seqdef) to determine STs.

ACKNOWLEDGMENTS

We thank the staff at the microbiology laboratory of the Research Institute for Tropical Medicine, Kazuhiro Yoshimura, Tetsuya Matsumoto, and Kokoro Nakagawa, for their excellent technical support. We also gratefully acknowledge Lyndon Lee Suy, Luis F. Cruz, and Nilo Marayag for their help in sample collection.

This study was supported by JSPS KAKENHI (grant numbers 16K09940 and 17K10027), the Japanese Agency for Medical Research and Development (grant number 15650264), and a Sasakawa Scientific Research Grant from the Japanese Science Society.

This work won an award at the 61st Annual Conference of the Japanese Association for Infectious Diseases, Central Regional Conference.

REFERENCES

1. Tzouvelekis LS, Markogiannakis A, Piperaki E, Souli M, Daikos GL. 2014. Treating infections caused by carbapenemase-producing *Enterobacteriaceae*. Clin Microbiol Infect 20:862–872. https://doi.org/10.1111/1469-0691.12697.

2. Logan LK, Weinstein RA. 2017. The epidemiology of carbapenem-resistant *Enterobacteriaceae*: the impact and evolution of a global menace. J Infect Dis 15:S28–S36. https://doi.org/10.1093/infdis/jiw282.
3. Bush K, Jacoby GA. 2010. Updated functional classification of β-lactamases. Antimicrob Agents Chemother 54:969–976. https://doi.org/10.1128/AAC.01009-09.
4. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR. 2009. Characterization of a new metallo-β-lactamase gene, bla_{NDM-1}, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. Antimicrob Agents Chemother 53:5046–5054. https://doi.org/10.1128/AAC.00774-09.
5. Walsh TR, Weeks J, Livermore DM, Toleman MA. 2011. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. Lancet Infect Dis 11:355–362. https://doi.org/10.1016/S1473-3099(11)70059-7.
6. Ludden C, Reuter S, Judge K, Gouliouris T, Blane B, Coll F, Naydenova P, Hunt M, Tracey A, Hopkins KL, Brown NM, Woodford N, Parkhill J, Peacock SJ. 2017. Sharing of carbapenemase-encoding plasmids between *Enterobacteriaceae* in UK sewage uncovered by MinION sequencing. Microb Genom 3:e000114. https://doi.org/10.1099/mgen.0.000114.
7. FAO, OIE, WHO, UN System Influenza Coordination, UNICEF and WORLD BANK. 2008. Contributing to One World, One Health: a strategic framework for reducing risks of infectious diseases at the animal-human-ecosystems interface. http://www.fao.org/docrep/011/aj137e/aj137e00.htm.
8. Caltagirone M, Nucleo E, Spalla M, Zara F, Novazzi F, Marchetti VM, Piazza A, Bitar I, De Cicco M, Paolucci S, Pilla G, Migliavacca R, Pagani L. 2017. Occurrence of extended spectrum β-lactamases, KPC-type, and MCR-1.2-producing *Enterobacteriaceae* from wells, river water, and wastewater treatment plants in Oltrepò Pavese area, Northern Italy. Front Microbiol 10:2232.
9. Woodford N, Wareham DW, Guerra B, Teale C. 2014. Carbapenemase-producing *Enterobacteriaceae* and non-*Enterobacteriaceae* from animals and the environment: an emerging public health risk of our own making? J Antimicrob Chemother 69:287–291. https://doi.org/10.1093/jac/dkt392.
10. Khan FA, Hellmark B, Ehricht R, Söderquist B, Jass J. 2018. Related carbapenemase-producing *Klebsiella* isolates detected in both a hospital and associated aquatic environment in Sweden. Eur J Clin Microbiol Infect Dis 37:2241–2251. https://doi.org/10.1007/s10096-018-3365-9.
