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HOUSEKEEPING GENES ANALYSIS OF FOODBORNE PATHOGENIC BACTERIA *Vibrio parahaemolyticus* ISOLATED FROM AQUATIC BIRDS IN THAILAND

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ABSTRACT

Background: *Vibrio parahaemolyticus is one of the leading causative agent of foodborne disease. Infection is caused by consumption of undercooked contaminated seafood. V. parahaemolyticus is commonly found in crustacean species and marine environment. Presence of this organism in avian host has been previously reported, however genetic analysis of avian V. parahaemolyticus is required for molecular epidemiological study of this organism. The aim of this study was to determine genetic profile of V. parahaemolyticus isolated from fecal aquatic bird samples by modified Multilocus Sequence Typing)MLST) method.*
Methods: *Three housekeeping genes fragments)dnaE, gyrB and pntA) of total 18 V. parahaemolyticus isolates from fecal aquatic bird samples at Bangpoo resort, Samut Prakarn province, Thailand, during 2016-2017, were amplified by using conventional PCR for nucleotide sequencing. Nucleotide sequences were analyzed and phylogenetic tree were constructed by MEGA 7.0 software. Comparative genetic analysis of avian isolates from Thailand and worldwide isolates were performed by using information from MLST database of V. parahaemolyticus (https://pubmlst.org /vparahaemolyticus/).*
Results: *Three housekeeping genes of 18 isolates were successfully amplified and purified for nucleotide sequencing. Phylogenetic tree analysis of concatenated nucleotide sequences indicated that 18 Thai avian isolates were genetically diverse. Five isolates (MUVP 9, 11, 22, 23 and 24) represented identical genetic profile with clinical isolates from China, India, Japan and Peru. Other examined isolates were closely related to environmental isolates from China and United Kingdom. These results showed that aquatic birds are natural reservoir of V. parahaemolyticus strains with multiple genetic background.*
Conclusion: *This study indicated that aquatic birds possessed potentially pathogenic V. parahaemolyticus and may play a role in transmission of this organism across the countries.*

Keywords: *Vibrio parahaemolyticus; housekeeping genes; aquatic bird, MLST*

INTRODUCTION

Vibrio parahaemolyticus is Gram-negative halophilic, facultative anaerobic bacteria. It is a leading foodborne pathogen causing outbreaks associated with consumption of undercooked contaminated seafood especially shrimp, crab, crawfish, oyster and shellfish [1]. This organism is ubiquitously found in seafood and estuarine environment. Pathogenic strains are responsible for acute diarrhoeal illness or acute gastroenteritis in human, can be recovered from 1-2% of environmental isolates. Moreover, *V. parahaemolyticus* can cause human wound infections from recreational aquatic activities. [2, 3].

Although, *V. parahaemolyticus* is commensal organism in marine animals and environment, evidence of avian host harboring *V. parahaemolyticus* was previously reported. Previous study showed that *Vibrio* spp. were isolated from fecal samples of gulls in Connecticut with detection rate of 51% and in Florida at the detected rate of 69% [4]. The *Vibrio* spp. were also isolated from other bird species including swans and Canadian geese with detection rates of 67% and 6%, respectively [4]. These findings indicated that aquatic birds are potential carriers for *Vibrio* spp. and may contribute to possible transmission of pathogens [5]. In Thailand, unpublished data from Chitrak *et al*.,)2017 (showed that *V. parahaemolyti- cus* was isolated from fecal aquatic bird samples in Thailand. Genetic determination of isolated *V. parahaemolyticus* can help investigating strain relatedness among isolates from aquatic birds, clinical samples and environmental samples.

