Epidemiological Studies on *Salmonella* Weltevreden of Wild Gecko in Southeast Asian countries

東南アジアのヤモリが保有する *Salmonella* Weltevreden に関する疫学的研究

2018

The United Graduate School of Veterinary Sciences, Gifu University

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CONTENTS

GENERAL INTRODUCTION

Salmonella is Gram-negative, facultatively anaerobic, rod-shaped bacilli belonging to the family *Enterobacteriaceae*. The genus *Salmonella* consists of three species (*S. enterica*, *S. bongori*, and *S. subterranea*), and six subspecies (*S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and *S. enterica* subsp. *indica* (VI)) (9, 24). *Salmonella enterica* comprises more than 2,600 serovars with different specificities for vertebrate hosts (9, 24). *Salmonella* can cause various symptoms ranging from asymptomatic infections, mild diarrhea to severe systemic disease resulting in the death of the hosts. *Salmonella enterica* subsp. *enterica* are known to be the important pathogens for warm-blooded animals. Other subspecies of *S. enterica* are primarily commensal organisms in cool-blooded hosts, such as reptiles, turtles, snakes, but can cause infection in human (24).

Salmonella is recognized worldwide as an important foodborne and human zoonotic pathogen. Salmonellosis is a significant public health problem because of the high burden of this disease involved with mortality in developing countries (71) and the presence of various animal reservoirs (22). Non-typhoidal *Salmonella* had the greatest impact on human health in

Africa, Southeast Asia, and Eastern Europe (71). Centers for Disease Control and Prevention (CDC, USA) estimates *Salmonella* causes more than 1.2 million illnesses, resulting in more than 23,000 hospitalizations and 450 deaths in the United States every year (12). Human salmonellosis was found in 0.6% to 7% in the total of human diarrhea cases in Southeast Asian countries such as Laos, Myanmar, and Vietnam (4, 36). *S.* Typhimurium and *S.* Enteritidis are known as the common human pathogens (27) and the predominant serovar of human salmonellosis in developed countries (26). In contrast, *S.* Weltevreden is known to be the predominant serovar of human salmonellosis in Southeast Asian countries (2, 26, 36, 68, 72), although *S.* Typhimurium and *S.* Enteritidis are also isolated from human patients at a high rate in this region as the same as in developed countries.

Salmonella has a wide prevalence in mammals, reptiles, birds, and the environment. Many of human salmonellosis cases are the zoonotic origin and can be transmitted from animal reservoirs (farm animal, wild animal, etc.) directly via feces or indirectly through food and the environment (53). The natural reservoirs for *Salmonella,* such as *S.* Typhimurium and *S.* Enteritidis, are determined as domestic animals, such as chicken, pig, and cattle. However, the natural reservoir and source of *S.* Weltevreden infection have not been identified yet in Southeast Asian countries. A few reports have been published that reptiles can harbor

Salmonella at a high rate (13, 20, 21, 40). Among of reptile species, wild geckos are widely distributing in residential areas in Southeast Asian countries (60), and they can excrete feces in the environment. Thus, wild geckos might also act as reservoirs and sources of *Salmonella* infection in these countries. However, no report about the role of the wild gecko as a reservoir and a source of *Salmonella* infection, especially *S*. Weltevreden in Southeast Asian countries has been published.

The infectious route of *Salmonella* is mainly via oral infection, person to person transmission (57). The consumption of *Salmonella*-contaminated food such as meat, vegetables, fruits, and drinking water is the available route to cause human salmonellosis. In recent years, the importance of foods originated from vegetables as the potential vehicles of enteropathogens, such as *Salmonella*, has been reported (25). Especially, some outbreaks of human *Salmonella* infection linked to fresh vegetables in developed countries have been announced (46). Vegetables are easily contaminated with many pathogens via direct or indirect contact with human, rodents, reptiles, manure, and irrigation water (46, 48, 58). In Southeast Asian countries including Vietnam, people usually have a habit of consuming raw vegetables sold in the wet markets. Therefore, the retail vegetable might be an important source of human *Salmonella* infection in Southeast Asian countries.

The main objective of this study is to clarify the epidemiology of *Salmonella* Weltevreden in wild gecko in Southeast Asian countries. Following subjects on *Salmonella* Weltevreden in the Southeast Asian countries were studied in the present study.

In chapter 1, the prevalence of *S.* Weltevreden in wild gecko in Southeast Asian countries was studied.

In chapter 2, the quantification and survival period of *Salmonella* in gecko feces were identified.

In chapter 3, the genetic diversity and relationship of *S.* Weltevreden isolates originated from wild geckos in Southeast Asian countries were clarified.

In chapter 4, a role of vegetables as the source of human *Salmonella* infection was determined.

CHAPTER 1

Prevalence of *Salmonella* Weltevreden in wild gecko living in Southeast Asian countries

1.1. INTRODUCTION

Reptiles are known to play an important role as a reservoir for *Salmonella* as well as a source of *Salmonella* infection (20, 21, 56, 69). Sumiyama *et al.* (54) reported that green anoles (*Anolis carolinensis*) harbored a high rate of *Salmonella* (27.1%) was a risk factor for human public health in Chichi Island, Japan. Callaway *et al.* (7) also indicated that Asian house geckos (*Hemidactylus frenatus*) harbored *Salmonella* might play a significant role in the epidemiology of sporadic salmonellosis in Northern Australia. Wild gecko is one of the important reptiles living widely in Southeast Asian countries (60). However, few reports have been published on the prevalence of *Salmonella* in wild gecko in these countries. Therefore, this study was carried out to clarify the prevalence of *Salmonella* in gecko in Southeast Asian countries as well as the importance of gecko as a natural reservoir for *Salmonella*.

1.2. MATERIALS AND METHODS

1.2.1. Sample collection

From 2012 to 2015, a total of 1,318 wild geckos were collected in Cambodia (n = 98), Thailand (n = 261), Hue in the central of Vietnam (n = 313), and the Mekong Delta in the South of Vietnam (n = 646). These geckos belonged to three species: common house gecko (*Hemidactylus frenatus*) (n = 794), flat-tailed house gecko (*Hemidactylus platyurus*) (n = 464) and four-clawed gecko (*Gehyra mutilata*) (n = 60). These geckos were caught and put separately in the sterilized plastic bags. In the laboratory, geckos were dissected, and the feces were collected from the rectum individually and aseptically after evisceration to avoid contamination from external sources.

1.2.2. Isolation and identification of *Salmonella* from wild gecko

In this study, about 0.1 g of gecko feces was suspended in 9 times volume of

enterobacteriaceae enrichment mannitol broth (EEM, Eiken, Tokyo, Japan) and was incubated at 37°C for 24 h for pre-enrichment. Then 1 m*l* of EEM enrichment broth was transferred into 9 m*l* of Hajna tetrathionate broth (Eiken) and was incubated at 37°C for 24 h. A loopful of Hajna broth culture from each sample was inoculated onto 2 selective media, mannitol lysine crystal violet brilliant agar (MLCB, Nissui, Tokyo, Japan) and desoxycholate hydrogen sulfide lactose agar (DHL, Nissui). The plates were incubated at 37°C for 24 h. The suspected *Salmonella* colonies grown on the selective agars were picked up and subcultured on trypticase soy agar (TSA, BD, USA). These suspected colonies were identified by biochemical characteristics with triple-sugar iron agar (TSI, Nissui), VP medium (Eiken), and lysine indol motility medium (LIM, Nissui). *Salmonella* isolates identified were serotyped according to the White–Kauffmann–Le Minor scheme (49) with commercial O and H antisera (Denka Seiken, Tokyo, Japan).

1.2.3. Antimicrobial susceptibility of *S.* Weltevreden isolates

Of *Salmonella* isolates from gecko samples, *S.* Weltevreden isolates were examined

for the antimicrobial susceptibility against the antibiotic agents. Disk diffusion method was carried out according to the Clinical Laboratory Standards Institute (CLSI) procedure M02-M07 (2014) (11). A total of 9 antibiotic agents that are regularly used for treatment to animals and humans in Southeast Asian countries, were used in this study including ampicillin (ABPC, 10 μg), oxytetracycline (OTC, 30 μg), chloramphenicol (CP, 30 μg), nalidixic acid (NA, 30 μg), cefazolin (CEZ, 30 μg), streptomycin (SM, 10 μg), kanamycin (KM, 30 μg), gentamycin (GM, 10 μg), and ofloxacin (OFLX, 5 μg). The antibiotic disks purchased from Becton Dickinson (BD, USA) were used in this study.

1.2.4. Data analysis

The data were tested by Chi-square (χ^2) method at the 95% confidence level ($p<0.05$).

1.3. RESULTS

Of 1,318 gecko samples in Southeast Asian countries, 293 samples (22.2%) were *Salmonella* positive. The *Salmonella* isolation rate from wild geckos in Thailand (46.0%) showed a significant higher than that in Vietnam (16.3%) and Cambodia (17.3%) ($p<0.01$) (Table 1-1). However, there was no significant difference in the isolation rate of *Salmonella* among 3 gecko species in these countries.

Of 293 *Salmonella* isolates, *S.* Weltevreden (32.1%) was the most predominant serovar isolated from wild geckos, followed by *S.* Brunei (5.5%), *S.* Lexington (4.4%), and *S.* Newport (3.4%). Moreover, of *Salmonella*-positive gecko samples in each country, *S.* Weltevreden occupied 94.1% (16/17) in Cambodia, 16.7% (20/120) in Thailand, and 37.2% (58/156) in Vietnam. The distribution of *Salmonella* serovars isolated from wild gecko was shown in Table 1-2 and Table 1-3.

All 94 *S.* Weltevreden isolates (100%) from wild geckos showed susceptibility against 9 antibiotics examined.

