The genus *Bartonella* is classified in the class *Alphaproteobacteria*, order *Rhizobiales* and family *Bartonellaceae* and, at the time of writing, comprised 19 recognized species and three subspecies. The bacteria in the genus *Bartonella* are small, fastidious, slow-growing Gram-negative rods (Brenner *et al.*, 1993; Birtles *et al.*, 1995). Species of the genus *Bartonella* are known to infect erythrocytes and endothelial cells of various mammals, such as humans, cats, dogs, ruminants, wild rabbits and wild rodents (Dehio, 2005). We previously described the prevalence of species of the genus *Bartonella* among 685 wild rodents in Japan (Inoue *et al.*, 2008). Based on phylogenetic trees constructed with the *rpoB* and *gltA* gene sequences and the criteria for the definition of a species of the genus *Bartonella* (La Scola *et al.*, 2003), two strains were identified as possible novel species of this genus. Strains Fuji 18-1^T and Fuji 23-1^T were isolated from *Apodemus argenteus* and *Apodemus speciosus* mice captured in the Mount Fuji forest in Japan. In the present study, these strains were characterized by biochemical, morphological and genetic approaches, including multilocus sequencing analysis of six housekeeping genes and the 16S–23S rRNA intergenic spacer region (ITS).

Strains Fuji 18-1^T and Fuji 23-1^T were grown on heart infusion agar plates (HIA; Difco) containing 5 % (w/v) defibrinated rabbit blood at 35 °C with 5 % CO_2 for 14 days. Gram staining was assessed by light microscopy (Olympus) at × 1000 magnification. Cell morphology was observed by transmission electron microscopy (model JEM1200EX; JEOL) at 100 kV with negative staining.

Biochemical characteristics were assessed by using a MicroScan Rapid Anaerobe Panel (Dade Behring Inc.) according to the manufacturer's instructions as described previously (Welch *et al.*, 1993). Cytochrome oxidase test strips (Nissui) were used for evaluating the oxidase activity of the strains. Catalase activity was examined by mixing fresh colonies which had been cultured for 14 days at 35 °C on 5 % rabbit blood chocolate HIA plates with 3 % H_2O_2 on a glass slide.

DNA G+C content was determined by HPLC (Mesbah & Whitman, 1989). Mean values of the G+C content (±SD) were calculated based on assays conducted in triplicate.

Genomic DNA was extracted using the Instagene Matrix (Bio-Rad) according to the manufacturer's instructions. Six housekeeping genes, 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB*, and ITS fragments were amplified by PCR as previously described (Birtles & Raoult, 1996; Heller *et al.*, 1997; Renesto *et al.*, 2001; Houpikian & Raoult, 2001; Zeaiter *et al.*, 2002a, b; Inoue *et al.*, 2009). The PCR products were purified using a Spin Column PCR Product Purification kit (Bio Basic). Direct DNA sequencing of the purified PCR products was performed using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) on a Genetic Analyzer (model 3130; Applied Biosystems). For the phylogenetic analysis, sequence data were aligned with those of type strains of other species of the genus *Bartonella* (Table 1) that were available in GenBank by using CLUSTAL W software (Thompson *et al.*, 1994) in the MEGA4 program (Tamura *et al.*, 2007). Phylogenetic trees based on six housekeeping genes and the ITS region were constructed using the neighbour-joining (NJ) method (Saitou & Nei, 1987). The nucleotide substitution rates were calculated by Kimura's two-parameter distance model (Kimura, 1980). Bootstrap analysis was carried out on 1000 replications of the dataset (Felsenstein, 1985). *Brucella melitensis* $16M^T$ was chosen as the outgroup. The phylogenetic trees of the concatenated sequence data for the 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* genes and the ITS region were constructed using the NJ and maximum-parsimony (MP) methods (Fitch, 1971) with the Jukes–Cantor parameter model (Jukes & Cantor, 1969) by using the MEGA4 program.

Strains Fuji 18-1^T and Fuji 23-1^T grown on HIA formed smooth, transparent to grey-whitish colonies of 1–2 mm in diameter. Gram-negative coccobacilli to short rod-shaped cells were observed by light microscopy after 14 days culture. The cell morphologies of both strains were similar and no flagella or pili were observed by electron microscopy (Fig. 1). Cell sizes were 0.74 μm in length and 0.36 μm in width for strain Fuji 18-1^T and 1.16 μm in length and 0.43 μm in width for strain Fuji 23-1^T.