The Multilocus sequence typing (MLST) is a portable molecular method that can be used to determine genetic background and study evolutionary relationships among bacteria. This method is based on nucleotide sequence analysis of housekeeping gene fragments. Obtained genetic profiles of studied isolates by MLST method from individual laboratory can be submitted and recorded in the online MLST database that is conveniently accessed via http://www.pubmlst.org. The MLST approach enables freely data sharing between distant laboratories which is beneficial for global epidemiological studies [6] . Previous studies of MLST for *V. parahaemolyticus* from clinical and environmental samples showed highly genetic diversity with evidence of restricted pandemic clonal complex [7]. However, the MLST approach has not been applied for studying *V. parahaemolyticus* from avian host. The established MLST scheme for *V. parahaemolyticus* was based on nucleotide sequence analysis of seven housekeeping genes [7]. Modified MLST scheme that use three housekeeping genes is useful for preliminary study prior to further detailed research. The present study aimed to determine genetic characterization of 18 *V. parahaemolyticus* isolates originating from aquatic birds in Thailand by modified MLST using three housekeeping genes, *dnaE*, *gyrB* and *pntA*, as well as to determine genetic relatedness of these isolates and worldwide isolates from online database.

MATERIAL AND METHODS

Protocol for modified MLST scheme in the present study was illustrated in Figure 1. Details for the method are provided as following.

Bacterial culture

The total 18 isolates of *V. parahaemolyticus* collected from aquatic bird, mostly *Chroicocephalus Brunnicephalus,* fecal samples at Bangpoo, Samut Prakarn province Thailand during 2016-2017 were used in this study. Bacterial isolation were previously performed by conventional culture method using thiosulfate citrate bile salt sucrose (TCBS) agar and the enrichment medium used was alkaline peptone water (APW) with 3% (w/v) NaCl and incubated at 37 °C for 18-24 hours (hr).

Suspected *V. parahaemolyticus* colonies were further examined by biochemical identification and detected for species-specific lecithin dependent hemolysin gene (*ldh*) by PCR method. In addition, detection of virulence factors including thermostable direct hemolysin gene (*tdh*), thermostable direct hemolysin-related hemolysin gene (*trh*), Type three secretion system 1 (T3SS1) and Type three secretion system 2 (T3SS2) were performed. All of *V. parahaemolyticus* isolates were confirmed and were stored in 20% (v/v) glycerol at -80 °C as cell suspension stocks.

DNA extraction

The genomic DNA of *V. parahaemolyticus* isolates were extracted by boiling method which was adapted from [8]. Bacterial culture from 20% (v/v) glycerol stock was streaked on tryptic soy agar (TSA) with 3% (w/v) NaCl and incubated at 37ºC, overnight. Few colonies from TSA plate were inoculated into 3 ml of Luria-Bertani (LB) broth containing 3% (w/v) NaCl and incubated at 37 ºC with shaking (~200 rpm). After overnight incubation, the 1 ml of cell culture was transferred into microcentrifuge tube for genomic DNA extraction. Cell culture was centrifuged at 5,000 g for 5 minute (min). The supernatant was discarded. Tris EDT (TE) (Sigma, US) buffer pH 8.0 solutions was added

400 µl into the cells and centrifuged at 5,000 g for 5 min. The supernatant was discarded. The 200 µl of 0.6% (w/v) chelex (Sigma, USA) was added into the cell pellet. Then, it was homogenized by vortex mixer. The cell suspension was incubated at 37 ºC for 30 min. After that, the cell suspension was boiled at 95ºC for 10 min and quickly put in ice for 5 min. Next, the cell suspension was centrifuged at 16,000 g for 10 min. Supernatant containing bacterial DNA was collected into new microcentrifuge tube. This DNA was stored at -20 ºC in a freezer until use as DNA template for polymerase chain reaction)PCR(.

Amplification of housekeeping genes fragments

The MLST scheme of *V. parahaemolyticus* in this study was adapted from previous study [7] by using three housekeeping genes including *dnaE* (DNA polymerase 3, alpha subunit) and *gyrB* (DNA gyrase, sub- unit B) from chromosome 1, and *pntA* (Transhydrogenase alpha subunit) from chromosome 2. Primers for PCR amplification and sequencing of housekeeping genes were adapted from previous study [9] which are shown in Table 1.