Table 1-1. Prevalence of *Salmonella* in wild geckos in Southeast Asian countries

Countries	No. of *Salmonella* positive samples/No. of samples examined (%)			
	H. frenatus	*H. cosymbotus*	*G. mutilata*	Total
Vietnam				
Hue	33/194 (17.0)	25/97 (25.8)	3/22 (13.6)	61/313 (19.5)
Mekong Delta	58/358 (16.2)	33/250 (13.2)	4/38 (10.5)	95/646 (14.7)
Subtotal	91/552 (16.5)	58/347 (16.7)	7/60 (11.7)	156/959 (16.3)
Cambodia	13/79 (16.5)	4/19 (21.1)		17/98 (17.3)
Thailand	81/163 (49.7)	39/98 (39.8)		120/261 (46.0)[a)]
Total	185/794 (23.3)	101/464 (21.8)	7/60 (11.7)	293/1,318 (22.2)

[a)] Thailand > Cambodia, Vietnam ($p < 0.01$)

Table 1-2. Serovars of *Salmonella* isolates from wild geckos by countries

Serovars	Countries			Total (%)	
	Cambodia	Thailand	Vietnam		
S. Weltevreden	16	20	58	94	(32.1)
S. Brunei		1	15	16	(5.5)
S. Lexington			13	13	(4.4)
S. Newport		1	9	10	(3.4)
S. Stanley		3		3	(1.0)
S. Vejle			2	2	(0.7)
S. Agona			1	1	(0.3)
S. Bovismorbificans			1	1	(0.3)
S. Dabou			1	1	(0.3)
S. Emek		1		1	(0.3)
S. Fillmore		1		1	(0.3)
S. Hindmarsh			1	1	(0.3)
S. Strathcona			1	1	(0.3)
S. Suberu	1			1	(0.3)
$O_{3,10}$: UT[a)]		28		28	(9.6)
O_4: UT		5		5	(1.7)
O_7: UT		1		1	(0.3)
O_8: UT		32		32	(10.9)
O_{13}: UT		1		1	(0.3)
Biovar IIIa			10	10	(3.4)
Biovar IV		26	20	46	(15.7)
Biovar V			1	1	(0.3)
Untyped			23	23	(7.8)
Total	17	120	156	293	(100.0)

[a)]UT: Untyped

Table 1-3. Serovars of *Salmonella* isolates from wild geckos by species

Serovars	Gecko species			Total (%)
	H. frenatus	*H. cosymbotus*	*G. mutilata*	
S. Weltevreden	55	38	1	94 (32.1)
S. Brunei	13	3		16 (5.5)
S. Lexington	6	6	1	13 (4.4)
S. Newport	6	4		10 (3.4)
S. Stanley		3		3 (1.0)
S. Vejle	2			2 (0.7)
S. Agona		1		1 (0.3)
S. Bovismorbificans		1		1 (0.3)
S. Dabou			1	1 (0.3)
S. Emek	1			1 (0.3)
S. Fillmore	1			1 (0.3)
S. Hindmarsh	1			1 (0.3)
S. Strathcona	1			1 (0.3)
S. Suberu	1			1 (0.3)
$O_{3,10}$: UT[a)]	16	12		28 (9.6)
O_4: UT	1	4		5 (1.7)
O_7: UT	1			1 (0.3)
O_8: UT	24	8		32 (10.9)
O_{13}: UT		1		1 (0.3)
Biovar IIIa	8	2		10 (3.4)
Biovar IV	35	8	3	46 (15.7)
Biovar V	1			1 (0.3)
Untyped	14	8	1	23 (7.8)
Total	187	99	7	293 (100.0)

[a)]UT: Untyped

1.4. DISCUSSION

Salmonella was isolated at a high rate (22.2%) from wild geckos living in Southeast Asian countries in the present study. A few reports about the prevalence of *Salmonella* in reptiles have been published. Cheng *et al.* (13) isolated *Salmonella* from captive and wild lizards in Malaysia, and found that 36% of fecal samples were positive for *Salmonella*. Geue *et al.* (21) reported that *Salmonella* was isolated from 54.1% (86/159) of reptiles including turtles, snakes, and lizards originated from Germany and Austria. With regards to gecko, Kallo *et al.* (31) found that 96.4% of Iraq geckos (*Hemidactylus turcicus*) were positive for *Salmonella* in Iraq. Nwachukwu *et al.* (45) also reported that 25.7% of the common house geckos (*Hemidactylus frenatus*) living in Nigeria were *Salmonella* positive. Jimenez *et al.* (30) also isolated *S.* Weltevreden from Asian common house gecko (*Hemidactylus frenatus*) in Costa Rica. In the present study, *S.* Weltevreden was the most predominant serovar (32.1%) isolated from wild geckos in 3 Southeast Asian countries. On the other hand, Tran *et al.* (61) indicated that the isolation rate of *S.* Weltevreden were very low from domestic animals such as chickens, ducks, and pigs in the Mekong Delta although the retail pork sold in wet markets were

contaminated with *Salmonella*, especially *S.* Weltevreden, at a high rate (62). Modarressi *et al.* (39) also reported that the retail raw chicken and beef in the wet market in Malaysia were contaminated with *S.* Weltevreden. Until now, the natural reservoir for *S.* Weltevreden has not been identified yet. These results indicate that wild geckos might be the natural reservoir for *S.* Weltevreden in Southeast Asian countries, such as Vietnam, Cambodia, and Thailand. The reason why wild geckos in Thailand harbored *Salmonella* at a high rate was not clear, it might be due to the sampling places in Thailand.

S. Weltevreden was often detected from diarrhea patients in Southeast Asian countries (2, 36, 59, 65, 68). Foodborne outbreaks due to *S.* Weltevreden were also reported in India (1) and in Réunion Island, France (17) where the same gecko species in Southeast Asian countries distributed. In addition, *Salmonella* serovar Agona, Bovismorficans, and Newport which serovars were isolated from wild geckos in the Mekong Delta, were also isolated from human diarrhea patients in this region (36). Further research should be carried out to determine the vehicle of *Salmonella* transmission from geckos to the human in these countries.

Lee *et al.* (33) reported that *S.* Weltevreden isolated from poultry and vegetables in Malaysia showed the resistance to streptomycin (15%), followed by cephalothin (12%),

ampicillin (9%), and gentamycin (9%). Truong *et al.* (63) isolated *S.* Weltevreden from the retail pork sold in the North of Vietnam, and found that this serovar was resistant to ampicillin, chloramphenicol, streptomycin, nalidixic acid, and neomycin. Tu *et al.* (64) also indicated that *S.* Weltevreden isolated from pigs, chickens, and ducks in the Mekong Delta, Vietnam showed the resistance to multiple antibiotics such as ampicillin, tetracycline, chloramphenicol, gentamycin, ciprofloxacin, and nalidixic acid. However, all 94 *S.* Weltevreden isolates from wild geckos were susceptible to 9 antibiotic agents in this study. The results indicate that *S.* Weltevreden seems to be maintained mainly in wild gecko in nature.

In conclusion, wild geckos in Southeast Asian countries harbored *Salmonella* at a high rate, and *S.* Weltevreden was the predominant serovar in wild geckos. Therefore, wild gecko seems to be an important natural reservoir for *S.* Weltevreden in these countries.

1.5. SUMMARY

From 2012 to 2015, a total of 1,318 wild geckos were collected in Cambodia, Thailand, and Vietnam (Hue and the Mekong Delta) to determine the prevalence of *S.* Weltevreden. The geckos belong to 3 species: common house gecko (*Hemidactylus frenatus*), flat-tailed house gecko (*Hemidactylus platyurus*), and four-clawed gecko (*Gehyra mutilata*). Of 1,318 gecko samples, *Salmonella* was positive for 293 samples (22.2%) in this study. The prevalence of *Salmonella* in geckos was 16.3% in Vietnam, 17.3% in Cambodia, and 46.0% in Thailand. Among of *Salmonella* isolates, *S.* Weltevreden was the most predominant serovar (32.1%) isolated from wild geckos in these countries. There was no significant difference in the prevalence of *Salmonella* among gecko species. All *S.* Weltevreden isolates (100%) were susceptible to the 9 antibiotics examined. The results indicate that wild gecko seems to be an important natural reservoir for *S.* Weltevreden in Southeast Asian countries.

CHAPTER 2

Quantification and survival period of *Salmonella* in gecko feces

2.1. INTRODUCTION

Few reports regarding on the survival of *Salmonella* in domestic animal feces have been published. Gray and Fedorka-Cray (23) indicated that *S.* Choleraesuis could be recovered from dry feces of pigs infected with *S.* Choleraesuis after 13 months of storage. You *et al.* (73) reported that *S.* Newport could persist for 184 days in the manure that made from dairy cattle feces. Thus, *Salmonella* could exist for a long time in animal feces. In Southeast Asian countries, wild gecko seems to be an important natural reservoir for *Salmonella,* especially *S.* Weltevreden shown in chapter 1. Wild geckos commonly live in the residential areas in those countries. They can excrete feces everywhere and be seen in close contact with humans. However, no report has been published about the quantification and survival analysis of *Salmonella* in gecko feces. Therefore, this study was carried out to clarify the viable number and survival periods of *Salmonella* in gecko feces.

2.2. MATERIALS AND METHODS

2.2.1. Sample collection

In this study, a total of 201 wild geckos (138 *Hemidactylus frenatus* and 63 *Hemidactylus platyurus*) were collected in the Mekong Delta, Vietnam. Geckos were dissected to collect the feces as described in chapter 1.2.1.

2.2.2. Determination of the number of *Salmonella* in gecko feces

Of 101 samples examined, about 0.1 g of feces in each sample was collected and suspended in 9 times volume of phosphate buffer saline (PBS, pH 7.2). Following this, 0.1 m*l* of the suspension diluted 10-fold with PBS was plated on MLCB and DHL (Nissui). The number of *Salmonella* colonies was counted on these selective agars, after incubation at 37^{o}C for 24 h. If suspected *Salmonella* colonies did not appear on the selective media, samples were enriched with Hajna tetrathionate broth (Eiken) at 37^{o}C for 24 h. A loopful of

enrichment broth was then streaked on the selective agars. The suspected *Salmonella* isolates grown on the selective agars were examined for biochemical characteristics and identified serovars following the methods described in chapter 1.2.2.

2.2.3. Determination of the survival period of *Salmonella* in gecko feces

Out of 201 gecko fecal samples, 101 that used for quantification analysis of *Salmonella* in gecko feces and 100 were divided into 2 groups. Fecal samples in each group were mixed and put into sterilized Erlenmeyer flasks (300 m*l*). Those fecal mixtures were kept at room temperature (25-30^{o}C) of Vietnam during 10 weeks. About 1 g of fecal sample has been taken from each mixture once a week for 10 weeks after storage. Isolation and identification of *Salmonella* from fecal samples were also done following the same methods described in chapter 1.2.2.

2.3. RESULTS

Of 101 gecko samples, 24 (23.8%) were *Salmonella* positive. Among these positive samples, 14 geckos excreted *Salmonella* more than 4 log CFU/g (CFU, Colonies Forming Units) in their feces. The highest number of *Salmonella* in gecko feces was 8.6 log CFU/g. The number of *Salmonella* in gecko feces excreted under 2 log CFU/g was calculated as 1 log CFU/g. The mean number of *Salmonella* in gecko feces was 4.5 ± 3.2 log CFU/g (Fig. 2-1).

Among of 24 *Salmonella* isolates, *S.* Weltevreden (37.5%) was the most predominant serovar, followed by *S.* Worthington (12.5%), *S.* Lexington (8.3%), *S.* Albany (4.2%) and *S.* Bellevue (4.2%) (Table 2-1).

Moreover, *Salmonella* was isolated from fecal mixtures of both groups for 6 weeks after storage. No *Salmonella* was detected from fecal samples for 7-10 weeks after storage.

