Title: Genetic characterization of coliform bacterial isolates from environmental water in Thailand

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Abstract

Introduction

In contrast to the study in other part of the world, information about characteristics of plasmids carrying antimicrobial resistance genes (ARGs) in *Enterobacteriaceae* derived from environmental water in tropical Asian countries including Thailand is limited. This study, therefore, aimed to gain insight into genetic information of antimicrobial resistance in environmental water in Thailand.

Methods

Coliform bacteria were isolated from environmental water collected at 20 locations in Thailand and identified. Then, susceptibility profiles to ampicillin, cefazoline, cefotaxime, kanamycin, ciprofloxacin, sulfamethoxazole, tetracycline, and nalidixic acid were assessed. In addition, antimicrobial resistant genes integrons, and replicon types were analyzed. And furthermore, plasmids carrying bla_{TEM} and *tetM* were identified by S1-PFGE analysis and confirmed transmissibility by transconjugation experiments.

Results

In 130 coliform bacteria isolated, 89 were resistant to cefazoline while 41 isolates were susceptible. Cefazoline-resistant coliform bacteria were found to be significantly resistant to cefotaxime and tetracycline as compared to susceptible isolates. Hence, bla_{TEM} and *tetM* correlating with β-lactam antibiotics and tetracycline, respectively, were analyzed found to co-localize on the IncFrepB plasmids in isolates from pig farms' wastewater by S1-PFGE analysis. And furthermore, transmissibility of the plasmids was confirmed.

Conclusions

Results obtained in this study suggested that ARGs in coliform bacteria may have been transmitted from one farm to the other via IncFrepB plasmids. Hence, appropriate use of antimicrobials and good hygiene management on the farm are required to prevent the emergence and spread of resistant bacteria.

Key words: *Enterobacteriaceae*, cefazoline-resistant, IncFrepB plasmid, *bla*$_{TEM}$, *tetM*

Introduction

Antimicrobial resistance (AMR) is a major issue posing a serious threat to global health. Currently, infectious diseases with AMR result in around 700,000 deaths every year globally. This is estimated to top 10 million lives per year in 2050, if no counter-measure will be taken [1]. In Thailand, infectious diseases with AMR have been responsible for the deaths of around 38,000 adults per year [2].

AMR can be transmitted among people, animals, and the environment via several different routes [3]. Many studies have highlighted the impact of the diverse nature of the reservoirs of antimicrobial resistance genes (ARGs) on promoting the emergence and transmission of AMR organisms [4]. This is because ARGs can be spread among microbial communities in the environment through horizontal gene transfer via mobile genetic elements, which is the main resistance acquisition mechanism in *Enterobacteriaceae* [5].

Mobile genetic elements including integrons and plasmids play a significant role in the dissemination of ARGs. Five classes of integrons have been reported, and among them is, class 1 integrons which have been extensively found in clinical isolates, and to which most of the known ARGs cassettes belong [6]. Plasmids are frequently categorized based on incompatibility groups (Inc), defined as the inability of two related plasmids to be propagated stably in the same cell. This phenomenon may be due to competition for the same replication or segregation sites caused by repression of replication initiation [7, 8]. Currently, there are 28 known plasmid types in *Enterobacteriaceae* distinguished by PCR-based replicon typing [9]. The frequency of *intl1* detection in the isolated strains from influent water, activated sludge and effluent water in one municipal sewage treatment plant in China was 20.4 %, 30.9%, and 38.9%, respectively [10]. Among 322 *E. coli* isolates, four of these isolates contained *intl1* and one isolate contained *intl2* from the river that separates the United States from Mexico [11]. The frequencies of *intl1*-positive *E. coli* isolates cultured from wastewater treatment plant effluent, river upstream, and downstream in Poland were reported to be

11.0%, 6.0%, and 14.0%, respectively [12]. Concerning plasmid, wastewaters in Portugal enclose a rich plasmid pool belonging to FrepB, FIC, FIA I1, HI1, and U replicons, associated with integron-carrying bacteria (*Aeromonas* spp. and *Enterobacteriaceae*), and capable of conjugating to different bacterial hosts [13]. Zurfluh et al showed that *bla*$_{\text{CTX-M-1}}$ genes encoded on IncHI1 plasmids were detected in isolates from rivers in Switzerland [14]. High levels of ARGs were detected in the sediments of the river in China and the conjugative plasmids with IncFII, IncP, and IncU played the role in horizontal transfer of ARGs in the environmental microbiome [15].

In contrast, information about characteristics of plasmids carrying ARGs in *Enterobacteriaceae* derived from environmental water in tropical Asian countries including Thailand is limited. This study, therefore, aimed to gain insight into genetic information of AMR in environmental water in Thailand.