Table 1 Properties of primers for PCR amplification and sequencing of three housekeeping gene fragments of *Vibrio parahaemolyticus*

Gene	Primer	Position	PCR size (bp)	Sequence (5' – 3')	Reference
dnaE	dnaE-F3	639-656	776	CGA GAT TCG TGT TGC GAT	[9]
	dnaE-R1	1414-1397		CTA GCG TCA TAC CCG GAT	
gyrB	gyrB-F1	582-599	758	GTT CTT GAA CTC AGG CGT	[9]
	gyrB-R1	1339-1322		GTG GTA GGA TTG CCT GAT	
pntA	pntA-F1	600-617	676	TGA CGT TCG TCC AGA AGT	[9]
	pntA-R3	1275-1258		TAC CGA TGC AAT CCA AGC	

Conditions for PCR amplification of three housekeeping genes including annealing temperature and concentration of $MgCl_2$ were modified from the previous study [9]. PCR amplification was performed on a final volume of 25 µl of 1 reaction system containing 1X PCR buffer (Thermo Scientific), 2.5 mM $MgCl_2$ (Thermo Scientific), 0.8 mM dNTP mixture (0.2 mM each of dATP, dTTP, dCTP and dGTP; Thermo Scientific), 0.5 µM of each forward and reverse primer (Macrogen) and 1 unit of Taq DNA polymerase (Thermo Scientific), 1µl of purified genomic DNA as DNA template. The final volume was adjusted to 25 µl with sterile deionized water. The 25 µl PCR mixture was mixed well for further step of PCR.

PCR parameter

The mixture of PCR reactions were subjected to Thermal Cycler (Thermo hybaid, USA) under the following conditions. All of isolates for each gene was started with initial denaturation at 95 ºC for 1 min, followed by 30 cycles of denaturation at 98 ºC for 10 sec, annealing temperature at 55 ºC for 5 sec, and extension at 72 ºC for 1 min. However, the optimal annealing temperature was individually adjusted for each gene. The PCR products were visualyzed by 1% (w/v) agarose gel electrophoresis followed by ethidium bromide staining.

DNA purification and sequencing

The amplified PCR products of housekeeping gene fragments were purified by using QIAquick Purification Kit (Qiagen, Germany) according to manufacturer's instructions with final eluted in 30 µl of sterile deionized water. All of purified products were quantified using a Biodrop Touch (Scientific promotion, US) UV visible spectrophotometer. Purified DNA and primers for sequencing were prepared according to the company's (Macrogen) guideline. DNA sequencing was performed by ABI PRISM 3130 platform (Applied Biosystems, Carlsbad, CA, USA).

Data analysis

Sequencing data was checked and edited by Geneious program version 11.0.5 Software [10]. Allele numbers of each gene for *V. parahaemolyticus* isolates were obtained by blasting the data against the pubMLST *V. parahaemolyticus* database (http://pubmlst.org/vparahaemolyticus/) [7]. For

phylogenetic analysis, concatenated sequences of *dnaE*, *gyrB* and *pntA* were constructed manually. To determine the confidence indices within the neighbor-joining (NJ), the bootstrap test(500 replicates) was used for in-frame concatenated sequences of three housekeeping genes from each strain (1,579 bp) using the Kimura two-parameter to estimate the evolutionary distances were constructed by MEGA 7.0.