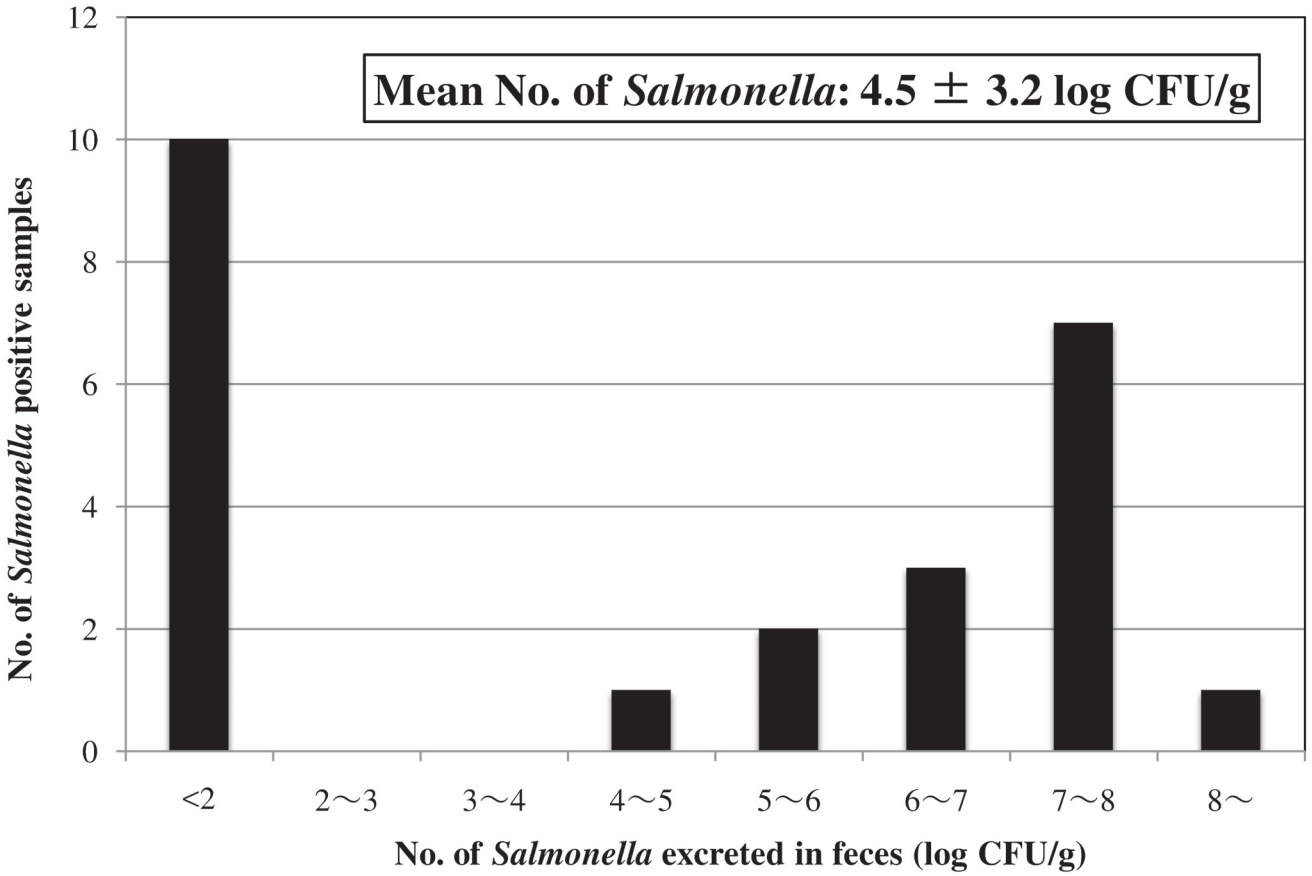


Fig. 2-1. The number of *Salmonella* excreted in gecko feces (log CFU/g)

Table 2-1. Serovars of *Salmonella* isolates from gecko feces (n = 24)

Serovar	No. of isolates (%)
S. Weltevreden	9 (37.5)
S. Worthigton	3 (12.5)
S. Lexington	2 (8.3)
S. Albany	1 (4.2)
S. Bellevue	1 (4.2)
Biovar III b	1 (4.2)
Biovar IV	3 (12.5)
Untyped	4 (16.7)
Total	24 (100.0)

2.4. DISCUSSION

In this study, wild gecko excreted a high viable number of *Salmonella* in their feces. Berghaus *et al.* (3) reported that chicken infected with *Salmonella* shed 1.56 log MPN/g (MPN, Most Probable Number) of *Salmonella* in their feces. Fegan *et al.* (19) indicated that *Salmonella* was excreted in cattle feces under 1 log MPN/g. Thus, the number of *Salmonella* shed in gecko feces seems to be higher than that in other animals. As the gecko shed *Salmonella* did not show clinical symptoms and not die, *Salmonella* seems to be the normal flora in gecko intestine. Furthermore, *S.* Weltevreden was also the most predominant serovar (37.5%) in this study as the same as the results in chapter 1. Ly *et al.* (36) reported human *S.* Weltevreden infection cases occurred in the Mekong Delta, Vietnam. Therefore, the results indicated that wild geckos seem to spread *Salmonella* to the environment in this region as well as in Southeast Asian countries, and be the potentially important source of *Salmonella* infection, especially by *S.* Weltevreden.

This study showed that *Salmonella* could survive for a long time in gecko feces under a normal environmental condition in Vietnam. Otokunefor *et al.* (47) reported that

Salmonella could survive longer in lizard feces in the dry environment from 6 to 8 weeks rather than in wet conditions in Nigeria. The long survival of *Salmonella* in gecko feces is a potential risk to cause human infection via direct or indirect contact with gecko feces. However, the mechanism involved in the survival of *Salmonella* for a long time in gecko feces in the environment is still unclear.

The present study indicates that wild gecko seems to play an important role as a natural reservoir and a source of human *Salmonella* infection in Southeast Asian countries.

2.5. SUMMARY

A total of 201 wild geckos in the Mekong Delta, Vietnam were collected to clarify the viable number and survival period of *Salmonella* in their feces. Of 101 samples examined, 24 samples (23.8%) were *Salmonella* positive. Those *Salmonella* positive geckos excreted *Salmonella* in their feces from 1 to 8.6 log CFU/g. The mean number of *Salmonella* in feces was 4.5 ± 3.2 log CFU/g. Among *Salmonella* serovars, *S.* Weltevreden was the most predominant serovar (37.5%). Moreover, *Salmonella* could survive for 6 weeks in gecko feces at the room temperature in Vietnam. These results indicate that wild gecko could play an important role as a reservoir for *Salmonella* and a source of human *Salmonella* infection in Southeast Asian countries.

CHAPTER 3

Genetic diversity of *Salmonella enterica* serovar Weltevreden isolated from wild gecko in Southeast Asian countries

3.1. INTRODUCTION

In Southeast Asian countries, *S.* Enteritidis and *S.* Weltevreden are known to be the predominant serovars (27, 68). Many reports have been published about the low genetic diversity of *S.* Enteritidis. Campioni *et al.* (8) reported that *S.* Enteritidis strains isolated from food and humans over a 24-year period in Brazil exhibited the high genetic similarity among these strains, and they might have descended from a common ancestor. *S.* Enteritidis isolates originated from human patients in Malaysia (42) and Thailand (66) also showed the limited genetic diversity because these isolates might have the same origin. However, the diversity and genetic relationship of *S.* Weltevreden has still not been identified clearly. Recently, molecular fingerprinting methods have been used for clarifying the genetic relationship of the pathogens from many sources. These methods are powerful tools for surveillance and outbreak investigation of pathogens. Among these methods, Pulsed-field Gel Electrophoresis (PFGE) is considered as a gold standard in research of bacteria epidemiology, and can obtain large fragments from DNA. Multiple Locus Variable-Number Tandem Repeat Analysis (MLVA) utilizes the naturally occurring variation in the number of tandem repeated DNA

sequences found in many different loci in the genome of a variety of organisms. PCR Binary Typing (P-BIT) is inexpensive, rapid, and highly portable and has a discriminatory power and stability similar to those of PFGE. Therefore, in the present study, 3 methods of PFGE, MLVA, and P-BIT were used to clarify the genetic diversity and relationship among *S.* Weltevreden isolates originated from wild geckos in Southeast Asian countries.

3.2. MATERIALS AND METHODS

3.2.1. Bacterial strains

A total of 77 *S.* Weltevreden isolates originated from wild geckos in Cambodia (n = 16), Thailand (n = 16), Hue in Vietnam (n = 19), the Mekong Delta in Vietnam (n = 24), and Okinawa Prefecture in Japan (n = 2) were analyzed to clarify the genetic diversity of this serovar in those regions. In addition, *S.* Weltevreden isolates originated from human patients (n = 3) in the Mekong Delta, Vietnam were also analyzed in this study.

3.2.2. Molecular genetic typing of *Salmonella* Weltevreden

3.2.2.1. Pulse-filed Gel Electrophoresis (PFGE)

Pulse-field Gel Electrophoresis (PFGE) was performed by clamped homogeneous electric field electrophoresis using a CHEF DR II apparatus (Biorad, USA) to compare the genetic characteristics of 80 *S.* Weltevreden isolates. The procedure of PFGE method was followed the guideline of PulseNet International (CDC, USA). In brief, *S.* Weltevreden isolates were cultured on TSA (BD, USA) and incubated at 37°C for 24 h. Bacterial colonies were then harvested and suspended into 2 m*l* of a buffer (100 mM Tris, 100 mM EDTA [pH 8.0]). The concentration of the cell suspension was adjusted to get an optical density of 1.45 to 1.55. For each sample, about 400 μ*l* of the adjusted cell suspension was mixed gently in Eppendorf tube with 20 μ*l* of Proteinase K (20 mg/m*l* stock) (Wako, Japan) and 400 μ*l* of melted 1% NA agarose (Amersham Pharmacia, UK) that was equilibrated to 50°C and prepared in 1X Tris-EDTA (TE) buffer and 10% sodium dodecyl sulfate solution. This mixture was dispensed immediately into the disposable plug mold to solidify at room temperature for 10 – 15 min. The plugs were removed from the molds and put in 5 m*l* of cell lysis buffer (50 mM

Tris, 50 mM EDTA [pH 8.0] - 1% N-Lauroylsarcosine) with 25 μ*l* of Proteinase K. In the plugs, bacterial cell wall were lysed in the cell lysis buffer at 54°C for 2 h in the water bath with shaking. After that, the lysis buffer was withdrawn, and the plugs were rinsed 4 times with the sterile deionized water and 1X TE buffer at 50°C for 1 h respectively. About 2 mm wide slice of each plug was used in the restriction step. Genomic DNA of *S.* Weltevreden isolates in gel plugs was restricted in the reaction mixture with *Xba*I enzyme (10 U/μ*l*) (Takara, Japan) at 37°C for 2 h in accordance with the manufacturer's instructions. *Salmonella* Braenderup H9812 was used as a reference strain. The DNA fragments were separated in 1% NA agarose gel that was prepared in 0.5X Tris-borate-EDTA buffer (TBE, Biorad, USA). Electrophoresis was conducted for 19 h at 14°C and 6 V/cm with pulsed times of 2.2 to 63.8 s. Thereafter, the gels were stained in ethidium bromide and photographed under UV light. The PFGE profiles were scanned and analyzed to clarify the diversity of those isolates using BioNumberics software (Applied Maths, Belgium). Cluster analysis was performed using UPGMA (the unweighted pair group method with arithmetic mean) to create the dendrogram.