Material and Methods

Sample collection

Water samples were collected in Thailand using sterile 1-liter bottle. Sample collections were as follows. At six canals in Bangkok (TBC1, 2, 3, 4, 5, and 6), two pig farms in Ratchaburi (TRP1 and TRP2), and two chicken farms in Ratchaburi (TRC1 and TRC2) in September 2014. At two sites in the Trang River (TSR1 and TSR2), two sites in the brackish water of the Parian River (TSE1 and TTE2), three pig farms (PF1, 2, and 3), and three chicken farms (BF1, 2 and 3) in Trang in September 2015. Since the pig farm effluent from Ratchaburi is treated by a biogas plant, pre-treatment (TRP1 pre, TRP2 pre) and post-treatment (TRP1 post, TRP2 post) water samples were collected. The locations of the samples collection sites are summarized in Figure 1. All samples were stored at 4°C after collection and used for culture within 12 hrs.

Bacterial Isolation

Deoxycholate-hydrogen sulfide-lactose (DHL) agar (Nissui Pharmaceutical, Tokyo, Japan) and Chromocult Coliform agar (Merck, Darmstadt, Germany) were used for the isolation of coliforms and

E. coli, respectively. DHL agar with 4 μg/ml of ciprofloxacin and Chromocult agar with 4 μg/ml of cefotaxime were also used for the selection of AMR bacteria efficiently. Each of 100 μL water samples was spread on the medium and incubated at 37°C for 24 h. A maximum of six colonies determined to be coliforms based on pink to red coloration and morphology on DHL agar and a maximum of three colonies determined to be *E. coli* based on dark blue to purple colonies on Chromocult agar were collected for the analysis. Colonies on DHL or Chromocult agar plates were picked up, suspended in 0.5 ml TE buffer and boiled at 95°C for 10 min. DNA were recovered in the supernatant of centrifuge at 20,000 g for 1 min and used for polymerase chain reaction (PCR).

Identification of bacterial isolates

Bacterial identification was conducted by matrix-assisted laser desorption ionization-time of flight mass spectrometry by using the Bruker MALDI Biotyper system (Bruker Daltonics, Bremen, Germany) according to the manufacturer's instructions and as outlined by Dierig et al. [16].

Drug susceptibility tests

Antimicrobial susceptibility was determined using the agar dilution method, according to the Clinical and Laboratory Standards Institute (CLSI) standards [17]. The following antimicrobials were used: ampicillin, cefazoline, cefotaxime, kanamycin, ciprofloxacin, sulfamethoxazole (Sigma-Aldrich, St Louis, MO), tetracycline, and nalidixic acid (Wako Pure Chemical Industries, Osaka, Japan). The breakpoints for each antimicrobial were in accordance with the CLSI standards [17]. *E. coli* ATCC25922, *Staphylococcus aureus* ATCC29213, *Enterococcus faecalis* ATCC29212 and *Pseudomonas aeruginosa* ATCC27853 were used as controls.

Detection of ARGs and integrons

The *bla*, *tet* genes, and integrons were detected by PCR as described previously using primers listed in Table 1 [18-23]. The PCR conditions were as follows: denaturation at 94 °C for 2 min, followed by 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 1 min and finalized by extension at 72 °C for 7 min for *tet*, denaturation at 95 °C

for 2 min, followed by 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min and finalized by extension at 72 °C for 7 min for *bla* and denaturation at 95 °C for 2 min, followed by 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 3 min and finalized by extension at 72 °C for 5 min for integrons.

Plasmid analysis

Plasmid profiling was performed according to previously described methods [24]. Inc groups were determined by PCR using primers shown in Table 2. To detect *bla*$_{TEM}$ and *tetM* encoding plasmid, pulsed-filed gel electrophoresis (PFGE) with S1 nuclease (Promega Corporation, Madison, WI) was performed as in previous studies [25]. Briefly, S1-digested slices were applied into wells in 13×14cm agarose gel and run in a CHEF-Mapper (Bio-Rad Laboratories, Inc., Hercules, CA) under the following conditions: run duration, 19h; temperature, 14 °C; Angle, 120°; Initial switch time, 2.16s; Final switch time, 63.8s; Gradient, 6.0v/cm. MidRange PFG Marker (New England BioLabs, Ipswich, MA) was used as a size marker. DNA probes for ARGs (*bla*$_{TEM}$ and *tetM*) and plasmid (with IncFrepB) detection were prepared using a PCR DIG Labeling Mix (Roche Diagnostic, Basel, Switzerland) according to the manufacturer's instructions. CSPD® (Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.13,7]decan}-4-yl)phenyl phosphate) was used for chemiluminescent substrate for detection of DIG-labeled probes (Roche Diagnostic). Amersham Imager 600 (GE Healthcare, Chicago, IL) was used to analyze the digital image to chemiluminescence exposed for 10s min.

Conjugation assay

Broth mating assays were performed with *E. coli* DH5α (rifampicin-resistant) and eight *E. coli* isolates containing *tetM* and *bla*$_{TEM}$ were used as recipient and donor strains, respectively. Transconjugants were selected in LB agar plate supplemented with rifampicin (50μg/ml), cefazoline (32μg/ml), and tetracycline (16μg/ml). DNA of transconjugants was extracted and PCR was performed

to determine the presence of *tetM*, *bla*$_{TEM}$ genes, and plasmid groups.