Both strains were oxidase- and catalase-negative and neither exhibited urease activity nor hydrolysed trehalose. They both hydrolysed bis-*p*-nitrophenyl phosphate, but not *p*-nitrophenyl *N*-acetyl *β*-D-glucosaminide. Both strains had amino acid arylamidase activity towards leucine, methionine, lysine (alkaline as well as acidic), glycine, arginine and tryptophan. Strain Fuji 23-1^T exhibited L-proline-*β*-naphthylamide activity but strain Fuji 18-1^T did not. Both strains had glycylglycylarylamidase activity, but not pyrrolidonyl arylamidase activity. These biochemical results are typical for members of the genus *Bartonella* (Bermond *et al.*, 2000, 2002; Maillard *et al.*, 2004); however, the profiles cannot be applied routinely and reliably for the differentiation of species of the genus *Bartonella* because of the relatively inert nature of bartonellae (Dehio *et al.*, 2001; Bermond *et al.*, 2002).

The DNA G+C contents of strains Fuji 18-1^T and Fuji 23-1^T were 40.1±0.6 mol% (mean±SD) and 40.4±0.5 mol% (mean±SD), respectively. These values were similar to those of other species of the genus *Bartonella* (Bermond *et al.*, 2000, 2002).

DNA fragments of all seven loci examined were sequenced and the sequence data were compared with the type strains of other species of the genus *Bartonella*. The 16S rRNA gene sequence similarities of strains Fuji 18-1^T and Fuji 23-1^T to other *Bartonella* species ranged from 96.5 % (*Bartonella bacilliformis*) to 98.8 % (*Bartonella grahamii*) and 96.7 % (*Bartonella bacilliformis*) to 98.8 % (*Bartonella koehlerae*), respectively (see Supplementary Table S1 in IJSEM Online).

Table 1. GenBank accession numbers of the seven loci for the *Bartonella* species used in this study

Species	GenBank accession numbers						
	16S rRNA	*ftsZ*	*gltA*	*groEL*	*ribC*	*rpoB*	ITS
Bartonella japonica sp. nov. Fuji 18-1^{T} (=JCM 15567^{T}=CIP 109861^{T})	AB440632	AB440633	AB242289	AB440634	AB242288	AB242288	AB498007
Bartonella silvatica sp. nov. Fuji 23-1^{T} (=JCM 15566^{T}=CIP 109862^{T})	AB440636	AB440637	AB242287	AB440638	AB440639	AB242292	AB498008
B. alsatica IBS 382^{T} (=CIP 105477^{T})	AJ002139	AF467763	AF204273	AF299357	AY116630	AF165987	AF312506
B. bacilliformis KC583^{T} (=ATCC 35685^{T})	Z11683	AB292602	AB292601	Z11683	AJ236918	AF165988	CP000524
B. birtlesii IBS 325^{T} (=CIP 106294^{T})	AF204274	AF467762	AF204272	AF355773	AY116632	AB196425	AY116640
B. bovis 91-4^{T} (=CIP 106692^{T})	AF293391	AF467761	AF293394	AF071194	AY116637	AY166581	AY116638
B. capreoli IBS 193^{T} (=CIP 106691^{T})	AF293389	AB290192	AF293392	AB290190	AB290194	AB290188	AB498009
B. chomelii A828^{T} (=CIP 107869^{T})	AY254309	AB290193	AY254308	AB290191	AB290195	AB290189	AB498010
B. clarridgeiae Houston-2 cat^{T} (=ATCC 51734^{T})	AB292603	AF141018	U84386	AF014831	AB292604	AF165990	AF312497
B. doshiae R18^{T} (=NCTC 12862^{T})	Z31351	AF467754	Z70017	AF014832	AY116627	AF165991	AJ269786
B. elizabethae F9251^{T} (=ATCC 49927^{T})	L01260	AF467760	Z70009	AF014834	AY116633	AF165992	L35103
B. grahamii V2^{T} (=NCTC 12860^{T})	Z31349	AF467753	Z70016	AF014833	AY166583	AF165993	AJ269785
B. henselae Houston-1^{T} (=ATCC 49882^{T})	BX897699	AF061746	BX897699	AF014829	AJ132928	AF171070	L35101
B. koehlerae C-29^{T} (=ATCC 700693^{T})	AF076237	AF467755	AF176091	AY116641	AY116634	AY166580	AF312490
B. quintana Fuller^{T} (=ATCC VR-358^{T})	M11927	AB292605	Z70014	AB290196	AJ236917	AF165994	L35100
B. schoenbuchensis R1^{T} (=NCTC 13165^{T})	AJ278187	AF467765	AJ278183	AY116642	AY116628	AY167409	AY116639
B. taylorii M6^{T} (=CIP 107028^{T})	Z31350	AF467756	Z70013	AF304017	AY116635	AF165995	AJ269788
B. tribocorum IBS 506^{T} (=CIP 105476^{T})	AJ003070	AF467759	AJ005494	AF304018	AB292600	AF165996	AF312505
B. vinsonii subsp. *arupensis* OK 94-513^{T} (=ATCC 700727^{T})	AF214558	AF467758	AF214557	AF304016	AY116631	AY166582	AF312504
B. vinsonii subsp. *berkhoffii* 93-CO1^{T} (=ATCC 51672^{T})	L35052	AF467764	U28075	AF014836	AY116629	AF165989	AF167988
B. vinsonii subsp. *vinsonii* Baker^{T} (=ATCC VR-152^{T})	M73230	AF467757	Z70015	AF014835	AY116636	AF165997	L35102