3.2.2.2. Multiple Locus Variable-Number Tandem Repeat Analysis (MLVA)

Of 80 *S.* Weltevreden isolates, 21 isolates showing different PFGE patterns were analyzed using MLVA assay. In brief, *S.* Weltevreden isolates were cultured on TSA (BD, USA) and incubated at 37^{o}C for 24 h. Then, *S.* Weltevreden isolates were extracted those DNA using FastGene Gel/PCR extraction kit (Nippon Genetics, Japan). Three target genes (*tol*A, *yoh*M, and intergenic) were amplified by PCR method. These genes localize on 4 different loci of *Salmonella* genome including STTR1, Sal16, SENTR5, and 3414090. PCR reaction was carried out using Takara EX *Taq* kit (Takara, Japan). The sequence and PCR condition of primers used in this method was shown in Table 3-1. After that, PCR products were purified, and the DNA sequences of those genes were analyzed in Eurofin Genomics Co., Ltd. (Tokyo, Japan). The number of tandem repeated sequences was determined using Tandem repeats Finder software (Boston University, USA). MLVA pattern was obtained by combining the number of tandem repeated sequences found in 4 loci. MLVA patterns were then compared to reveal the genetic relationship among *S.* Weltevreden isolates.

3.2.2.3. PCR Binary Typing (P-BIT)

Twenty-one *S*. Weltevreden isolates that were used in MLVA analysis were also used in the P-BIT method. A total of 14 pathogenic genes of *Salmonella* were applied in this method. These genes localize on the genome such as *Salmonella* Pathogenic Island (SPI) from SPI-1 to SPI-17, prophage, fimbrial operon or on the plasmid. Bacterial culture and DNA extraction were done as the methods described in the MLVA assay. The sequence and PCR condition of primers used in this study was shown in Table 3-2. PCR reaction was carried out using Takara EX *Taq* kit (Takara, Japan). Then, PCR products were run electrophoresis in 1.5% ME agarose gel (Wako, Japan) at 50V for 1 h. After that, the gels were stained in ethidium bromide and captured the imagines under UV light. The binary profiles of *S*. Weltevreden isolates were obtained depending on the presence of pathogenic genes. Those profiles were analyzed to know the genetic relationship of *S*. Weltevreden isolates.

Table 3-1. Sequence and PCR condition of oligonucleotide primers used in MLVA assay

Locus	Target gene	Sequences (5'-3')	Size of product (bp)	Reaction temperature (°C)			References
				Denaturation	Annealing	Extension	
STTR1	*tolA*	CAGCAGTACAACCGTCAGCAGGAT GCCCCACCGTTAGCGCCCGATGTA	770	94 (30)[a)]	63 (90)	72 (90)	Lindstedt *et al.* (34)
Sal16	*yohM*	CCATGGCTGCAGTTAATTTCT TGATACGCTTTTGACGTTGC	224	96 (30)	62 (60)	72 (60)	Ramisse *et al.* (51)
SENTR5	*yohM*	CACCGCACAATCAGTGGAAC GCGTTGAATATCGGCAGCATG	270	94 (30)	55 (90)	72 (90)	Malorny *et al.* (37)
3414090	Intergenic	AATTAATTGCCGGATGGTGA AGCGATTGCTGGCCTAGAT	841	96 (60)	55 (60)	72 (30)	Witonski *et al.* (70)

[a)] The value in parenthesis is the reaction time (in seconds)

Table 3-2. Sequence and PCR condition of oligonucleotide primers used in P-BIT assay

Locus	Target gene	Sequences (5'-3')	Size of product (bp)	Reaction temperature (°C)			References
				Denaturation	Annealing	Extension	
SPI-1[a)]	*sopE1*	CGGGCAGTGTTGACAAATAAAG TGTTGGAATTGCTGTGGAGTC	422	95 (30)[b)]	58 (30)	72 (30)	Huehn *et al.* (28)
	hilD	AGCAGGTTACCATCAAAAATCTTTATG TGAGCCGAGCTAAGGATGATC	509	94 (60)	58 (60)	72 (60)	Khoo *et al.* (32)
SPI-2	*sseC*	TATGGTAGGTGCAGGGGAAG CTCATTCGCCATAGCCATTT	121	95 (60)	50 (60)	72 (60)	Fazl *et al.* (18)
	sifA	ATGCCGATTACTATAGGCAATGG TTATAAAAAACAACATAAACAGCCG	1,011	94 (30)	58 (30)	72 (60)	Campioni *et al.* (8)
	ssrB	ATGAAATCATCATTAACGGCATTAT ACAGAACTTGCTGACTACTGCTTTT	310	94 (60)	55 (60)	72 (60)	Khoo *et al.* (32)
SPI-5	*pipA*	CTCTTGGATGATTTTCTTCTTTA CTTATCTCAGGCGCGGGTGG	406	94 (60)	55 (60)	72 (60)	Khoo *et al.* (32)
	sopB	GATGTGATTAATGAAGAAATGCC GCAAACCATAAAAACTACACTCA	1,170	94 (60)	55 (60)	72 (60)	Khoo *et al.* (32)

[a)]SPI: *Salmonella* Pathogenic Island
[b)]The value in parenthesis is the reaction time (in seconds)

Table 3-2. Sequence and PCR condition of oligonucleotide primers used in P-BIT assay (cont.)

Locus	Target gene	Sequences (5'-3')	Size of product (bp)	Reaction temperature (°C)			References
				Denaturation	Annealing	Extension	
Prophage	*gipA*	ACGACTGAGCAGGCTGAG TTGGAAATGGTGACGGTAGAC	518	95 (30)[a)]	58 (30)	72 (30)	Huehn *et al.* (28)
	sodC1	CCAGTGGAGCAGGTTTATCG GGTGCGCTCATCAGTTGTTC	424	95 (30)	58 (30)	72 (30)	Huehn *et al.* (28)
	gtgB	TGCACGGGGAAAACTACTTC TGATGGGCTGAAACATCAAA	436	90 (30)	58 (30)	72 (60)	Capuano *et al.* (10)
	sspH1	TGCAGAAAAAGGGGAATACG GCAGCCTGAAGGTCTGAAAC	246	95 (30)	58 (30)	72 (60)	Capuano *et al.* (10)
Fimbrial operon	*agfA*	TCCGGCCCGGACTCAACG CAGCGCGGCGTTATTACCG	261	94 (30)	58 (30)	72 (60)	Craciunas *et al.* (15)
Plasmid	*spvC*	ACTCCTTGCACAACCAAATGCGGA TGTCTTCTGCATTTCGCCACCATCA	570	94 (30)	56 (30)	72 (120)	Capuano *et al.* (10)
	pefA	TTGCACTGGGTGTTCTGG TGTAAGCCACTGCGAAAG	486	95 (30)	58 (30)	72 (60)	Borriello *et al.* (5)

[a)]The value in parenthesis is the reaction time (in seconds)

3.3. RESULTS

Twenty-one different PFGE patterns were obtained from 80 *S.* Weltevreden isolates in Southeast Asian countries and Japan (Fig. 3-1). These PFGE patterns of *S.* Weltevreden isolates were divided into 2 clusters A (12 patterns) and B (9 patterns) at a cut-off value of 40% (Fig. 3-2). Almost all *S.* Weltevreden isolates originated from the same region or country showed similar PFGE patterns. Only 2 PFGE patterns, F1 and F13, were found in different places of the Mekong Delta and Hue, Vietnam. The pattern F13 was identified from both wild gecko and human isolates in Vietnam in this study. Furthermore, *S.* Weltevreden isolated from wild gecko in Japan showed 60% similarity to those isolates in Vietnam.

Sixteen MLVA types were obtained from 21 *S.* Weltevreden isolates in this study. Among 4 primers used, primer Sal16 could make more the number of repeated sequences in the locus than other primers do. The number of repeated sequences in 4 loci was shown in Table 3-3. Of 16 MLVA patterns, 12 MLVA patterns except M4, M5, M8 and M11 were obtained from single *S.* Weltevreden isolates. However, the patterns M4, M5, and M8 were obtained from multiple *S.* Weltevredem isolates originated from Vietnam, Thailand, and Japan

respectively (Table 3-6).

Of 21 *S*. Weltevreden isolates, all isolates harbor 7 pathogenic genes (*hilD*, *ssrB*, *pipA*, *sopB*, *pefA*, *gtgB*, and *sspH1*). *spvC* gene was not detected from any *S.* Weltevreden isolates. The prevalence rate of other 6 pathogenic genes (*sopE1*, *sseC*, *sifA*, *gipA*, *sodC1*, *agfA*) was from 4.8% to 95.2% (Table 3-4). Therefore, the binary profile of *S*. Weltevreden isolates was obtained by combining presence of these 6 pathogenic genes. A total of 10 gene types was obtained from 21 *S.* Weltevreden isolates. Among of 10 profiles, type P1 is the most popular type (33.3%), followed by type P6 (19.0%), type P2 (9.5%), and type P4 (9.5%) (Table 3-5). The PCR binary typing results of 21 *S*. Weltevreden isolates originated from geckos and human was shown in Table 3-6.