Data analysis

Statistical analysis was performed by the chi-square test. Differences were considered to be significant if the *p*-value was < 0.05.

Results

Bacterial isolation

One hundred-thirty coliform bacteria were isolated from environmental water collected at 20 locations in Thailand. Of them, 89 and 41 were resistant and susceptible to cefazoline, respectively. The resistant isolates were distributed among five bacterial species as follows; *E. coli* (n = 59), *Enterobacter* spp. (n = 16), *Klebsiella* spp. (n = 8), *Pantoea* spp. (n = 5) and *Kluyvera* spp. (n = 1).

Antimicrobial susceptibility

A comparison of antimicrobial resistance rates in cefazoline-resistant (n=89) and cefazoline-susceptible (n=41) colifroms is shown in Figure 2. Cefazoline-resistant coliform bacteria tended to be resistant to cefotaxime ($p < 0.0001$), tetracycline ($p = 0.0015$) and kanamycin ($p = 0.0019$) with significance. Hence, further analysis focused on β-lactamase and tetracycline resistance is required.

Prevalence of ARGs and integrons

Table 3 shows the prevalence of β-lactamase and tetracycline resistance genes and integrons detected in cefazoline-resistant coliform bacteria. *bla*$_{CTX-M1}$, *bla*$_{CTX-M9}$, *bla*$_{TEM}$ and *bla*$_{CMY-2}$ were widely distributed in environmental water. In particular, *bla*$_{TEM}$ was frequently detected (60.0%) in pig farm pre-treated wastewater in Ratchaburi, while *bla*$_{SHV}$ was not detected from all environmental samples. Among the tetracycline genes, *tetA* was found in the highest number of coliforms, 87% in Ratchaburi pig farms (before treatment), and 52% in Bangkok canals. *tetM* was subsequently found in many environmental waters, with 60% in the Ratchaburi pig farm (before treatment). In contrast, *tetD* was not detected in any sample. Comparing wastewater before and after biogas plant treatment at pig

farms, the percentage of coliforms with ARGs and *intl1* was reduced in the treated water. *intl1* was detected from all environmental samples except the rural river and estuary. *intl3* was found in one strain from brackish water in the rural area. As shown in Table 4, the highest number of *bla* pattern was a single $bla_{\text{CTX-M}}$, which accounted for about 16%. Although coliforms carrying three *bla* genes were found at 2.2%, more than half of coliforms did not contain any *bla* genes. The pattern of $bla_{\text{CTX-M}}$ groups in cefazoline-resistant coliform bacteria and the prevalence of $bla_{\text{CTX-M1}}$ and $bla_{\text{CTX-M9}}$ was 61.5% (16/26) and 38.5% (10/26), respectively. From the result of the presence of antimicrobial resistance genes, statistical analysis shows that bla_{TEM} was more likely to coexist with *tetM* (p = 0.0011) than *tetA* (p = 0.0097). Hence, further analysis focused on the eight *E. coli* isolates harboring bla_{TEM} and *tetM* was conducted.

Prevalence of plasmid replicons

The characteristics of 8 *E. coli* isolates harboring bla_{TEM} and *tetM* are shown in Table 5. Each isolate possessed at least 2 plasmids, and a variety of inc types were found. Briefly, IncFrepB, IncY and IncFIB were detected in 7, 5 and 5 isolates, respectively.

S1-PFGE and Southern blot hybridization

Among 7 *E. coli* isolates harboring FrepB plasmid shown in Table 5, the results of S1-PFGE and Southern blot hybridization elucidated the size of each *tetM* and bla_{TEM} gene-encoding plasmid (Figure 3). *tetM* and bla_{TEM} that were co-localized on FrepB plasmids ranged from around 45kbp to 150kbp in lane 1 to 7 except lane 3. *tetM* was found on the FrepB plasmid but bla_{TEM} was not found on the FrepB plasmid in lane 3.

Transferability of β-lactamase encoding genes and plasmid

The results of the conjugation experiment on seven strains carrying FrepB plasmid, $bla_{\text{TEM,}}$ and *tetM* are shown in Table 6. ARGs from six of these strains were transferred to the recipient strains. Of the six transconjugant strains, FrepB plasmid was identified in all of them. All transconjugant strains carried *tetM* whereas four strains carried bla_{TEM}.

Discussion

In this study, we analyzed co-resistance to some antimicrobials among cefazoline-resistant environmental coliform strains, since multi drug-resistant strains can result from the co-selection of several ARGs in the same genetic platform or from cross-resistance due to mechanisms responsible for resistance to different antimicrobials [26].