11. Islam MA, Islam M, Hasan R, Hossain MI, Nabi A, Rahman M, Goessens WHF, Endtz HP, Boehm AB, Faruque SM. 2017. Environmental spread of New Delhi metallo-β-lactamase-1-producing multidrug-resistant bacteria in Dhaka, Bangladesh. Appl Environ Microbiol 17:e00793-17. https://doi.org/10.1128/AEM.00793-17.
12. Piedra-Carrasco N, Fàbrega A, Calero-Cáceres W, Cornejo-Sánchez T, Brown-Jaque M, Mir-Cros A, Muniesa M, González-López JJ. 2017. Carbapenemase-producing *Enterobacteriaceae* recovered from a Spanish river ecosystem. PLoS One 12:e0175246. https://doi.org/10.1371/journal.pone.0175246.
13. Zurfluh K, Hachler H, Nuesch-Inderbinen M, Stephan R. 2013. Characteristics of extended-spectrum β-lactamase- and carbapenemase-producing *Enterobacteriaceae* isolates from rivers and lakes in Switzerland. Appl Environ Microbiol 79:3021–3026. https://doi.org/10.1128/AEM.00054-13.
14. Velasco JM, Valderama MT, Peacock T, Warawadee N, Nogrado K, Navarro FC, Chua D, Jr, Apichai S, Sirigade R, Macareo LR, Swierczewski B. 2017. Carbapenemase-producing *Enterobacteriaceae* and nonfermentative bacteria, the Philippines, 2013–2016. Emerg Infect Dis 23:1597–1598. https://doi.org/10.3201/eid2309.161237.
15. Chou A, Ro M, Evangelista MA, Sulit AK, Lagamayo E, Torres BC, Klinzing DC, Daroy ML, Navoa-Ng J, Sucgang R, Zechiedrich L. 2016. Emergence of *Klebsiella pneumoniae* ST273 carrying bla_{NDM-7} and ST656 carrying bla_{NDM-1} in Manila, Philippines. Microb Drug Resist 22:585–588. https://doi.org/10.1089/mdr.2015.0205.
16. Woodford N, Ellington MJ, Coelho JM, Turton JF, Ward ME, Brown S, Amyes SG, Livermore DM. 2006. Multiplex PCR for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. Int J Antimicrob Agents 27:351–353. https://doi.org/10.1016/j.ijantimicag.2006.01.004.
17. Jean SS, Lee WS, Hsueh PR, SMART Asia-Pacific Group. 2018. Ertapenem non-susceptibility and independent predictors of the carbapenemase production among the *Enterobacteriaceae* isolates causing intra-abdominal infections in the Asia-Pacific region: results from the Study for Monitoring Antimicrobial Resistance Trends (SMART). Infect Drug Resist 11:1881–1891. https://doi.org/10.2147/IDR.S181085.
18. Yano H, Uemura M, Endo S, Kanamori H, Inomata S, Kakuta R, Ichimura S, Ogawa M, Shimojima M, Ishibashi N, Aoyagi T, Hatta M, Gu Y, Yamada M, Tokuda K, Kunishima H, Kitagawa M, Hirakata Y, Kaku M. 2013. Molecular characteristics of extended-spectrum β-lactamases in clinical isolates from *Escherichia coli* at a Japanese tertiary hospital. PLoS One 8:e64359. https://doi.org/10.1371/journal.pone.0064359.
19. Cai JC, Zhang R, Hu YY, Zhou HW, Chen GX. 2014. Emergence of *Escherichia coli* sequence type 131 isolates producing KPC-2 carbapenemase in China. Antimicrob Agents Chemother 58:1146–1152. https://doi.org/10.1128/AAC.00912-13.
20. Dorado-Garcia A, Smid JH, van Pelt W, Bonten MJM, Fluit AC, van den Bunt G, Wagenaar JA, Hordijk J, Dierikx CM, Veldman KT, de Koeijer A, Dohmen W, Schmitt H, Liakopoulos A, Pacholewicz E, Lam TJGM, Velthuis AG, Heuvelink A, Gonggrijp MA, van Duijkeren E, van Hoek AHAM, de Roda Husman AM, Blaak H, Havelaar AH, Mevius DJ, Heederik DJJ. 2018. Molecular relatedness of ESBL/AmpC-producing *Escherichia coli* from humans, animals, food and the environment: a pooled analysis. J Antimicrob Chemother 73:339–347. https://doi.org/10.1093/jac/dkx397.