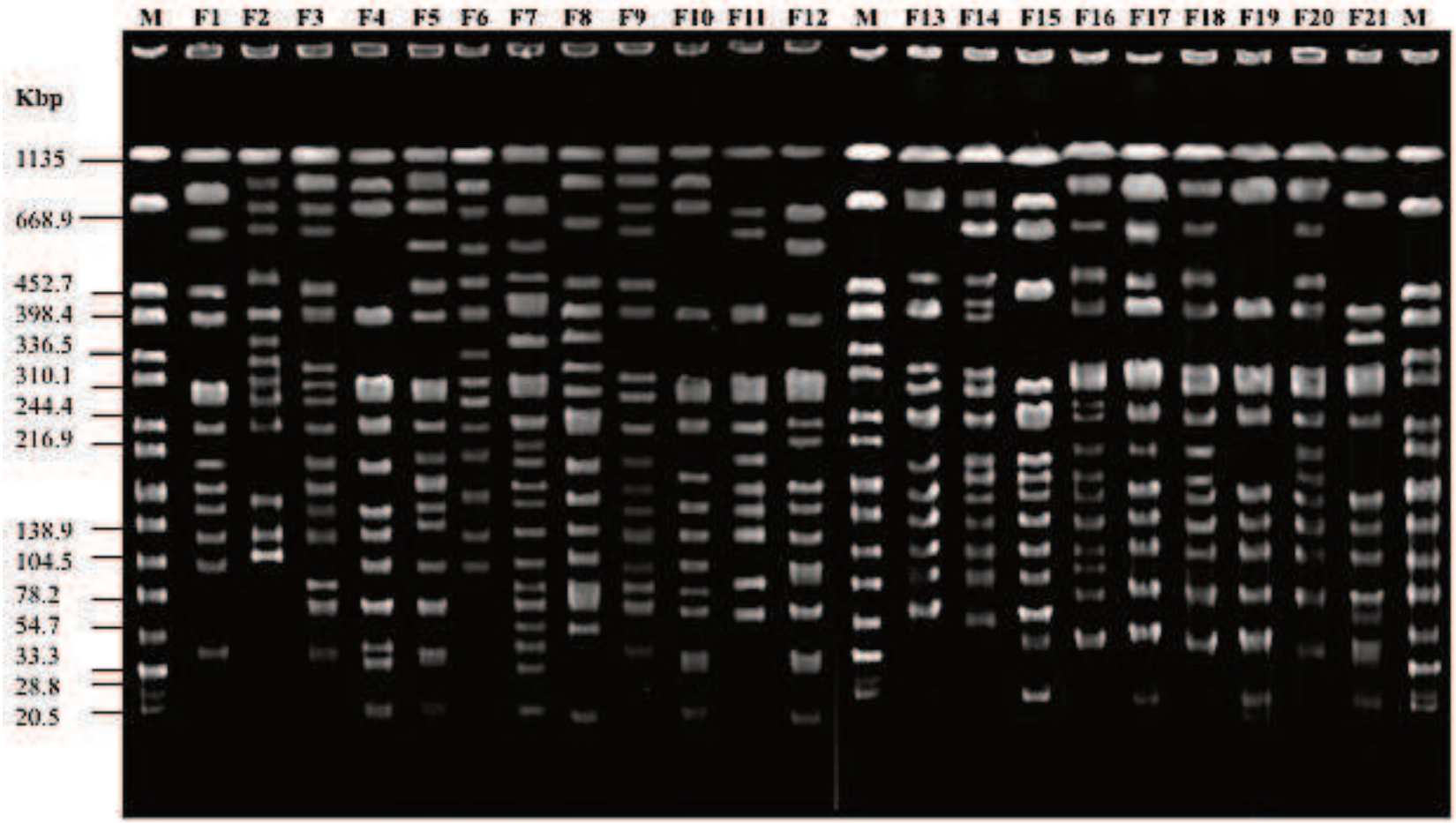
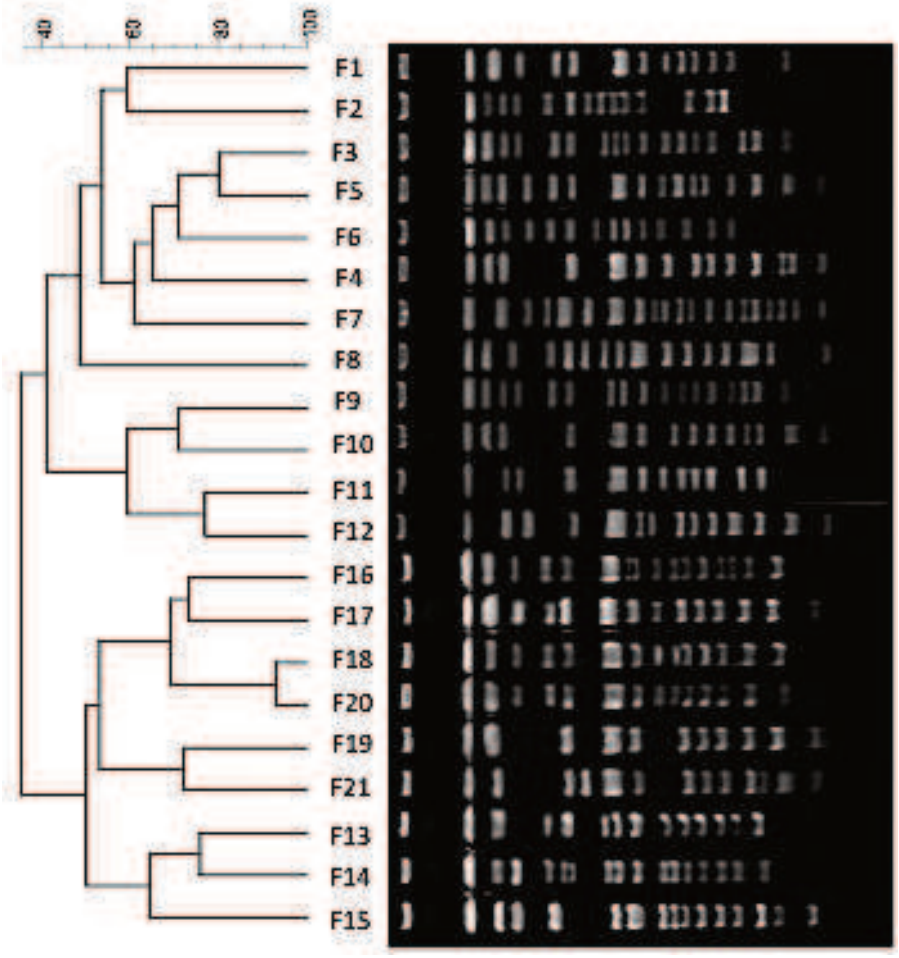


Fig. 3-1. PFGE patterns of *S.* Weltevreden strains isolated from wild geckos and humans

M: reference marker (*S.* Braenderup H9812) F1~10: Vietnam (Mekong Delta) F11: Cambodia F1,F12~14: Vietnam (Hue)
F15: Japan (Okinawa) F16~F20: Thailand F13, F21: human patient (Vietnam)



	Source	No. of isolates	Region	
F1	Gecko	12	Mekong Delta -Hue	Cluster A
F2	Gecko	1	Mekong Delta	
F3	Gecko	1	Mekong Delta	
F5	Gecko	4	Mekong Delta	
F6	Gecko	1	Mekong Delta	
F4	Gecko	1	Mekong Delta	
F7	Gecko	1	Mekong Delta	
F8	Gecko	1	Mekong Delta	
F9	Gecko	2	Mekong Delta	
F10	Gecko	1	Mekong Delta	
F11	Gecko	16	Cambodia	
F12	Gecko	1	Hue	
F16	Gecko	12	Thailand	Cluster B
F17	Gecko	1	Thailand	
F18	Gecko	1	Thailand	
F20	Gecko	1	Thailand	
F19	Gecko	1	Thailand	
F21	Human	1	Mekong Delta	
F13	Gecko - Human	3	Hue - Mekong Delta	
F14	Gecko	16	Hue	
F15	Gecko	2	Okinawa	

Fig. 3-2. The dendrogram showing the results of cluster analysis of 21 PFGE patterns of *S.* Weltevreden by UPGMA

Table 3-3. MLVA profiles of 21 *S.* Weltevreden isolates

MLVA pattern	No. of repeated alleles				No. of isolates (%)
	Sal16	STTR1	SENTR5	3414090	
M1	22	1	1	1	1 (4.8)
M2	14	1	1	1	1 (4.8)
M3	20	1	1	2	1 (4.8)
M4	22	2	1	1	3 (14.3)
M5	21	2	1	1	2 (9.5)
M6	15	2	1	2	1 (4.8)
M7	14	1	0	1	1 (4.8)
M8	16	2	1	1	2 (9.5)
M9	18	1	1	2	1 (4.8)
M10	23	1	2	1	1 (4.8)
M11	25	2	0	1	2 (9.5)
M12	27	1	0	1	1 (4.8)
M13	15	2	1	1	1 (4.8)
M14	20	1	1	1	1 (4.8)
M15	21	0	1	1	1 (4.8)
M16	20	2	1	1	1 (4.8)

Table 3-4. Prevalence of pathogenic genes in 21 *S.* Weltevreden isolates

Gene localization	Pathogenic genes	No. of positive isolates (%)
Chromosome		
Salmonella Pathogenic Island (SPI)		
SPI-1	*sopE1*	1 (4.8)
	hilD	21 (100.0)
SPI-2	*sseC*	9 (42.9)
	sifA	8 (38.1)
	ssrB	21 (100.0)
SPI-5	*pipA*	21 (100.0)
	sopB	21 (100.0)
Prophage	*gipA*	20 (95.2)
	sodC1	20 (95.2)
	gtgB	21 (100.0)
	sspH1	21 (100.0)
Fimbrial operon	*agfA*	17 (81.0)
Plasmid	*spvC*	0 (0.0)
	pefA	21 (100.0)

Table 3-5. Gene profiles of 21 *S.* Weltevreden isolates

Gene types	Gene profiles	No. of isolates (%)
P1	*gipA-sodC1-agfA*	7 (33.3)
P2	*gipA-sodC1-agfA-sifA*	2 (9.5)
P3	*gipA-agfA*	1 (4.8)
P4	*gipA-sodC1-agfA-sseC*	2 (9.5)
P5	*sodC1-sseC*	1 (4.8)
P6	*gipA-sodC1-agfA-sseC-sifA*	4 (19.0)
P7	*gipA-sodC1-agfA-sseC-sifA-sopE1*	1 (4.8)
P8	*gipA-sodC1-sifA*	1 (4.8)
P9	*gipA-sodC1*	1 (4.8)
P10	*gipA-sodC1-sseC*	1 (4.8)

Table 3-6. Typing results of 21 *S.* Weltevreden isolates originated from wild gecko and human in Southeast Asian countries and Japan

No.	Strain	Source	Region	PFGE pattern	MLVA pattern	Gene pattern
1	CRI4a	Wild gecko	Mekong Delta	F1	M1	P1
2	OM9b	Wild gecko	Mekong Delta	F2	M2	P1
3	OM14a	Wild gecko	Mekong Delta	F3	M3	P1
4	OM20b	Wild gecko	Mekong Delta	F4	M4	P2
5	OM29b	Wild gecko	Mekong Delta	F5	M5	P3
6	CMI24a	Wild gecko	Mekong Delta	F6	M6	P1
7	CMII43b	Wild gecko	Mekong Delta	F7	M7	P1
8	KGII30a	Wild gecko	Mekong Delta	F8	M8	P1
9	KGII31a	Wild gecko	Mekong Delta	F9	M9	P4
10	KGIII53a	Wild gecko	Mekong Delta	F10	M10	P1
11	C-4	Wild gecko	Cambodia	F11	M11	P4
12	H-62.1	Wild gecko	Hue	F12	M12	P5
13	H-137.1	Wild gecko	Hue	F13	M13	P6
14	H-244.3	Wild gecko	Hue	F14	M14	P6
15	Ishi-G5D	Wild gecko	Okinawa	F15	M8	P7
16	T-6k	Wild gecko	Thailand	F16	M4	P6
17	T-32k	Wild gecko	Thailand	F17	M4	P6
18	T-136k	Wild gecko	Thailand	F18	M5	P2
19	T-149k	Wild gecko	Thailand	F19	M15	P8
20	T-150k	Wild gecko	Thailand	F20	M16	P9
21	199A	Human patient	Mekong Delta	F21	M11	P10

3.4. DISCUSSION

In the PFGE method, *S.* Weltevreden isolates showed a high genetic diversity with a total of 21 PFGE patterns obtained. Although *S.* Weltevreden isolates showed similar PFGE patterns in the same region, these isolates originated from different regions exhibited different PFGE patterns in Southeast Asian countries. The results indicate that *S.* Weltevreden originated from wild geckos shows a high heterogenicity and the specific PFGE patterns in each region. Thong *et al.* (58, 59) also reported that *S.* Weltevreden isolated from human patients and the environment in Malaysia showed a high genetic diversity. In this study, PFGE patterns of *S.* Weltevreden isolated from wild gecko in Japan and Vietnam showed 60% similarity, it indicated a relatively close relationship of this serovar between these regions. The reason of this similarity should be clarified in further research.