The β-lactamase gene, bla_{TEM}, was detected in pig farm effluent (before treatment) in Ratchaburi at a high rate of 60% (Table 3). As bla_{TEM} has also been previously reported from pig feces in Sa Kaeo province, Thailand [27], bla_{TEM} in coliforms isolated from pig farm effluent in this study may be derived from pig feces. High prevalence of tetracycline resistance genes were observed in pig farm wastewater in Ratchaburi than those in Trang (Table 3). Indeed, chlortetracycline were used in pig farm in Ratchaburi for growth promotion, while tetracyclines were not used in pig farm in Trang. It is possible that selective pressure of tetracycline in Ratchaburi lead to higher prevalence of tetracycline resistant bacteria and resistance genes comparing to Trang. Various β-lactamase genes were detected in the canals of Bangkok (Table 3). The percentage was considerably higher than the β-lactamase genes ($bla_{\mathrm{CTX\text{-}M}}$ group and bla_{TEM}) detected in canals in the United States [28] and lower than that of the $bla_{\mathrm{CTX\text{-}M1}}$ group detected in the aquatic environment including rivers and canals in the Phitsanulok and Nakhon Sawan provinces of Thailand [29]. The diversity and abundance of ARGs is thought to be mainly influenced by local/national parameters related to sanitation and health [30]. The results of $bla_{\mathrm{CTX\text{-}M1}}$ and *tetA*, which showed high resistance rates in the canals of Bangkok, were not found from the rivers and brackish waters of Trang (Table 3). The difference of results between region was considered by the prevalence of antimicrobial resistant bacteria selected by residue antimicrobials in environmental water. There are hospitals and human residences nearby canals and the discharge of antimicrobials could flow into the canal, while there are few hospitals and houses around river and estuary in rural area in this study.

Among plasmid replicons found in this study, IncFrepB was the most common type (Table 5). IncFrepB belongs to IncF and is a different replicon type from IncI and IncH, which were often found in the European environment [9]. *Enterobacteriaceae* isolates with IncFrepB plasmid were reported from human patients in India, China, and Italy [31-34] and no report of IncFrepB from the environment in Asia has been found. The origin of IncFrepB found from the environment in this study should be further elucidated.

Although the incompatible plasmids are not usually present in one cell, there are several FrepB plasmids observed in one isolate in present study. In a previous study, *E. coli* strains carrying more than 2 plasmids with the same inc type were reported and the transconjugant obtained from donor strains were shown to have only one plasmid [25]. Hence, the observation on the carriage of more than two plasmid with IncFrepB in this study might be possible. Only one of a few FrepB plasmids detected in this study would finally be maintained and contribute to transmission.

Six *E. coli* strains with bla_{TEM} and *tetM* were co-localized on the IncFrepB plasmid (Figure 3). The co-selection of ARGs due to selective pressure of antimicrobials might cause this phenomenon. IncFrepB plasmid harboring bla_{TEM} and *tetM* may be spreading in a pig farm, therefore appropriate use of antimicrobials and good hygiene management at farms are required to prevent the emergence and spread of resistant bacteria. Further research is required to investigate if IncFrepB plasmids carry other genes conferring resistance to aminoglycoside, quinolone and sulfonamide.

Biogas plant treatment significantly reduced the percentage of resistance genes (Table 3). The same biogas plant as this study was previously reported to result in decreased rates of resistance to six antimicrobials, as well as reduced concentrations of antimicrobials [35]. Thus, biogas plant treatment is a promising way to avoid ARGs spread in the aquatic environment.

In conclusion, IncFrepB plasmids in pig farms effluent might be playing a significant role in the spread of ARGs. Hence further studies focused on the plasmids are necessary to elucidate their origin including human and prevent further dissemination of ARGs.

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Transparency declaration

None to declare.

ICMJE Statement

Risa Tsunoda: Conceptualization, Methodology, Analysis, Original draft preparation

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Table 1. Primers for the amplification of antimicrobial resistance genes and integrons.

Genes	Nucleotide sequence(5'－3')	Length(bp)	Reference
Antimicrobial resistance genes			
tetA	GCGCTNTATGCGTTGATGCA ACAGCCCGTCAGGAAATT	387	【18】
tetB	GCGCTNTATGCGTTGATGCA TGAAAGCAAACGGCCTAA	171	【18】
tetC	GCGCTNTATGCGTTGATGCA CGTGCAAGATTCCGAATA	631	【18】
tetD	GCGCTNTATGCGTTGATGCA CCAGAGGTTTAAGCAGTGT	484	【18】
tetE	GCGCTNTATGCGTTGATGCA ATGTGTCCTGGATTCCT	246	【18】
tetG	GCGCTNTATGCGTTGATGCA ATGCCAACACCCCCGGCG	803	【18】
tetM	GTTAAATAGTGTTCTTGGAG CTAAGATATGGCTCTAACAA	656	【19】
bla_{TEM}	ATGAGTATTCAACATTTTCG TTACCAATGCTTAATCAGTG	861	【20】
bla_{SHV}	ATGCGTTATATTCGCCTGTG TTAGCGTTGCCAGTGCTCGA	841	【20】
$bla_{\mathrm{CMY\text{-}2}}$	GACAGCCTCTTTCTCCACA TGGACACGAAGGCTACGTA	1000	【20】
$bla_{\mathrm{CTX\text{-}M\text{-}1group}}$	GCGTGATACCACTTCACCTC TGAAGTAAGTGACCAGAATC	260	【21】
$bla_{\mathrm{CTX\text{-}M\text{-}9group}}$	ATCAAGCCTGCCGATCTGGTTA GTAAGCTGACGCAACGTCTGC	293	【21】
Integrons			
Intl1	CCTCCCGCACGATGATC TCCACGCATCGTCAGGC	280	【22】
Intl2	TTATTGCTGGGATTAGGC ACGGCTACCCTCTGTTATC	233	【23】
Intl3	AGTGGGTGGCGAATGAGTG TGTTCTTGTATCGGCAGGTG	600	【23】