La Scola *et al.* (2003) reported that *rpoB* and *gltA* were the most appropriate genes for discriminating species of the genus *Bartonella* and proposed that gene sequence similarities <95.4 % in *rpoB* and <96.0 % in *gltA* between recognized species of the genus *Bartonella* could be used as cut-off values for the designation of novel *Bartonella* species. The highest sequence similarities between strains Fuji 18-1^{T}, Fuji 23-1^{T} and other species of the genus *Bartonella* showed values considerably lower than 91.4 % (strain Fuji 18-1^{T} compared with *Bartonella alsatica*) and 89.9 % (strain Fuji 23-1^{T}/*B. alsatica*) for the *rpoB* gene and 89.1 % (strain Fuji 18-1^{T}/*Bartonella vinsonii* subsp. *arupensis*) and 90.4 % (strain Fuji 23-1^{T}/*Bartonella taylorii*) for the *gltA* gene. Thus, strains Fuji 18-1^{T} and Fuji 23-1^{T} fulfil the requirements for classification as novel species of the genus *Bartonella*.

Strains Fuji 18-1^{T} and Fuji 23-1^{T} also showed considerably lower gene sequence similarities for the remaining four loci, *ftsZ*, *groEL*, *ribC* and ITS, when compared with other recognized species of the genus *Bartonella* (Supplementary Table S1). In the phylogenetic tree based on the merged set of concatenated sequences of seven loci, strains Fuji 18-1^{T} and Fuji 23-1^{T} formed a distinct clade with other species of the genus *Bartonella* (Fig. 2). The MP tree based on the concatenated sequence of the seven loci and the NJ trees based on sequence analyses of the seven loci also revealed that strains Fuji 18-1^{T} and Fuji 23-1^{T} were clearly separated from all other species of the genus *Bartonella* (see Supplementary Figs S1 and S2 in IJSEM Online).

In conclusion, sequence similarities of the *rpoB* and *gltA* genes and the phylogenetic analyses of seven different loci support the classification of strains Fuji 18-1^{T} and Fuji 23-1^{T} as novel species of the genus *Bartonella*, for which we propose the names *Bartonella japonica* sp. nov. and *Bartonella silvatica* sp. nov., respectively.

Description of *Bartonella japonica* sp. nov.

Bartonella japonica [ja.po'ni.ca. N.L. fem. adj. *japonica* of Japan, where the host rodent, the small Japanese field mouse (*Apodemus argenteus*), from which the strain was isolated, is widely distributed].