Jimenez *et al.* (30) reported that *S.* Weltevreden isolated from the common house gecko (*Hemidactylus frenatus*) had the same PFGE pattern obtained from human salmonellosis patients, and geckos could play a role in the epidemiology of human salmonellosis in Costa Rica. In the present study, although the PFGE pattern of F13 was detected from both gecko

and human *S.* Weltevreden isolates, these isolates were obtained in different regions of Hue and the Mekong Delta, Vietnam. Further research should be done to clarify the relationship of *S.* Weltevreden originated from geckos and human salmonellosis patients at the same region in Southeast Asian countries.

Of 21 *S.* Weltevreden isolates, 16 MLVA patterns were obtained in Southeast Asian countries. MLVA showed a less discriminatory power in genotyping *S.* Weltevreden than PFGE in this study. Ngoi *et al.* (43) supposes that MLVA shows a moderate discriminatory power because this method depends on the specific loci and serovar-specific assay. However, Boxrud *et al.* (6) reported that MLVA showed higher discriminatory power than that of PFGE in differentiating *S.* Enteritidis isolates from human patients in USA. Cho *et al.* (14) also indicated that MLVA had a higher discriminatory power than PFGE in classifying *S.* Enteritidis isolates from human and non-human sources in USA. Davis *et al.* (16) also reported the same result in discriminating *S.* Newport isolates from humans and bovine. Until now, no reports have been published regarding about the specific loci for *S.* Weltevreden in MLVA assay. Another research should be done to find the specific loci in *S.* Weltevreden genome to increase the discriminatory power of MLVA.

PCR Binary Typing (P-BIT) seems to be a quick and simple method to clarify the genetic relationship of *Salmonella.* Of 21 *S.* Weltevreden isolates, 10 gene patterns were obtained by combining the presence of 6 pathogenic genes (*sopE1*, *sseC*, *sifA*, *gipA*, *sodC1*, *agfA*). The pattern P1 and P6 were predominant among *S.* Weltevreden isolates in Southeast Asian countries (Table 3-5). It reveals that those pathogenic genes might be dominated in this serovar in this region. Khoo *et al.* (32) identified that *S. enterica* subsp. *enterica* from vegetables and poultry meat in Malaysia was positive to *pipB* and *sopD* (100%), followed by *hilD* and *sopB* (95%), *pipA* (94%), and *sopE* (83%). Huehn *et al.* (28) found *S.* Enteritidis and *S.* Typhimurium isolates from humans and animals in Europe were positive to *sodC1* (100%), and *gipA* with 8.9% and 49.7% respectively. Craciunas *et al.* (15) reported that 100% of *S.* Enteritidis isolates in patients in Romania was positive to *agfA* and *spvC*. These results show that those pathogenic genes are widely found in many *Salmonella* serovars, including *S.* Weltevreden. Therefore, the specific pathogenic genes of *S.* Weltevreden should be clarified to increase the discriminatory power of P-BIT assay in genotyping this serovar.

In a comparison of the discriminatory power of PFGE, MLVA, and P-BIT, PFGE showed the highest discriminatory power among them. However, all these methods seem to be

useful tools for clarifying the genetic diversity and relationship of *S.* Weltevreden isolates because multiple genotypes of *S.* Weltevreden could be obtained by these methods. The great diversity of *S.* Weltevreden isolates revealed that this serovar might be prevalent in wild geckos in Southeast Asian countries since the ancient times.

3.5. SUMMARY

A total of 80 *S.* Weltevreden isolates from wild geckos and human patients in Southeast Asian countries and Japan were characterized by molecular methods to clarify their genetic diversity and relationship among these isolates. The PFGE assay by *Xba*I enzyme identified 21 different patterns from 80 *S.* Weltevreden isolates. Almost all *S.* Weltevreden isolates originated from the same region showed similar PFGE patterns. On the other hand, MLVA method created 16 MLVA types, and the P-BIT method yielded 10 binary profiles. The discriminatory power of PFGE was higher than that of MLVA and P-BIT method. These results indicate that *S.* Weltevreden has been prevalent since the ancient times in Southeast Asian countries because several genetic types of *S.* Weltevreden are prevalent in wild geckos in this region.

CHAPTER 4

Contamination of *Salmonella* in retail vegetables in the Mekong Delta, Vietnam

4.1. INTRODUCTION

The raw vegetables are considered as an important source of foodborne pathogens, including *Salmonella* (46, 48). Losio *et al.* (35) indicated that fresh leafy vegetables and "ready-to-eat" vegetables retailed in supermartkets or farm markets in Italy were contaminated with multiple pathogens including *Salmonella*, *Listeria*, *E. coli* O157:H7, thermotolerant *Campylobacter*, *Y. enterocolitica*, and norovirus. Human could be infected with these pathogens via eating raw these vegetables. Many *Salmonella* outbreaks associated with vegetables have also been reported in developed countries (46). As peoples in Southeast Asian countries have a habit to eat raw vegetables, they could be infected with *Salmonella* via consumption of *Salmonella*-contaminated vegetables. However, few reports have been published regarding to the role of vegetables as a source of human *Salmonella* infection in these countries. In the present study, the contamination with *Salmonella,* especially *S.* Weltevreden, in retail vegetables was examined to know the role of retail vegetable as the vehicle of *Salmonella* transmission in the Mekong Delta, Vietnam.

4.2. MATERIALS AND METHODS

4.2.1. Sample collection

From July 2017 to March 2018, a total of 358 retail vegetables were collected in the wet markets of Cantho city in the Mekong Delta, Vietnam. Among the total of 358 samples, 235 vegetable samples were collected in the rainy season (from April to November) and 123 samples were in the dry season (from December to March). Reatail vegetable were purchased in wet markets and brought to the laboratory in the cold condition. The contents of the retail vegetable samples was shown in Table 4-1.

4.2.2. Isolation and identification of *Salmonella* in retail vegetables

About 25 g of each vegetable sample was suspended in 225 m*l* of EEM broth (Eiken) for pre-enrichment. The pre-enrichment suspension was incubated at 37^{o}C for 24 h. After that, the procedure of *Salmonella* isolation and identification was followed the methods

described in chapter 1.2.2.

4.2.3. Antimicrobial susceptibility of *S.* Weltevreden isolates

S. Weltevreden isolates from retail vegetables were examined for the antimicrobial susceptibility. The method and antibiotic agents were used as described in chapter 1.2.3.

4.2.4. Data analysis

The data were tested by Chi-square (χ^2) and Fisher's exact test.

4.3. RESULTS

The isolation rate of *Salmonella* in retail vegetables was shown in Table 4-1. Of 358 vegetable samples, *Salmonella* was detected from 58 samples (16.2%). No significant difference in the prevalence of *Salmonella* among vegetable species was observed. However, a significant difference in the prevalence of *Salmonella* was observed between the rainy

(20.9%) and the dry season (9.1%) ($p<0.01$).

The distribution of *Salmonella* serovars from retail vegetables was shown in Table 4-2. Of 61 *Salmonella* isolates from retail vegetables, 9 *Salmonella* serovars were identified. Among them, the most predominant serovar was *S.* Weltevreden (29.5%), followed by *S.* Derby (8.2%), *S.* Lexington (3.3%), and *S.* Worthington (3.3%).

All 18 *S.* Weltevreden isolates originated from retail vegetables showed susceptibility against 9 antibiotics examined as the same in chapter 1.2.3 in this study.

Table 4-1. Contamination of *Salmonella* in retail vegetables in the Mekong Delta, Vietnam

Season	Vegetable species			No. of samples	No. of *Salmonella* positive samples (%)	*Salmonella* serovars (No. of strains)
		Common name	Nomenclature			
Rainy season	Jul-2017	Mustard green	*Brassica juncea*	31	7 (22.6)	*S.* Weltevreden (5); *S.* Lexington (1); *S.* London (1); *S.* Virchow (1); *S.* Bareilly (1); O3,10 (2); O7 (1); O8 (1)
		Water spinach	*Ipomoea aquatica*	29	6 (20.7)	
	Oct-2017	Long coriander	*Eryngium foetium*	30	5 (16.7)	*S.* Weltevreden (9); *S.* Worthington (2); *S.* Derby (3); O3,10 (1); O7 (1); O9 (1); Untyped (6)
		Rice paddy herb	*Limnophila aromatica*	28	8 (28.6)	
		Mint	*Mentha arvensis*	29	5 (17.2)	
		Sweet basil	*Ocimum basilicum*	28	5 (17.9)	
	Nov-2017	Green leaf lettuce	*Lactuca sativa*	20	3 (15.0)	*S.* Weltevreden (3); *S.* Typhimurium (1); *S.* Derby (1); *S.* Saintpaul (1); O4 (4); O7 (2); O9 (1); Untyped (2)
		Water dropwort	*Oenanthe javanica*	20	6 (30.0)	
		Watercress	*Nasturium officinale*	20	4 (20.0)	
	Subtotal			235	49 (20.9)[a)]	
Dry season	Jan-2018	Crisphead lettuce	*Lactuca sativa var. acephala*	31	3 (9.7)	*S.* Lexington (1); O3,10 (2); O4 (1); O9(1)
		Cutting lettuce	*Lactuca sativa var. capitata*	19	1 (5.3)	
	Mar-2018	Crisphead lettuce	*Lactuca sativa var. acephala*	29	2 (6.9)	*S.* Weltevreden (1); *S.* Derby (1); O3,10 (1); O7 (1); 08 (1)
		Cutting lettuce	*Lactuca sativa var. capitata*	44	3 (6.8)	
	Subtotal			123	9 (7.3)	
Total				358	58 (16.2)	

[a)] Rainy season > Dry season ($p < 0.01$)