Table 2. Primers for replicon typing.

Replicon type	Nucleotide sequence(5'－3')	Length(bp)
B/O-F	GCGGTCCGGAAAGCCAGAAAAC	159
B/O-R	TCTGCGTTCCGCCAAGTTCGA	
FIC-F	GTGAACTGGCAGATGAGGAAGG	262
FIC-R	TTCTCCTCGTCGCCAAACTAGAT	
A/C-F	GAGAACCAAAGACAAAGACCTGGA	465
A/C-R	ACGACAAACCTGAATTGCCTCCTT	
P-F	CTATGGCCCTGCAAACGCGCCAGAAA	534
P-R	TCACGCGCCAGGGCGCAGCC	
T-F	TTGGCCTGTTTGTGCCTAAACCAT	750
T-R	CGTTGATTACACTTAGCTTTGGAC	
W-F	CCTAAGAACAACAAAGCCCCCG	242
W-R	GGTGCGCGGCATAGAACCGT	
FIA-F	CCATGCTGGTTCTAGAGAAGGTG	462
FIA-R	GTATATCCTTACTGGCTTCCGCAG	
FIB-F	GGAGTTCTGACACACGATTTTCTG	702
FIB-R	CTCCCGTCGCTTCAGGGCATT	
K/B-F	GCGGTCCGGAAAGCCAGAAAAC	160
K/B-R	TCTTTCACGAGCCCGCCAAA	
FIIA-F	CTGTCGTAAGCTGATGGC	270
FIIA-R	CTCTGCCACAAACTTCAGC	
Y-F	AATTCAAACAACACTGTGCAGCCTG	765
Y-R	GCGAGAATGGACGATTACAAAACTTT	
I1-F	CGAAAGCCGGACGGCAGAA	139
I1-R	TCGTCGTTCCGCCAAGTTCGT	
X-F	AACCTTAGAGGCTATTTAAGTTGCTGAT	376
X-R	TGAGAGTCAATTTTTATCTCATGTTTTAGC	
HI1-F	GGAGCGATGGATTACTTCAGTAC	471
HI1-R	TGCCGTTTCACCTCGTGAGTA	
N-F	GTCTAACGAGCTTACCGAAG	559
N-R	GTTTCAACTCTGCCAAGTTC	
HI2-F	TTTCTCCTGAGTCACCTGTTAACAC	644
HI2-R	GGCTCACTACCGTTGTCATCCT	
L/M-F	GGATGAAAACTATCAGCATCTGAAG	785
L/M-R	CTGCAGGGGCGATTCTTTAGG	
FrepB-F	TGATCGTTTAAGGAATTTTG	270
FrepB-R	GAAGATCAGTCACACCATCC	

Table 3. Prevalence of β-lactamase and tetracycline resistance genes and integrons detected in cefazoline-resistant coliform bacteria.

		β-lactamase genes					Tetracycline resistance genes						Integrase		
		bla$_{CTX-M1}$ group	*bla*$_{CTX-M9}$ group	*bla*$_{TEM}$	*bla*$_{SHV}$	*bla*$_{CMY-2}$	*tetA*	*tetB*	*tetC*	*tetD*	*tetE*	*tetM*	*int1*	*int2*	*int3*
Sampling sites	n	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
PFW-pre in Ratchaburi	15	26.7	26.7	60.0	0	26.7	86.7	0	0	0	0	60.0	100.0	0	0
PFW-post in Ratchaburi	9	0	0	22.2	0	11.1	66.7	0	0	0	0	33.3	55.6	0	0
PFW in Trang	12	8.3	16.7	0	0	41.7	25.0	25.0	0	0	16.7	8.3	50.0	0	0
CFW in Ratchaburi	2	0	0	0	0	0	50.0	0	0	0	0	0	100.0	0	0
CFW in Trang	14	0	0	7.1	0	7.1	7.1	7.1	0	0	0	0	21.4	0	0
City canal in Bangkok	27	40.7	14.8	14.8	0	3.7	51.9	14.8	7.4	0	0	18.5	70.4	0	0
River in Trang	4	0	0	0	0	25.0	0	0	0	0	0	0	0	0	0
Estuary in Trang	6	0	0	0	0	0	0	0	0	0	0	0	0	0	16.7

PFW; pig farm wastewater, CFW; chicken farm wastewater, pre; pre-treatment, post; post-treatment

Table 4. Pattern of *bla* among cefazoline-resistant coliform bacteria.