After 14 days incubation on HIA at 35 °C in a moist atmosphere under 5 % CO_2, colonies appear small (1–2 mm in diameter), round, grey-whitish and smooth. Cells are small bacilli without flagella or pili and are 0.74×0.36 µm. Oxidase- and catalase-negative, does not exhibit urease activity or hydrolyse trehalose. Hydrolyses bis-*p*-nitrophenyl phosphate but not *p*-nitrophenyl *N*-acetyl *β*-D-glucosaminide. Exhibits arylamidase activity

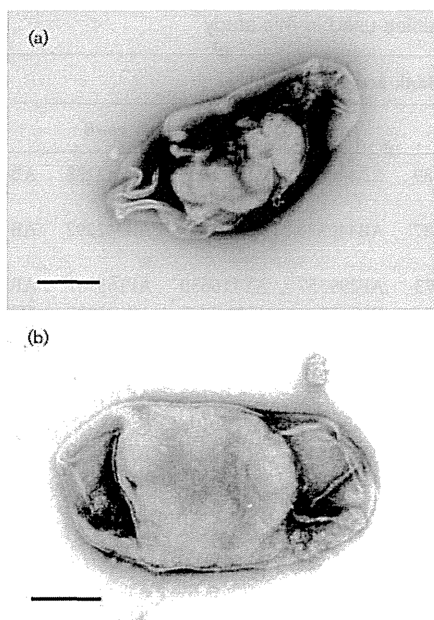

Fig. 1. Transmission electron micrograph of cells of (a) *Bartonella japonica* sp. nov. strain Fuji 18-1^T and (b) *Bartonella silvatica* sp. nov. strain Fuji 23-1^T. Bars, 200 nm.

towards leucine, methionine, lysine (alkaline as well as acidic), glycine, arginine and tryptophan, but not to proline. Glycylglycylarylamidase activity is present, but no pyrrolidonyl arylamidase activity. Can be distinguished from other species of the genus *Bartonella* by the 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* gene and ITS region sequences.

The type strain, Fuji 18-1^T (=JCM 15567^T=CIP 109861^T), was isolated from the blood of *Apodemus argenteus* mice. The DNA G+C content of the type strain is 40.1 mol%.

Description of *Bartonella silvatica* sp. nov.

Bartonella silvatica [sil.va'ti.ca. L. fem. adj. *silvatica* of the forest where the host rodent, the large Japanese field mouse (*Apodemus speciosus*), from which the strain was isolated, was captured].

After 14 days incubation on HIA at 35 °C in a moist atmosphere under 5 % CO_2, colonies appear small (1–2 mm in diameter), round, grey-whitish and smooth. Cells are small bacilli without flagella or pili and are 1.16 × 0.43 μm. Oxidase- and catalase-negative. Does not exhibit urease activity or hydrolyse trehalose. Hydrolyses bis-*p*-nitrophenyl phosphate but not *p*-nitrophenyl *N*-acetyl *β*-D-glucosaminide. Exhibits arylamidase activity towards leucine, methionine, lysine (alkaline as well as acidic), glycine, proline, arginine and tryptophan. Glycylglycylarylamidase activity, but no pyrrolidonyl arylamidase activity. Can be distinguished from other species of the genus *Bartonella* by the 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* gene and ITS region sequences.

The type strain, Fuji 23-1^T (=JCM 15566^T=CIP 109862^T), was isolated from the blood of *Apodemus speciosus* mice. The DNA G+C content of the type strain is 40.4 mol%.

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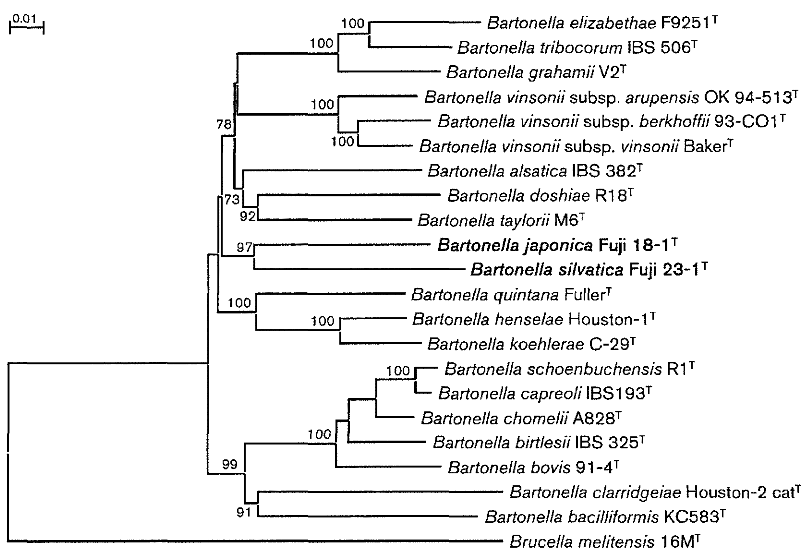