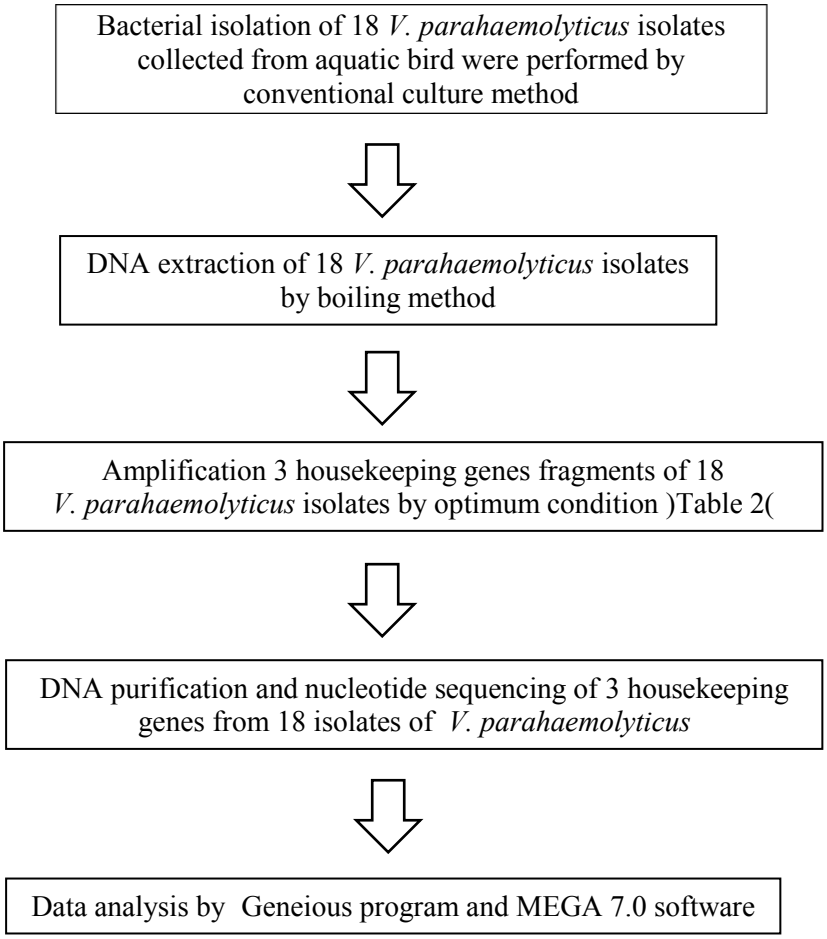

Figure 1 Workflow for modified MLST scheme of *Vibrio parahaemolyticus* in the present study

RESULTS

Amplification of housekeeping gene fragments

The present study examined total 18 *V. parahaemolyticus* isolates from fecal aquatic bird samples from Bangpoo resort, Samutprakarn province, Thailand during 2016-2017. PCR amplification of three housekeeping genes including *dnaE*, *gyrB* and *pntA* were performed by using conventional PCR. Gene fragments were successfully amplified by optimizing PCR condition for each gene (Figure 2). The results showed that *dnaE* gene fragments of all isolates were amplified by annealing temperature at 55˚C with 2 mM $MgCl_2$ in PCR mixture. However, various annealing temperatures and $MgCl_2$ concentrations were used for successful amplification of *gyrB* and *pntA* (Table 2). The *gyrB* gene fragments were amplified by annealing temperature at 55˚C (MUVP 2, 5, 6, 7, 9, 11, 16, 17, 18, 19, 21 and 23), 53˚C (MUVP 12, 13, 14 and 25) and 51˚C (MUVP 22 and 24) with 2 mM $MgCl_2$ for all isolates. The *pntA* gene fragments were amplified by annealing temperature at 55˚C (MUVP 6, 16, 17, 18, 21, 22, 23 and 24), 51˚C (MUVP 2, 5, 7, 9, 11, 12, 13 and 14) and 49˚C (MUVP 19 and 25) with 1.5 mM $MgCl_2$ for all isolates. (mM)

Table 2 Optimal amplification condition of three housekeeping genes for 18 *Vibrio parahaemolyticus* isolates

No.	Isolates	*dnaE*		*gyrB*		*pntA*	
		Annealing temperature (°C)	Concentration of $MgCl_2$ (mM)	Annealing temperature (°C)	Concentration of $MgCl_2$ (mM)	Annealing temperature (°C)	Concentration of $MgCl_2$ (mM)
1	MUVP2	55	2	55	2	51	1.5
2	MUVP5	55	2	55	2	51	1.5
3	MUVP6	55	2	55	2	55	1.5
4	MUVP7	55	2	55	2	51	1.5
5	MUVP9	55	2	55	2	51	1.5
6	MUVP11	55	2	55	2	51	1.5
7	MUVP12	55	2	53	2	51	1.5
8	MUVP13	55	2	53	2	51	1.5
9	MUVP14	55	2	53	2	51	1.5
10	MUVP16	55	2	55	2	55	1.5
11	MUVP17	55	2	55	2	55	1.5
12	MUVP18	55	2	55	2	55	1.5
13	MUVP19	55	2	55	2	49	1.5
14	MUVP21	55	2	55	2	55	1.5
15	MUVP22	55	2	51	2	55	1.5
16	MUVP23	55	2	55	2	55	1.5
17	MUVP24	55	2	51	2	55	1.5
18	MUVP25	55	2	53	2	49	1.5