Patterns of *bla* genes	No. of isolates	Percentage
bla_{TEM} + bla_{CMY-2} + bla_{CTX-M}	2	2.2
bla_{TEM} + bla_{CMY-2}	3	3.4
bla_{TEM} + bla_{CTX-M}	8	9.0
bla_{CMY-2} + bla_{CTX-M}	1	1.1
bla_{TEM} only	3	3.4
bla_{CMY-2} only	7	7.9
bla_{CTX-M} only	15	16.9
bla_{SHV}	0	0
Negative for bla_{TEM}, bla_{CMY-2}, bla_{CTX-M1}, bla_{CTX-M9}, bla_{SHV}	50	56.2
Total	89	100

Table 5. Characteristics of eight *E. coli* isolates harboring *bla*$_{TEM}$ and *tetM*.

Isolate No.	Species	Site	Resistance phenotype	*bla*/*tet* genes	Integron	Replicon typing	Transconjugant
1	*E. coli*	TRP1 pre in Ratchaburi	**ABPC**, **CEZ**, **CTX**, KM, **TET**, NA, SMX	***bla*$_{TEM}$**, *bla*$_{CMY-2}$, *bla*$_{CTX-M9}$, *tetA*, ***tetM***	*intl1*	FIB, Y, FrepB	+
2	*E. coli*	TRP1 pre in Ratchaburi	**ABPC**, **CEZ**, **CTX**, KM, **TET**, NA, CPFX, SMX	***bla*$_{TEM}$**, *bla*$_{CTX-M1}$, *tetA*, ***tetM***	*intl1*	P, B/O, FIB, N, FrepB	+
3	*E. coli*	TRP1 pre in Ratchaburi	**ABPC**, **CEZ**, **CTX**, KM, **TET**, NA, CPFX, SMX	***bla*$_{TEM}$**, *bla*$_{CMY-2}$, ***tetM***	*intl1*	P, FrepB	+
4	*E. coli*	TRP1 pre in Ratchaburi	**ABPC**, **CEZ**, **CTX**, KM, **TET**, NA, CPFX, SMX	***bla*$_{TEM}$**, *bla*$_{CTX-M9}$, *tetA*, ***tetM***	*intl1*	Y, N, FrepB	+
5	*E. coli*	TRP1 pre in Ratchaburi	**ABPC**, **CEZ**, **CTX**, KM, **TET**, NA, CPFX, SMX	***bla*$_{TEM}$**, *bla*$_{CTX-M9}$, *tetA*, ***tetM***	*intl1*	P, FIB, Y, FrepB	-
6	*E. coli*	City canal in Bangkok	**ABPC**, **CEZ**, **CTX**, KM, **TET**, NA, CPFX, SMX	***bla*$_{TEM}$**, *bla*$_{CTX-M9}$, *tetA*, *tetB*, ***tetM***	*intl1*	P, FIB, Y, FrepB	+
7	*E. coli*	TRP2 pre in Ratchaburi	**ABPC**, **CEZ**, **CTX**, KM, **TET**, NA, SMX	***bla*$_{TEM}$**, *bla*$_{CMY-2}$, *bla*$_{CTX-M9}$, *tetA*, ***tetM***	*intl1*	FIB, FrepB	+
8	*E. coli*	TRP2 post in Ratchaburi	**ABPC**, **CEZ**, **CTX**, **TET**, SMX	***bla*$_{TEM}$**, *tetA*, ***tetM***	*intl1*	Y, L/M	+

TRP; Ratchaburi pig farm, pre; pre-treatment, post; post-treatment

Table 6. Characteristics of transconjugants.

Isolate no.	***bla*$_{TEM}$**	***tetM***	**IncFrepB**
TC1	-	+	+
TC2	+	+	+
TC3	-	+	+
TC4	+	+	+
TC6	+	+	+
TC7	+	+	+