21. Kittinger C, Lipp M, Folli B, Kirschner A, Baumert R, Galler H, Grisold AJ, Luxner J, Weissenbacher M, Farnleitner AH, Zarfel G. 2016. *Enterobacteriaceae* isolated from the River Danube: antibiotic resistances, with a focus on the presence of ESBL and carbapenemases. PLoS One 11:e0165820. https://doi.org/10.1371/journal.pone.0165820.
22. Gomi R, Matsuda T, Matsumura Y, Yamamoto M, Tanaka M, Ichiyama S, Yoneda M. 2017. Whole-genome analysis of antimicrobial-resistant and extraintestinal pathogenic *Escherichia coli* in river water. Appl Environ Microbiol 83:e02703-16. https://doi.org/10.1128/AEM.02703-16.
23. Zahra R, Javeed S, Malala B, Babenko D, Toleman MA. 2018. Analysis of *Escherichia coli* STs and resistance mechanisms in sewage from Islamabad, Pakistan indicates a difference in *E. coli* carriage types between South Asia and Europe. J Antimicrob Chemother 73:1781–1785. https://doi.org/10.1093/jac/dky109.
24. Lee CR, Lee JH, Park KS, Kim YB, Jeong BC, Lee SH. 2016. Global dissemination of carbapenemase-producing *Klebsiella pneumoniae*: epidemiology, genetic context, treatment options, and detection methods. Front Microbiol 13:895.
25. Zhang R, Liu L, Zhou H, Chan EW, Li J, Fang Y, Li Y, Liao K, Chen S. 2017. Nationwide surveillance of clinical carbapenem-resistant *Enterobacteriaceae* (CRE) strains in China. EBioMedicine 19:98–106. https://doi.org/10.1016/j.ebiom.2017.04.032.
26. Becker L, Kaase M, Pfeifer Y, Fuchs S, Reuss A, von Laer A, Sin MA, Korte-Berwanger M, Gatermann S, Werner G. 2018. Genome-based analysis of carbapenemase-producing *Klebsiella pneumoniae* isolates from German hospital patients, 2008–2014. Antimicrob Resist Infect Control 7:62. https://doi.org/10.1186/s13756-018-0352-y.
27. Global Water Intelligence. 2010. Global Water Market 2011: meeting the world's water and wastewater needs until 2016. Media Analytics Ltd, Oxford, United Kingdom.
28. Partridge SR, Kwong SM, Firth N, Jensen SO. 2018. Mobile genetic elements associated with antimicrobial resistance. Clin Microbiol Rev 31:e00088-17. https://doi.org/10.1128/CMR.00088-17.
29. Mouftah SF, Pál T, Darwish D, Ghazawi A, Villa L, Carattoli A, Sonnevend Á. 2019. Epidemic IncX3 plasmids spreading carbapenemase genes in the United Arab Emirates and worldwide. Infect Drug Resist 12:1729–1742. https://doi.org/10.2147/IDR.S210554.
30. Sugawara Y, Akeda Y, Sakamoto N, Takeuchi D, Motooka D, Nakamura S, Hagiya H, Yamamoto N, Nishi I, Yoshida H, Okada K, Zin KN, Aye MM, Tomono K, Hamada S. 2017. Genetic characterization of bla_{NDM}-harboring plasmids in carbapenem-resistant *Escherichia coli* from Myanmar. PLoS One 12:e0184720. https://doi.org/10.1371/journal.pone.0184720.
31. Li Y, Luo L, Xiao Z, Wang G, Li C, Zhang Z, Zhou Y, Zhang L. 2019. Characterization of a carbapenem-resistant *Kluyvera cryocrescens* isolate carrying bla_{ndm-1} from hospital sewage. Antibiotics (Basel) 8:149. https://doi.org/10.3390/antibiotics8030149.
32. Huang W, Wang G, Sebra R, Zhuge J, Yin C, Aguero-Rosenfeld ME, Schuetz AN, Dimitrova N, Fallon JT. 2017. Emergence and evolution of multidrug-resistant *Klebsiella pneumoniae* with both bla_{KPC} and bla_{CTX-M} integrated in the chromosome. Antimicrob Agents Chemother 61:e00076-17. https://doi.org/10.1128/AAC.00076-17.
33. Rodríguez I, Thomas K, Van Essen A, Schink AK, Day M, Chattaway M, Wu