Table 4-2. Serovars of *Salmonella* isolates from retail vegetables in the Mekong Delta, Vietnam

Serovar	No. of isolates	(%)
S. Weltevreden	18	(29.5)
S. Derby	5	(8.2)
S. Lexington	2	(3.3)
S. Worthington	2	(3.3)
S. Bareilly	1	(1.6)
S. London	1	(1.6)
S. Saintpaul	1	(1.6)
S. Typhimurium	1	(1.6)
S. Virchow	1	(1.6)
O3,10:UT[a)]	6	(9.8)
O4:UT	5	(8.2)
O7:UT	5	(8.2)
O8:UT	2	(3.3)
O9:UT	3	(4.9)
Untyped	8	(13.1)
Total	61	(100.0)

[a)]UT: Untyped

4.4. DISCUSSIONS

A few reports concerning about *Salmonella* contamination in vegetables have been published. Ni *et al.* (44) reported that 4.5% of lettuce sold in markets was positive for *Salmonella* in Shanghai, China. Quiroz-Santiago *et al.* (50) reported that *Salmonella* was isolated from 7% of lettuce and watercress in the supermarket in Mexico. In Malaysia, *Salmonella* was contaminated in 100% of water dropwort, 83.3% of Asiatic pennywort and 32% of water spinach in the wet markets (41, 52). Vital *et al.* (67) also reported that 22% of cabbage and 24% of lettuce sold at the open-air markets in the Philippines was contaminated with *Salmonella*. In the present study, retail vegetables in the wet markets in the Mekong Delta were contaminated with *Salmonella* at a relatively high rate (16.2%). Moreover, *Salmonella* was detected from all type of retail vegetables and all wet markets in this study. These results indicated retail vegetables sold in the wet markets were widely contaminated with *Salmonella* in the Mekong Delta, Vietnam.

Until now, the source of *Salmonella* contamination in retail vegetables in wet markets of the Mekong Delta has not been identified yet. Vegetables could be contaminated

with *Salmonella* from many sources including from wild animals living in the markets (29, 38, 52, 55). Especially, wild geckos are commonly living widely in the environment including wet markets in this region. Wild geckos living in Southeast Asian countries harbor *Salmonella*, especially *S.* Weltevreden, at a high rate as described in chapter 1. Moreover, the predominant serovar in retail vegetables was also *S.* Weltevreden as the same as in wild geckos in this study. These results indicate that wild gecko seems to be an important source of *Salmonella* contamination in retail vegetables in these wet markets. Furthermore, the isolation rate of *Salmonella* in retail vegetables in the rainy season was higher than that in the dry season in this study. The activity of gecko in this region becomes lower in the dry season than in the rainy season because of the low temperature and a decrease of insects as a feed of gecko in this season. Although the reason why this phenomenon occurred is not determined clearly, activities of gecko might affect the seasonal difference of *Salmonella* isolation rate. Further research should be done to clarify this phenomenon in this region.

S. Typhimurium or *S.* Enteritidis was the most common serovar isolated from vegetables in China and Mexico (44, 50). In contrast, *S.* Weltevreden was the predominant serovar (29.5%) in retail vegetables in the Mekong Delta. Salleh *et al.* (52) also reported

that *S.* Weltevreden was the predominant serovar in retail vegetables in Malaysia. Moreover, all *S.* Weltevreden isolates in vegetables in the Mekong Delta show susceptibility to 9 antibiotics examined as the same as *S.* Weltevreden isolates from wild geckos shown in chapter 1. These results support strongly that retail vegetables might be contaminated with *S.* Weltevreden from wild geckos in the Mekong Delta. Thong *et al.* (58) reported that the similar PFGE patterns of *S.* Weltevreden isolates were observed in human and vegetable isolates in Malaysia. As fresh raw vegetables such as lettuce are usually eaten in Southeast Asian countries, vegetables are considered as the important source of *Salmonella* infection to humans in the Mekong Delta. Further research should be carried out to clarify the genetic relationship of *S.* Weltevreden isolates from vegetables and geckos collected in the same wet market as well as from human salmonellosis patients in this region.

4.5. SUMMARY

From July 2017 to March 2018, a total of 358 retail vegetables were collected to clarify the contamination of *Salmonella* in the Mekong Delta, Vietnam. *Salmonella* was isolated from 58 (16.2%) of 358 samples. The isolation rate of *Salmonella* from retail vegetables in the rainy season was significantly higher than that in the dry season, 20.9% and 7.3% respectively. Among of *Salmonella* isolates, *Salmonella* Weltevreden was the most predominant serovar (29.5%) identified from retail vegetable in all of the wet markets. All *S.* Weltevreden isolates (100%) were susceptible to 9 antibiotics examined. Moreover, retail vegetables seem to be contaminated with *S.* Weltevreden from wild geckos. Thus, retail vegetables were considered as the important vehicle of *Salmonella* transmission to humans in the Mekong Delta.

GENERAL CONCLUSIONS

The main objective of this study is to clarify the epidemiological aspects of *Salmonella* Weltevreden originated from wild geckos in Southeast Asian countries. The main findings of this study are summarized as follows:

1. *Salmonella* was isolated from 293 samples (22.2%) of 1,318 wild geckos living in Southeast Asian countries. The prevalence of *Salmonella* in geckos was the highest in Thailand (46.0%), followed by Vietnam (16.3%), and Cambodia (17.3%). Among of *Salmonella* isolates, *S.* Weltevreden was the most predominant serovar (32.1%) isolated from wild geckos in these countries. All *S.* Weltevreden isolates (100%) were susceptible to 9 antibiotics examined. The results indicated that wild geckos might be an important natural reservoir for *S.* Weltevreden in Southeast Asian countries.

2. Wild geckos in the Mekong Delta, Vietnam were collected to clarify the viable number and survival period of *Salmonella* in their feces. Of 101 gecko examined, those *Salmonella* positive geckos excreted *Salmonella* in their feces from 1 to 8.6 log CFU/g. The mean number of *Salmonella* in feces was 4.5 ± 3.2 log CFU/g. *Salmonella* Weltevreden was

the most predominant serovar (37.5%) isolated from wild geckos. Moreover, *Salmonella* could survive for 6 weeks in gecko feces at the room temperature (25-30^{o}C) in Vietnam. These results suggested that wild gecko might play an important role as a reservoir for *Salmonella* and a source of human *Salmonella* infection in Southeast Asian countries.

3. PFGE, MLVA, and P-BIT assay were used to clarify the genetic diversity and relationship of *S.* Weltevreden isolates in Southeast Asian countries. The PFGE by *Xba*I enzyme identified 21 different patterns from 80 *S.* Weltevreden isolates. Almost all *S.* Weltevreden isolates originated from the same region showed similar PFGE patterns. On the other hand, MLVA method created 16 MLVA types, and the P-BIT method yielded 10 binary profiles. The discriminatory power of PFGE was higher than that of MLVA and P-BIT method. Several genetic types of *S.* Weltevreden were prevalent in wild geckos in Southeast Asian countries. It indicates that *S.* Weltevreden has been prevalent since the ancient times in this region.

4. Retail vegetables were collected in the wet markets to determine the contamination of *Salmonella* in the Mekong Delta, Vietnam. *Salmonella* was isolated from 58 (16.2%) of 358 vegetable samples. The isolation rate of *Salmonella* from retail

vegetables in the rainy season (20.9%) was higher than that in the dry season (7.3%). Among of *Salmonella* isolates, *S.* Weltevreden was the most predominant serovar (29.5%) identified from retail vegetable in all of the wet markets. All *S.* Weltevreden isolates (100%) were susceptible to 9 antibiotics examined. Moreover, vegetables seem to be contaminated with *S.* Weltevreden from wild geckos. Therefore, retail vegetables were considered as an important source of *Salmonella* infection to humans in the Mekong Delta.

5. These findings might be useful for understanding the epidemiology and ecology of *S.* Weltevreden in Southeast Asian countries, and developing preventive measures against human *Salmonella* infection.

ACKNOWLEDGMENTS

I would like to express my extreme gratitude to my supervisor, Associate Professor Dr. Hideki HAYASHIDANI, Department of Veterinary Medicine, Tokyo University of Agriculture and Technology, Japan for his instructions, stimulus, support and valuable advice and criticisms through the experiments and in preparation of this thesis.

I also would like to be grateful to Associate Professor Dr. Takahide TANIGUCHI, Tokyo University of Agriculture and Technology, Japan; Professor Dr. Hiroshi ASAKURA, National Institute of Health Science, Japan; Professor Dr. Haruko OGAWA, Ohibiro University of Agriculture and Veterinary Medicine, Japan; Professor Dr. Jun TERAJIMA, Iwate University, Japan; Professor Dr. Hiroshi FUJIKAWA, Department of Veterinary medicine, Tokyo University of Agriculture and Technology, Japan; Professor Dr. Tetsuo ASAI, The United Graduate School of Veterinary Sciences, Gifu University, Japan for their valuable suggestions and critical reading of this manuscript.

I wish to express my sincere gratitude to Associate Professor Dr. LY Thi Lien Khai, Department of Veterinary Medicine, College of Agriculture and Applied Biology, Can Tho

University, Vietnam for her help, support and encouragement.

I also thank all of the students in Animal Health Laboratory, Department of Veterinary Medicine, Tokyo University of Agriculture and Technology, Japan and Food Hygiene Laboratory, Department of Veterinary Medicine, College of Agriculture and Applied Biology, Can Tho University, Vietnam for their help, kindness, and their friendship.

I would like to sincerely thank Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan for supporting me the Monbukagakusho Scholarship to study Doctor course in The United Graduate School of Veterinary Sciences, Gifu University and Tokyo University of Agriculture and Technology, Japan.

Finally, I dedicate this thesis to my parents and family who always support and wish the best things for me.

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ABSTRACT

Salmonella is recognized worldwide as an important foodborne and zoonotic human pathogen. *Salmonella* has a wide prevalence in mammals, reptiles, birds, and the environment. In Southeast Asian countries, *S.* Weltevreden is known to be the predominant serovar of human salmonellosis such as Vietnam, Thailand, and Malaysia. However, the natural reservoir of *S.* Weltevreden has not been identified yet. Reptile is known to be one of natural reservoirs for *Salmonella*. Wild geckos distribute widely in the human residential areas and contact closely with the humans in these countries. A few reports have been published regarding the prevalence of *Salmonella* in wild gecko in Southeast Asian countries. Therefore, the main objectives in this study are to identify the epidemiology of *Salmonella* Weltevreden in wild geckos in Southeast Asian countries.