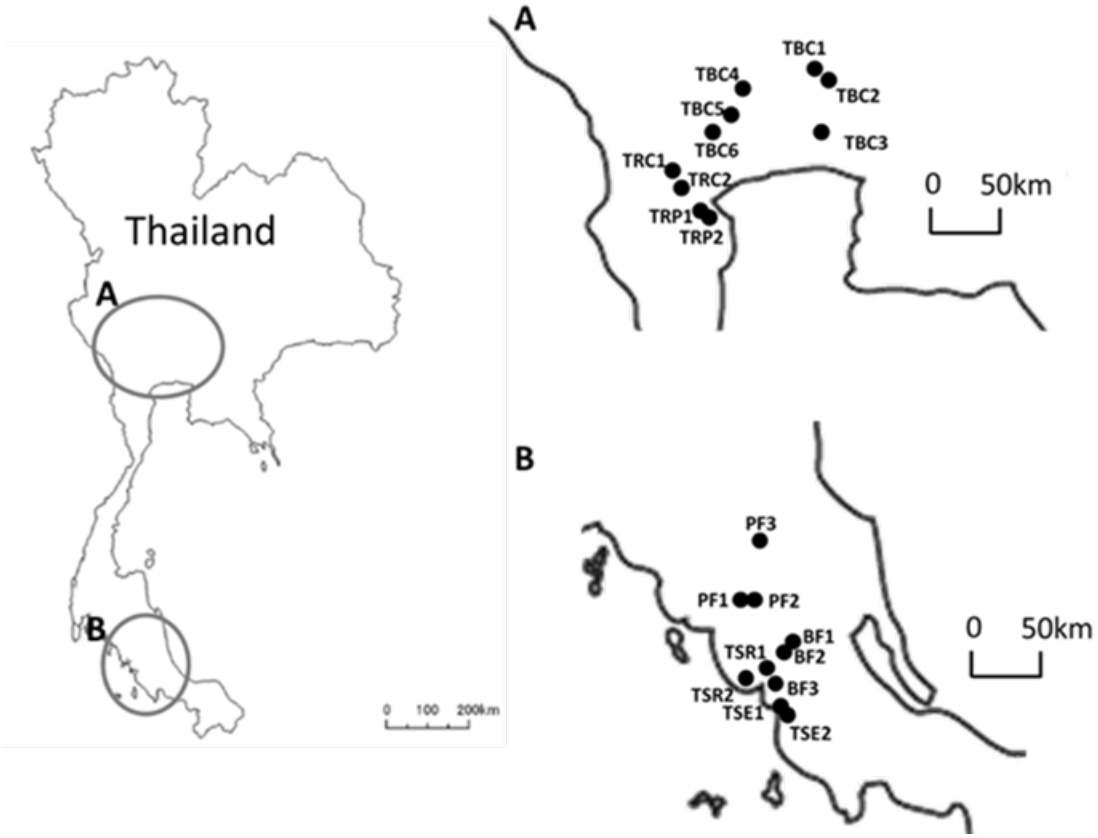
Legends for figures

Figure 1. Sampling sites in Thailand. A: 6 sites (TBC1, 2, 3, 4, 5 and 6) at the city canals in Bangkok, 2 sites (TRP1 and TRP2) and 2 sites (TRC1 and TRC2) from pig and chicken farms, respectively, in Ratchaburi. B: 2 sites (TSR1 and TSR2) in Trang river, 2 sites (TSE1 and TTE2) from brackish water in Palian river, 3 sites (PF1, 2 and 3) and 3 sites (BF1, 2 and 3) from pig and chicken farms, respectively, in Trang.

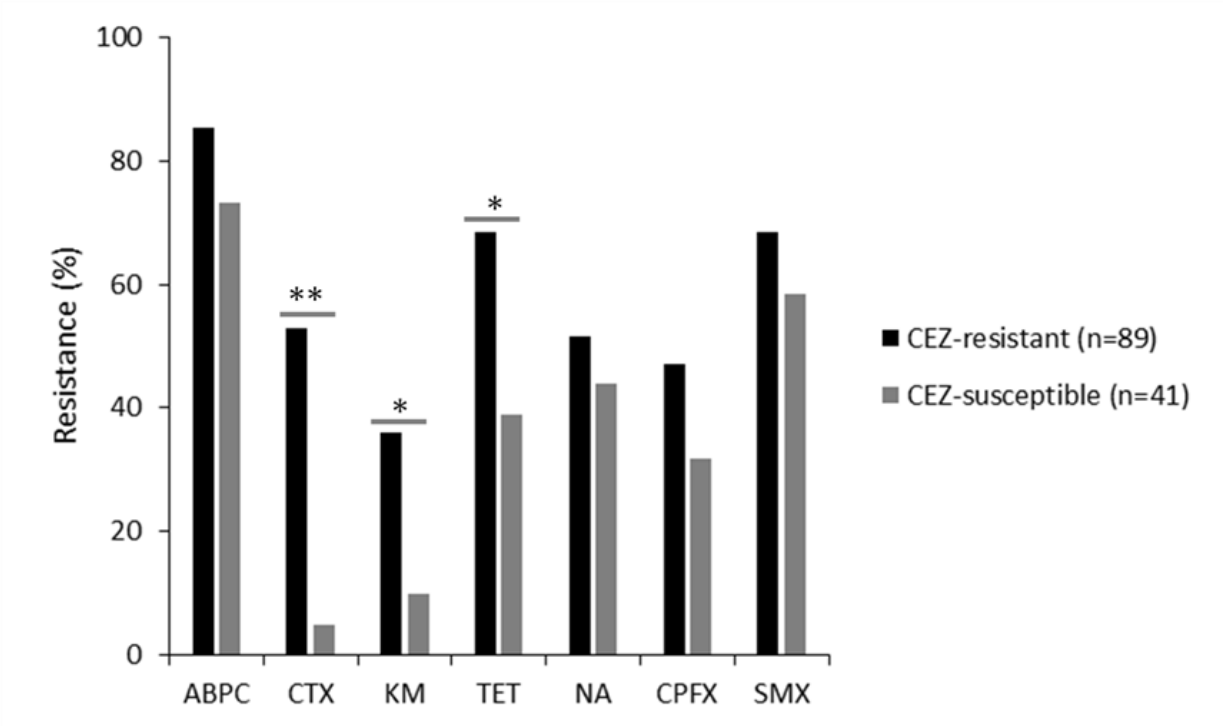
Figure 2. Phenotypic characteristics of CEZ (cefazoline) resistant and susceptible bacteria. Differences were considered significantly if the *p*-value was < 0.01(*) and < 0.001(**). AMBC, ampicillin; CTX, cefotaxime; KM, kanamycin; TET, tetracycline; NA, nalidixic acid; CPFX, ciprofloxacin; SMX, sulfamethoxazole.