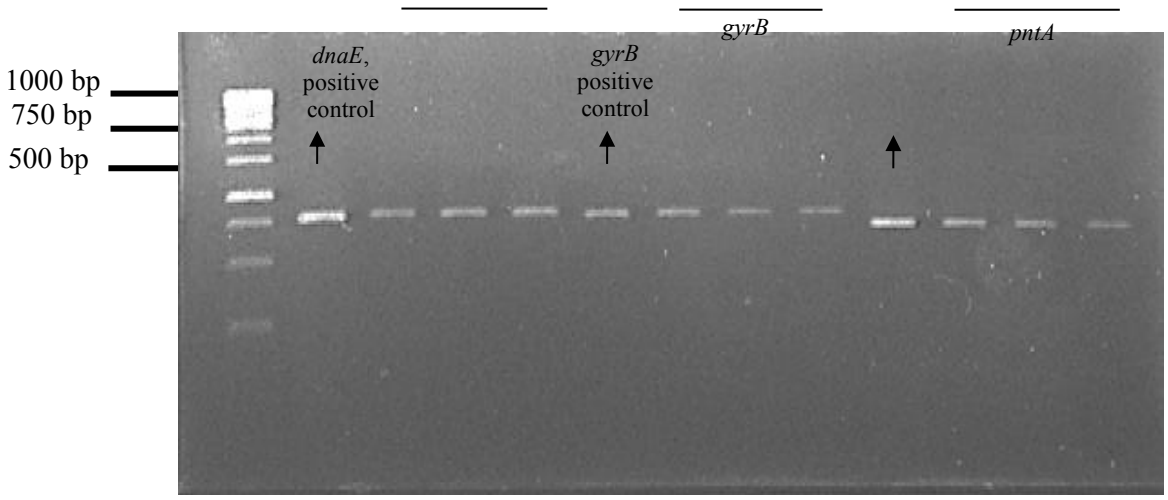

Figure 2 The 1%(w /v) agarose gel electrophoresis of *V. parahaemolyticus* PCR products corresponding to the PCR optimal condition of three housekeeping genes. Lane M: 1 kb ladder (Thermo Scientific, USA); Lane 1: *dnaE* (776 bp) PCR product of *dnaE* positive control VP902; Lane 2-4: *dnaE* positive PCR product of studied isolates. Lane 5: *gyrB* (758 bp) PCR product of *gyrB* positive control VP902; Lane 6-8: *gyrB* positive PCR product of studied isolates. Lane 9: *pntA* (676 bp) PCR product of *pntA* positive control VP902; Lane 10-12: *pntA* positive PCR product of studied isolates.

Analysis of nucleotide sequence for housekeeping gene fragments

Total allele number for each gene represented *dnaE* 8 alleles, *gyrB* 8 alleles and *pntA* 7 alleles (Table 3). Phylogenetic tree analysis of concatenated nucleotide sequences of three housekeeping genes showed that the avian isolates are not clonal representation. These isolates showed diverse genetic background while a number of isolates showed identical typing groups i.e. Group A, B, C and D (Figure 3).

Isolates in Group A (MUVP 2, 5, 6, 7 and 18), Group C (MUVP 16 and 17) and Group D (MUVP 12, 13 and 14) represented identical genetic profile to the isolates from environmental samples in China, including isolates bjciq-VP-607, VP898 and 2012-77, respectively. In contrast, isolates in Group B (MUVP 22, 23 and 24) closely related to clinical samples including isolates 26795 from Peru, GZ052 from China, VP200 from Thailand, S030_1379 from India, S031_1441 from Japan and CDC_K5439 from USA. In addition, isolates MUVP 19, 21 and 25 represented identical genetic profile to environmental isolates from Thailand, including isolates VP22, VP156 and VP-E9, respectively. Furthermore, the MUVP 11 showed identical genetic profile to isolate 235118 from clinical sample with unknown source. Lastly, the MUVP 9 showed similar genetic profile to isolates VP07015 and VN-0084 from clinical sample in China and environmental sample in UK, respectively (https://pubmlst.org /vparahaemolyticus/).