Fig. 2. Phylogenetic relationship of species of the genus *Bartonella* inferred from concatenated sequences of 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* genes and the ITS region. The phylogenetic tree was constructed by using the NJ method with the Jukes–Cantor parameter model. The tree was rooted by the use of *Brucella melitensis* 16M^T as an outgroup. Bootstrap values (percentages of 1000 replications) above 70 % are indicated at the nodes. Bar, 0.01 estimated nucleotide substitutions per site.

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ORIGINAL ARTICLES

Detection of *Bartonella tamiae* DNA in Ectoparasites from Rodents in Thailand and Their Sequence Similarity with Bacterial Cultures from Thai Patients

Hidenori Kabeya,[1,2] James M. Colborn,[1] Ying Bai,[1] Kriangkrai Lerdthusnee,[3]
Jason H. Richardson,[3] Soichi Maruyama,[2] and Michael Y. Kosoy[1]

Abstract

Ectoparasites, including chigger mites (genera *Leptotrombidium*, *Schoengastia*, and *Blankarrtia*) and one tick (genus *Haemaphysalis*) collected from wild-caught rodents in Thailand, were assessed for the presence of *Bartonella* DNA by using a polymerase chain reaction assay targeting the 16S–23S intergenic spacer region and citrate synthase gene (*gltA*). Of the 41 pooled samples tested, 34 were positive for *Bartonella* DNA. Sequence analysis demonstrated that DNA detected in 33 chigger mite pools and one tick pool was similar to *Bartonella tamiae* sequences previously isolated from three patients in Thailand. This is the first report of the detection of *B. tamiae* DNA in chigger mites; additional field and experimental investigations are required to determine the role of chigger mites as potential vectors of *B. tamiae*.

Key Words: *Bartonella tamiae*—Chigger mite—Ectoparasites—Rodent—Thailand—Tick.

Introduction

ORGANISMS OF THE GENUS *Bartonella* are aerobic, fastidious, gram-negative, and slow-growing bacteria, many of which have been isolated from the blood of mammals. This genus currently consists of at least 20 named species and 3 subspecies (Chomel et al. 2006); of these, at least 13 species have been reported to be potential causative agents for a wide spectrum of human diseases (Chomel et al. 2009). A variety of *Bartonella* species have been isolated from wild rodents, four of which were suspected to have caused clinical manifestations in humans (Daly et al. 1993, Kerkhoff et al. 1999, Welch et al. 1999, Kosoy et al. 2003, Fenollar et al. 2005). Previous reports indicating a high prevalence of *Bartonella* infection in wild rodents makes these bacteria a potential public health concern (Inoue et al. 2008).

Bloodsucking arthropods have been shown to be involved in the transmission of *Bartonella* species among their hosts (Billeter et al. 2008a). Sandflies (*Lutzomyia* spp.), the human body louse (*Pediculus humanus humanus*), and the cat flea (*Ctenocephalides felis*) have been implicated in the transmission of *B. bacilliformis*, *B. quintana*, and *B. henselae*, respectively (Chomel et al. 1996, Raoult and Roux 1999, Karem et al. 2000). Ticks were suspected to be involved in the transmissions of *B. vinsonii* subsp. *berkhoffii* and *B. vinsonii* subsp. *arupensis* (Pappalardo et al. 1997, Welch et al. 1999, Chang et al. 2001), and recent experimental study suggests that *Ixodes ricinus* may be involved in the transmission of *B. henselae* (Cotté et al. 2008). The DNA of *Bartonella* species has been detected in fleas collected from diverse wild rodents (Stevenson et al. 2003, Marié et al. 2006, Morway et al. 2008). In addition, *Bartonella* DNA was found in keds, biting flies, mites, and miscellaneous arthropods (Billeter et al. 2008a). These results demonstrated the potential role of these various insects as vectors for the transmission of *Bartonella* sp. among mammalian hosts.