G, Mevius D, Helmuth R, Guerra B, SAFEFOODERA-ESBL consortium. 2014. Chromosomal location of bla_{CTX-M} genes in clinical isolates of *Escherichia coli* from Germany, The Netherlands and the UK. Int J Antimicrob Agents 43:553–557. https://doi.org/10.1016/j.ijantimicag.2014.02.019.
34. Hirai I, Fukui N, Taguchi M, Yamauchi K, Nakamura T, Okano S, Yamamoto Y. 2013. Detection of chromosomal $bla_{CTX-M-15}$ in *Escherichia coli* O25b-B2-ST131 isolates from the Kinki region of Japan. Int J Antimicrob Agents 42:500–506. https://doi.org/10.1016/j.ijantimicag.2013.08.005.
35. Zhang J, van Hung P, Hayashi M, Yoshida S, Ohkusu K, Ezaki T. 2011. DnaJ sequences of *Bacillus cereus* strains isolated from outbreaks of hospital infection are highly similar to *Bacillus anthracis*. Diagn Microbiol Infect Dis 70:307–315. https://doi.org/10.1016/j.diagmicrobio.2011.02.012.
36. Clinical and Laboratory Standards Institute. 2015. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 10th ed, M07-A10. CLSI, Wayne, PA.
37. Clinical and Laboratory Standards Institute. 2015. Performance standards for antimicrobial susceptibility testing; 20th informational supplement M100-S25. CLSI, Wayne, PA.
38. Poirel L, Walsh TR, Cuvillier V, Nordmann P. 2011. Multiplex PCR for detection of acquired carbapenemase genes. Diagn Microbiol Infect Dis 70:119–123. https://doi.org/10.1016/j.diagmicrobio.2010.12.002.
39. Hong SS, Kim K, Huh JY, Jung B, Kang MS, Hong SG. 2012. Multiplex PCR for rapid detection of genes encoding class A carbapenemases. Ann Lab Med 32:359–361. https://doi.org/10.3343/alm.2012.32.5.359.
40. Bradford PA, Bratu S, Urban C, Visalli M, Mariano N, Landman D, Rahal JJ, Brooks S, Cebular S, Quale J. 2004. Emergence of carbapenem-resistant *Klebsiella* species possessing the class A carbapenem-hydrolyzing KPC-2 and inhibitor-resistant TEM-30 β-lactamases in New York City. Clin Infect Dis 39:55–60. https://doi.org/10.1086/421495.
41. Kaase M, Nordmann P, Wichelhaus TA, Gatermann SG, Bonnin RA, Poirel L. 2011. NDM-2 carbapenemase in *Acinetobacter baumannii* from Egypt. J Antimicrob Chemother 66:1260–1262. https://doi.org/10.1093/jac/dkr135.
42. Poirel L, Le Thomas I, Naas T, Karim A, Nordmann P. 2000. Biochemical sequence analyses of GES-1, a novel class A extended-spectrum β-lactamase, and the class 1 integron In52 from *Klebsiella pneumoniae*. Antimicrob Agents Chemother 44:622–632. https://doi.org/10.1128/aac.44.3.622-632.2000.
43. Poirel L, Bonnin RA, Nordmann P. 2012. Genetic features of the widespread plasmid coding for the carbapenemase OXA-48. Antimicrob Agents Chemother 56:559–562. https://doi.org/10.1128/AAC.05289-11.
44. Nakano A, Nakano R, Suzuki Y, Saito K, Kasahara K, Endo S, Yano H. 2018. Rapid identification of bla_{IMP-1} and bla_{IMP-6} by multiplex amplification refractory mutation system PCR. Ann Lab Med 38:378–380. https://doi.org/10.3343/alm.2018.38.4.378.
45. Dallenne C, Da Costa A, Decre D, Favier C, Arlet G. 2010. Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in *Enterobacteriaceae*. J Antimicrob Chemother 65:490–495. https://doi.org/10.1093/jac/dkp498.
46. Mena A, Plasencia V, Garcia L, Hidalgo O, Ayestarán JI, Alberti S, Borrell N, Pérez JL, Oliver A. 2006. Characterization of a large outbreak by CTX-M-1-producing *Klebsiella pneumoniae* and mechanisms leading to *in vivo* carbapenem resistance development. J Clin Microbiol 44:2831–2837. https://doi.org/10.1128/JCM.00418-06.
47. Berçot B, Poirel L, Nordmann P. 2011. Updated multiplex polymerase chain reaction for detection of 16S rRNA methylases: high prevalence among NDM-1 producers. Diagn Microbiol Infect Dis 71:442–445. https://doi.org/10.1016/j.diagmicrobio.2011.08.016.
48. Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ. 2005. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods 63:219–228. https://doi.org/10.1016/j.mimet.2005.03.018.
49. Johnson TJ, Bielak EM, Fortini D, Hansen LH, Hasman H, Debroy C, Nolan LK, Carattoli A. 2012. Expansion of the IncX plasmid family for improved identification and typing of novel plasmids in drug-resistant *Enterobacteriaceae*. Plasmid 68:43–50. https://doi.org/10.1016/j.plasmid.2012.03.001.
50. Nakano R, Okamoto R, Nakano Y, Kaneko K, Okitsu N, Hosaka Y, Inoue M. 2004. CFE-1, a novel plasmid-encoded AmpC β-lactamase with an *ampR* gene originating from *Citrobacter freundii*. Antimicrob Agents Chemother 48:1151–1158. https://doi.org/10.1128/aac.48.4.1151-1158.2004.
51. Jørgensen RL, Nielsen JB, Friis-Møller A, Fjeldsøe-Nielsen H, Schønning K. 2010. Prevalence and molecular characterization of clinical isolates of *Escherichia coli* expressing an AmpC phenotype. J Antimicrob Chemother 65:460–464. https://doi.org/10.1093/jac/dkp484.
52. Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S. 2005. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. J Clin Microbiol 43:4178–4182. https://doi.org/10.1128/JCM.43.8.4178-4182.2005.