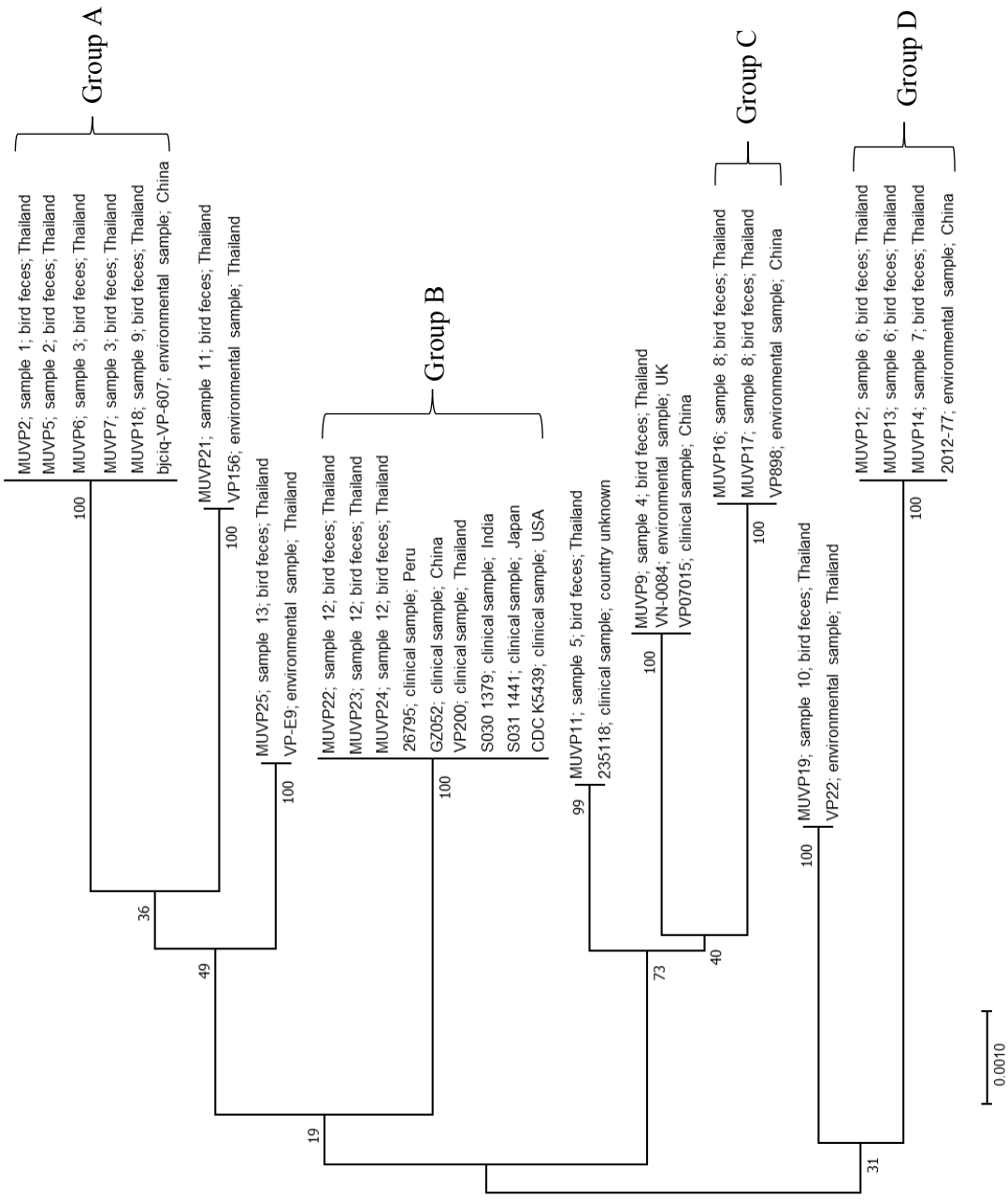

Figure 3 Phylogenetic tree of concatenated nucleotide sequences of three housekeeping genes of *V. parahaemolyticus* isolates from aquatic bird fecal samples in Thailand and other international isolates from MLST database