Kosoy and colleagues (2008) recently isolated a novel *Bartonella* sp., *B. tamiae*, from three patients in Thailand. The patients showed symptoms typical for bartonellosis, such as fever, mild anemia, and ocular disorder. Although these patients had reported contact with rats, the source and/or possible vectors of the infection remains unknown. Recent detection of *B. tamiae*-like DNA in *Amblyomma americanum*

[1]Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado.
[2]Laboratory of Veterinary Public Health, Department of Veterinary Medicine, College of Bioresource Science, Nihon University, Fujisawa, Kanagawa, Japan.
[3]Department of Entomology, U.S. Army Medical Component, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand.

ticks (Acari: Ixodidae) collected in Virginia also suggests a potential role of ticks in the transmission of *B. tamiae* (Billeter et al. 2008).

More than 250 species of mites are associated with health-related problems for humans and domestic animals (Mullen and Durden 2002). Probably foremost among these problems are temporary irritation of the skin, dermatitis, and allergies. There are very few human diseases caused by pathogens transmitted by mites, with two significant exceptions: rickettsialpox and scrub typhus (Mullen and Durden 2002). Several studies have implicated mites of the superfamily Dermanyssoidea in the transmission of bacterial pathogens, such as *Salmonella*, *Pasteurella*, and *Borrelia* (Netusil et al. 2005, Valiente et al. 2005). *Orientia tsutsugamushi*, the etiological agent of scrub typhus, is the only known pathogen transmitted by trombiculid mites (genus *Trombicula*) (Lerdthusnee et al. 2003).

The aim of this study was to evaluate mites and other ectoparasites for the presence of *Bartonella*-specific DNA.

Materials and Methods

Ectoparasites were collected from 41 rodents of five species: *Rattus rattus* (29), *Rattus argentiventer* (2), *Bandicota indica* (5), *Bandicota savilei* (3), and *Mus cervicolor* (2). These rodents were captured from five different regions of Thailand: the northern region (Nam province, $n = 7$), the northeastern region (Ubon Ratchathani and Ubon Thani provinces, $n = 8$), the eastern region (Chon Buri province, $n = 1$), the central region (Nonthaburi and Pha Nakon Si Ayutthaya provinces, $n = 3$), and the southern region (Nakhon Si Thammarat, Surat Thani, and Yala provinces, $n = 22$).

The ectoparasites were morphologically identified to genus. The 209 collected mites belonged to the genera *Leptotrombidium* (130), *Schoengastia* (67), and *Blankarrtia* (12), and eight collected ticks belonged to the genus *Haemaphysalis*. The mites collected from each animal were pooled for testing by mite genus. The number of mites in each pool was from 1 to 9 (mean, 5.3). The eight ticks were collected from one mouse and were also pooled together. In total, we tested 40 pools of 209 mites and one pool of eight ticks. The 40 tested mite pools belonged to the genera *Leptotrombidium* (24), *Schoengastia* (14), and *Blankarrtia* (2). DNA was extracted using the Qiagen Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol for blood and body fluid, with minor modifications. Briefly, polymerase chain reaction (PCR) was performed in 25 μL of mixtures containing 12.5 μL of 2×iQ SYBR Green Supermix (BioRad, Hercules, CA), 0.5 pmol of each primer, and 2.5 μL of template DNA. *Bartonella* DNA was amplified using primers for intergenic spacer (ITS) (Billeter et al. 2008b) and for *gltA* specifically designed for detection of *B. tamiae* (BtGLT5′: TTC CTG AGT TTG TAG CAA AA; BtGLT3′: GGA TCA TCT TTA ATG CCC AA). The amplification of DNA was performed on a thermal cycler (iCycler; BioRad). Briefly, for ITS, 1 cycle for 3 min at 95°C was followed by 55 cycles for 30 s at 95°C, 30 s at 57°C, and 30 s at 72°C, and a final extension cycle for 7 min at 72°C; and for *gltA*, 1 cycle for 4 min at 94°C, followed by 45 cycles for 30 s at 95°C, 60 s at 49°C, and 30 s at 72°C, and a final extension cycle for 7 min at 72°C. Ten microliters of each PCR product was run on a 1.5% agarose gel (Agarose; Promega, Madison, WI).