[Chapter 1] Prevalence of *Salmonella* Weltevreden in wild gecko living in Southeast Asian countries

From 2012 to 2015, a total of 1,318 intestinal contents of wild geckos were

collected in Cambodia (n = 98), Thailand (n = 261), and Vietnam (Hue and the Mekong Delta) (n = 959) to determine the prevalence of *Salmonella* in wild gecko. These geckos belonged to three species: common house gecko (*Hemidactylus frenatus*) (n = 794), flat-tailed house gecko (*Hemidactylus platyurus*) (n = 464), and four-clawed gecko (*Gehyra mutilata*) (n = 60). *Salmonella* was isolated from 293 (22.2%) of 1,318 gecko samples. The prevalence of *Salmonella* in geckos was 16.3% in Vietnam, 17.3% in Cambodia, and 46.0% in Thailand. However, there was no significant difference in the prevalence of *Salmonella* among these gecko species. Of 293 *Salmonella* isolates, *S.* Weltevreden was the most predominant serovar (32.1%) isolated from wild geckos in all of those countries. All *S.* Weltevreden isolates (100%) were susceptible to 9 antibiotics examined. These results indicate that wild geckos seem to be a natural reservoir for *S.* Weltevreden in Southeast Asian countries.

[Chapter 2] Quantification and survival period of *Salmonella* in gecko feces

Wild geckos harbor *Salmonella* in their intestine and excrete their feces everywhere. Gecko feces could become the infectious source of *Salmonella* to humans. However, the

number and survival period of *Salmonella* in gecko feces have been still unknown. A total of 201 wild geckos in the Mekong Delta, Vietnam were collected to clarify the viable number and survival period of *Salmonella* in their feces. Of 101 samples examined, 24 (23.8%) were positive for *Salmonella*. Of 24 positive samples, the number of *Salmonella* was excreted in feces from 1 to 8.6 log CFU/g. The mean number of *Salmonella* in feces was 4.5 ± 3.2 log CFU/g. Among of *Salmonella* serovars, *S.* Weltevreden was the most predominant serovar (37.5%). Moreover, *Salmonella* could survive for 6 weeks in gecko feces at the room temperature (25-30^{o}C) in Vietnam. These results indicate that gecko excretes the high number of *Salmonella* in their feces and *Salmonella* can survive in the feces for long time in the environment. Therefore, wild gecko seems to be an important reservoir for *Salmonella* and a source of human *Salmonella* infection in Southeast Asian countries.

[Chapter 3] Genetic diversity of *Salmonella enterica* serovar Weltevreden isolated from wild gecko in Southeast Asian countries

S. Weltevreden has been known as the predominant serovar in Southeast Asian countries. However, a little information has been reported regarding to the genetic diversity

of this serovar originated in those countries. A total of 80 *S.* Weltevreden isolates from wild geckos and human patients in Southeast Asian countries and Japan were characterized by molecular methods to clarify their genetic diversity and relationship among these isolates. The PFGE (Pulse-filed Gel Electrophoresis) by *Xba*I enzyme identified 21 different patterns from 80 *S.* Weltevreden isolates. Almost all *S.* Weltevreden isolates originated from the same region showed similar PFGE patterns. On the other hand, MLVA method (Multiple Locus Variable-Number Tandem Repeat Analysis) created 16 MLVA types, and the P-BIT method (PCR - Binary Typing) yielded 10 binary profiles. The discriminatory power of PFGE was higher than that of MLVA and P-BIT method. These results indicate that *S.* Weltevreden has been prevalent since the ancient times in Southeast Asian countries because several genetic types of *S.* Weltevreden are prevalent in wild gecko in this region.

[Chapter 4] Contamination of *Salmonella* in retail vegetables in the Mekong Delta, Vietnam

From July 2017 to March 2018, a total of 358 retail vegetables were collected in wet markets to know the contamination with *Salmonella* in the Mekong Delta, Vietnam. *Salmonella* was isolated from 58 (16.2%) of 358 vegetable samples. The isolation rate of

Salmonella from retail vegetables in the rainy season (20.9%) was higher than that in the dry season (7.3%). Among *Salmonella* isolates, *S.* Weltevreden (29.5%) was the most predominant serovar identified from retail vegetable in all of the wet markets. All *S.* Weltevreden isolates (100%) were susceptible to 9 antibiotics examined. These results indicate that retail vegetables in wet markets are considered as an important vehicle of *Salmonella* transmission to humans in the Mekong Delta.

In the present study, wild geckos were considered as the natural reservoir for *S.* Weltevreden and a source of human *Salmonella* infection in Southeast Asian countries. Moreover, *S.* Weltevreden originated from wild geckos showed a high genetic diversity. It indicates that *S.* Weltevreden has existed since the ancient times in these countries. Furthermore, retail vegetables seem to be one of the important vehicles of *Salmonella* transmission, especially *S.* Weltevreden to humans in these countries. These findings might be useful for understanding the epidemiology and ecology of *S.* Weltevreden in Southeast Asian countries, and developing preventive measures against human *Salmonella* infection.

東南アジアのヤモリが保有する *Salmonella* Weltevreden に関する疫学的研究

Epidemiological Studies on *Salmonella* Weltevreden of Wild Gecko in Southeast Asian countries

Nguyen Khanh Thuan

Salmonella は，重要な食中毒または人獣共通感染症の原因菌として知られている。*Salmonella* は哺乳類，爬虫類，鳥及び環境に広く分布する。ベトナム，タイ，マレーシアなどの東南アジア諸国において，*S.*Weltevreden は，人のサルモネラ症において分離頻度の高い血清型として知られている。しかし，その自然界におけるレゼルボアは明らかになっていない。爬虫類は，*Salmonella* の自然界におけるレゼルボアの一つとして知られている。ヤモリは東南アジアにおいて広く人の環境に分布し，人との接触が多い。ヤモリにおける *Salmonella* の保有状況に関する報告は少ない。そこで，本研究では，東南アジアのヤモリにおける *S.*Weltevreden の疫学を明らかにするために調査を実施した。

[第 1 章]東南アジアの野生ヤモリにおける *S.*Weltevreden の分布

2012-2015 年に，カンボジア（98 匹），タイ（261 匹），ベトナム（959 匹）において計 1,318 匹の野生ヤモリを捕獲し，直腸内容物を採取し，*Salmonella* の保有状況を調べた。捕獲したヤモリは，ホオグロヤモリ（*Hemidactylus frenatus*）794 匹，ヒラオヤモリ（*Hemidactylus platyurus*） 464 匹およびオンナダケヤモリ（*Gehyra mutilata*）60 匹である。*Salmonella* は，ベトナムでは 16.3% ，カンボジアでは 17.3% ，タイでは 46.0% のヤモリから分離された。ヤモリの種類により，分離率に差は認められなかった。分離された *Salmonella* 293 株のうち，*S.* Weltevreden は，最も分離率が高く(32.1%)，いずれの国においても同様の傾向であった。分離されたすべての *S.* Weltevreden 菌株は，供試した 9 種類の抗生物質すべてに感受性を示した。これらの結果から，東南アジアにおいて，野生のヤモリは *S.* Weltevreden の自然界におけるレゼルボアになっているものと思われた。

[第 2 章] ヤモリの糞便中の *Salmonella* の排菌量と生存期間

野生ヤモリは, *Salmonella*を腸管内に保菌し, 糞便とともに環境中に排出している。従って，ヤモリは，人のサルモネラ症の感染源となり得る。しかし，ヤモリの糞便中

の*Salmonella* の排菌量と環境中の糞便における生存期間は明らかになっていない。そこで, ベトナム・メコンデルタにおいてヤモリ201匹を捕獲し, 糞便中の*Salmonella*菌数を定量するとともに, 糞便中における*Salmonella*の生存性を調べた。調べた糞便101検体中, 24検体(23.8%)から*Salmonella*が分離された。*Salmonella*が分離された24検体からの糞便中の*Salmonella*菌数は, 1 ~ 8.6 log CFU/gで, 平均の排菌数は4.5 ± 3.2 log CFU/gであった。分離された*Salmonella* の血清型では, *S.* Weltevreden (37.5%)が最も分離頻度が高かった。また, 糞便中において, *Salmonella*は, ベトナムにおける室温 (25-30°C)の環境下で, 6週間生存した。 これらの結果から, ヤモリは高い菌量の*Salmonella*を糞便中に排菌し, また, 環境に排出された糞便中で, *Salmonella* は長期間にわたって生存することが明らかになった。従って, 野生ヤモリは, 東南アジアにおいては, ヤモリは *Salmonella* の自然界における重要なレゼルボアのひとつであり, 人への感染源になっている可能性が高いものと思われた。

[第 3 章] 東南アジアのヤモリから分離された *Salmonella* Weltevreden の遺伝的多様性

S. Weltevreden は東南アジアにおいて, 分離頻度の高い血清型として知られている。しかし, これらの国に分布する本血清型菌の遺伝的多様性に関する報告はほとんどみられない。本研究では, 東南アジアと日本においてヤモリと人から分離された *S.* Weltevreden 80 株について, 遺伝的多様性を調べるとともに, 菌株間の遺伝的関連性を解析した。*Xba*I による PFGE (Pulse-filed Gel Electrophoresis)により, *S.* Weltevreden 80 株から 20 の PFGE パターンが得られた。ほとんどの供試菌株は, 地域特異的な PFGE パターンを示した。 一方, MLVA (Multiple-Locus variable number tandem repeat analysis)解析では 16 の MLVA タイプが, P-BIT (PCR - binary typing) 解析では 10 の binary profiles が得られた。PFGE の菌株識別能は, MLVA と P-BIT 解析に比べて高かった。以上のように, 東南アジアのヤモリから分離された *S.* Weltevreden は遺伝的多様性を示し, 地域特異的であったことから, 本血清型菌は古くから本地域に分布していたものと思われた。

[第 4 章] ベトナム・メコンデルタの市販野菜における *Salmonella* の汚染状況

2017 年 7 月から 2018 年 3 月に, ベトナム・メコンデルタの市場で販売されていた野菜 358 検体における *Salmonella* の汚染状況を調査した。*Salmonella* は調査した野菜 358 検体中 58 検体(16.2%) から分離された。分離率は, 雨季 (20.9%) は乾季

(7.3%)よりも有意に高かった。調査したいずれの市場からも *Salmonella* は分離され，*S.* Weltevreden (29.5%)の分離頻度が最も高かった。分離されたすべての *S.* Weltevreden は 9 種類の抗生物質すべてに感受性を示した。これらの結果から，ベトナム・メコンデルタでは市場で販売されている野菜は，*Salmonella* の重要な媒介物になっていると思われた。

本研究から，東南アジアに生息する野生ヤモリは，*S.* Weltevredenden の自然界における重要なレセルボアのひとつであり，人のサルモネラ症の感染源になっていることが明らかになった。また，本地域のヤモリから分離された *S.* Weltevreden は遺伝的に多様性を示し，古くから本地域に分布しているものと思われた。さらに，野菜は *Salmonella*，特に *S.* Weltevreden の人への重要な媒介者の一つになっていることが推測された。得られた成績は，東南アジアにおける *S.* Weltevreden の疫学を理解するうえで重要な知見を提供し，本地域における *Salmonella* 感染症の予防対策の確立に資するものと考える。