Table 3 Allele profile of analyzed three housekeeping genes in this study

No.	Isolates	Allele number of housekeeping genes		
		dnaE	*gyrB*	*pntA*
1	MUVP2	158	23	66
2	MUVP5	158	23	66
3	MUVP6	158	23	66
4	MUVP7	158	23	66
5	MUVP9	28	106	18
6	MUVP11	5	106	50
7	MUVP12	234	285	61
8	MUVP13	234	285	61
9	MUVP14	234	285	61
10	MUVP16	35	154	26
11	MUVP17	35	154	26
12	MUVP18	158	23	66
13	MUVP19	5	84	26
14	MUVP21	3	82	30
15	MUVP22	11	48	26
16	MUVP23	11	48	26
17	MUVP24	11	48	26
18	MUVP25	42	147	66
Total allele number		8	8	7

DISCUSSION

In the present study, the total 18 *V. parahaemolyticus* isolates from aquatic bird feces were analyzed by modified MLST scheme. All of isolates for three housekeeping genes (*dnaE*, *gyrB*, *pntA*) were successfully amplified with different conditions. The PCR optimization depends on several parameters such as annealing temperature and magnesium concentration [11]. In this study, most genes from each isolate were performed by annealing temperature at 55 ˚C with 2 mM $MgCl_2$ while a number of isolates required particular condition (Table 2). Since *V. parahaemolyticus* is relatively diverse organism, nucleotide variation in genome may cause difficulty for genes amplification [9, 12].

Analyzing genetic relationships of *V. parahaemolyticus* isolated from fecal aquatic bird samples and international isolates from MLST database revealed that isolates from bird closely related to isolates from both clinical and environmental samples from other countries. Particularly, isolates in Group B had identical allele profile to clinical isolates from many countries including China, India, Japan, Peru, Thailand and USA. These findings indicated that aquatic birds are potential carriers for possible pathogenic *V. parahaemolyticus* and may play role in transmission across countries. Furthermore, the Thai avian isolates represented identical genetic profile with environmental isolates from other countries. Thus, it is possible that aquatic birds are vehicles of transmission of domestic environmental *V. parahaemolyticus* over geographical distance. Although prevalence of *V. parahaemolyticus* in

environmental [13-15] and marine mammals [16-18] were previously reported, this study revealed that avian host is a natural reservoir for *V. parahaemolyticus* with various genetic background. .

Noteworthy, isolates in Group A represented identical profile although they were collected from different samples)Figure 3(. The same situation was also occurred in Group D. These findings implied common genetic types that were frequently recovered from bird feces. This information suggested further study with the larger sampling size to determine dominant genetic type of *V. parahaemolyticus* isolates in aquatic birds.

CONCLUSION

In conclusion, the genetic characterization of *V. parahaemolyticus* isolates originating from aquatic bird feces in Thailand was investigated by modified MLST method. Nucleotide analysis of Thai avian isolates represented genetic diversity with similar background to clinical and environmental isolates from other countries. Regarding the presence of isolates showing closely related to global clinical isolates (Figure 3; Group B), the present study implied that monitoring scheme for this organism in aquatic birds is required to evaluate the risk of transmission for potentially pathogenic *V. parahaemolyticus* in aquatic environment.

CONFLICT OF INTEREST

There is no conflict of interest.

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ABBREVIATIONS

bp	Base pair
dNTP	Deoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
H_2O	Dihydrogen Monoxide (water)
kb	Kilobase
LB	Luria-Bertani
$MgCl_2$	Magnesium Chloride
Min	Minute
MLST	Multilocus sequence typing
mM	Milimolar
NaCl	Sodium chloride
NJ	Neighbor-joining
^oC	Degrees Celsius
PCR	Polymerase chain reaction
rpm	Revolutions per minute
TE	Tris-EDTA
TSA	Tryptic Soy Agar
v/v	Volume per volume
w/v	Weight per volume
UK	United Kingdom
µl	Microlitre
µM	Micromolar
US	United States
USA	United States of America
UV	Ultraviolet