Figure 3. Detection of plasmids carrying *tetM*, bla_{TEM}, and IncFrepB by S1-PFGE and Southern blot hybridization.

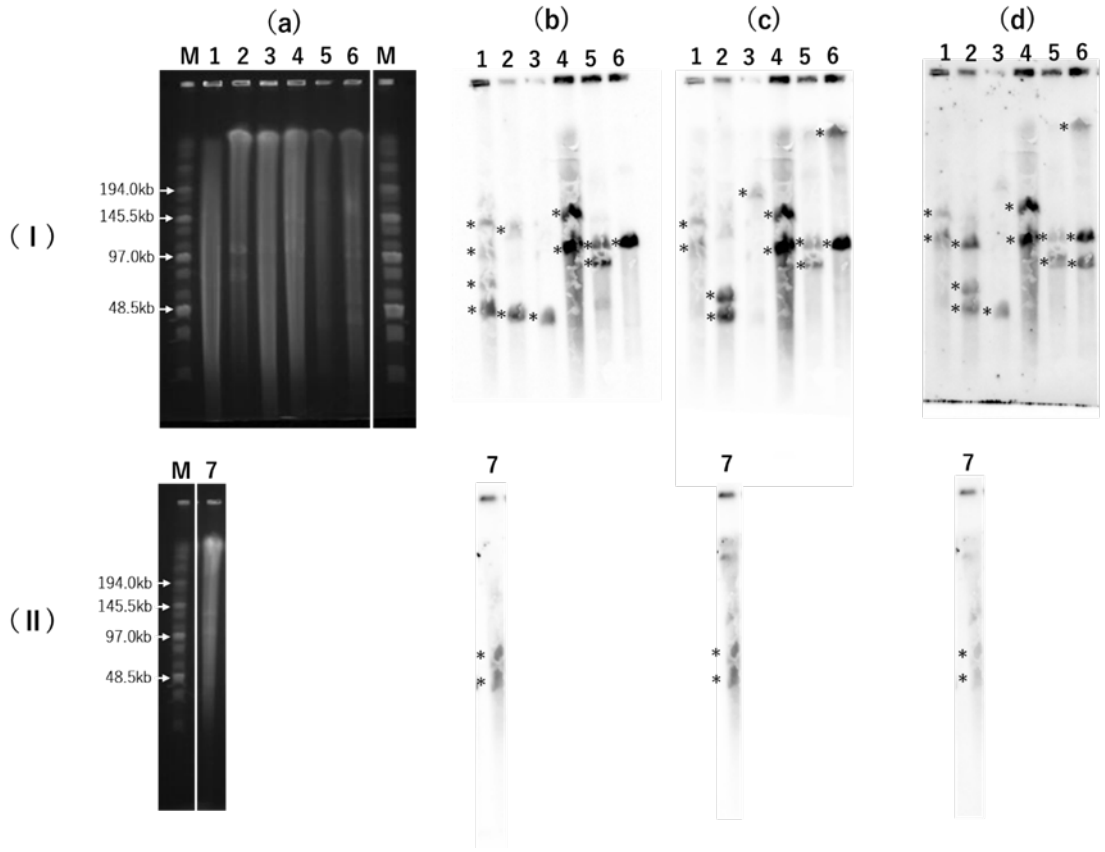
Ia, IIa: S1-PFGE pattern stained by EtBr, Ib-d, IIb-d: Southern blot hybridization with a *tetM*-specific (b), bla_{TEM}-specific (c) and IncFrepB-specific (d) probe. Lane M: MidRange PFG Marker. Lane 1-5 & 7: *E. coli* from Ratchaburi pig farms (TRP) pre-treatment (pre), Lane 6: *E. coli* from city canal in Bangkok. * shows the positive band of each specific probe.



Tsunoda et al. Figure 1.



Tsunoda et al. Figure 2.



Tsunoda et al. Figure 3.