Positive PCR products were sequenced using an Applied Biosystems Model 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The CLUSTAL-X program was used for the phylogenetic analysis of the obtained sequences. The neighbor-joining method by Kimura's two-parameter distance method and bootstrap calculation was carried out for 1000 resamplings. MEGA 4.0.1 software (The Biodesign Institute, Tempe, AZ) was used for these analyses.

Results

Of the 40 pooled mite samples, 29 (72.5%) and 9 (22.5%) were positive for *B. tamiae* based on ITS and *gltA*, respectively (Table 1). Among these, five samples were positive for both genes (12.5%). One tick sample was also positive for *B. tamiae* for ITS. The length of the PCR amplicons for ITS and *gltA* were 230–242 and 114–116 bp, respectively.

Sequence analysis of the ITS amplicons revealed two distinct sequence groups, one of which formed a cluster with *B. tamiae* strain Th239 (ITS239) and the other with strains Th307 and Th339 (ITS307/339), which were previously isolated from humans (Kosoy et al. 2008) (Fig. 1). Fourteen of the 30 ITS sequences (34.2% of tested) grouped with the ITS239 sequence, whereas the other 16 (39.0%) grouped with the ITS307/339 sequence (Table 2). These groups can be distinguished by the presence of 12-bp insertion in the amplified ITS region; this insertion was present only in ITS239 but not in ITS307/339 (data not shown). Analysis of the *gltA* sequences also revealed two distinct groups, and both groups were closer to strains Th239/307 than to Th339 (Fig. 2).

The sequence groups of ITS239 and ITS307/339 were 29.2% (7/24) and 37.5% (9/24), respectively, among mites of genus *Leptotrombidium*, 35.7% (5/14) and 42.9% (6/14) among mites of genus *Schoengastia*, and 100% (2/2) and 0% (0/2) among mites of genus *Blankarrtia*, respectively (Table 2). The two sequence groups were detected in 20.7% (6/29) and 44.8% (13/29) of mites collected from *R. rattus*, 50.0% (1/2) and 0% (0/2) of mites from *R. argentiventer*, 80.0% (4/5) and 20.0% (1/5) of mites from *B. indica*, 66.7% (2/3) and 33.3% (1/3) of mites *B. savilei*, and 50% (1/2) and 50% (1/2) of mites from *M. cervicolor*, respectively (Table 3). Of the 14 total sequence positives found in the ITS239 group, 4 were isolated from ectoparasites collected in the northern region of Thailand, whereas the other 10 came from the southern region of Thailand. Of the 16 total ITS sequence positives in the ITS307/339 group, 3 were found in the northern region of Thailand, 6 from the northeastern region, 2 from the central region, and 5 from the southern region (Table 4).

TABLE 1. DETECTION OF *BARTONELLA TAMIAE* DNA IN CHIGGER MITES AND TICKS COLLECTED FROM THAI RODENTS BY POLYMERASE CHAIN REACTION TARGETING OF INTERGENIC SPACER (ITS) AND *GLTA*

		ITS		
		Positive number (%)	*Negative number (%)*	*Total number (%)*
gltA	Positive	5 (12.2)	4 (9.8)	9 (22.0)
	Negative	25[a] (61.0)	7 (17.1)	32 (78.1)
	Total	30[a] (73.2)	11 (26.8)	41[a] (100)

[a]This includes a tick pool.

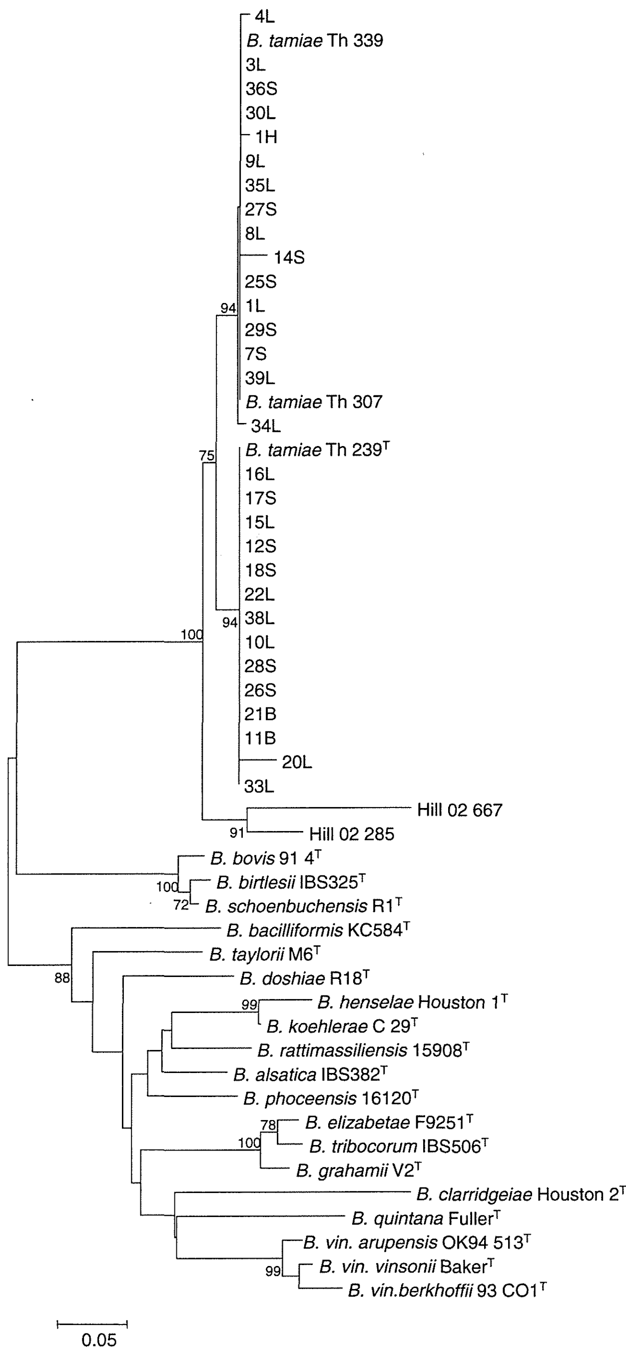

FIG. 1. Phylogenetic analysis of the sequences of intergenic spacer (ITS) from *Bartonella* detected in ectoparasites collected from Thai rodents. The phylogenetic tree was constructed by the neighbor-joining method; bootstrap values were estimated with 1000 replicates. Only bootstrap replicates >70% are noted. The 30 samples that were positive for ITS were classified into two (ITS239 and ITS307/339) clusters. The sequence detected from an *Amblyoma americanum* tick (Hill-02-28, Hill-02-66; Billeter et al. 2008b) was also included. Sample ID represents sample no., followed by the genus of ectoparasite (L for *Leptotrombidium*, S for *Schoengastia*, B for *Blankarrtia*, and H for *Haemaphysalis*).

TABLE 2. DETECTION OF *BARTONELLA* DNA INTERGENIC SPACER (ITS) FROM MITES AND TICKS INFESTING WILD RODENTS IN THAILAND: COMPARISON BY ECTOPARASITE

Ectoparasites	Number tested	*Number positive (%) for* Bartonella tamiae *sequence group*		
		I-239	*I-307/339*	*Total*
Leptotrombidium	24	7 (29.2)	9 (37.5)	16 (66.7)
Schoengastia	14	5 (35.7)	6 (42.9)	11 (8.6)
Blankarrtia	2	2 (100)	0 (0)	2 (100)
Haemaphysalis	1	0 (0)	1 (100)	1 (100)
Total	41	14 (34.1)	16 (39.0)	30 (73.2)

Discussion

In our study, we detected DNA specific for *B. tamiae* in mites collected from Thai rodents; this, therefore, represents one of the few reports showing a potential role of mites in the transmission of *Bartonella* (Durden et al. 2004, Billeter et al. 2008a). Percentage of the sequence homologies between the detected DNA in this study and those of the known *B. tamiae* were 97.8–100% for ITS307/339 group and 97.7–99.6% for ITS239 group, respectively, and 98.3–100% for the *gltA* gene. The first study on mites in the transmission of *Bartonella* species was reported more than 60 years ago when Baker (1946) showed that hamsters inoculated with suspension of mites collected from *Microtus* voles from Canada became bacteremic with an agent that was later identified as *B. vinsonii*. More recently, Kim et al. (2005) tested 21 Mesostigmatid mite pool samples and found 4 (19%) positive pools by PCR targeting the 16S rRNA gene; the DNA sequence showed a high homology (99.2%) with *B. doshiae*. Reeves et al. (2006) demonstrated that a *Steatonyssus* sp., a mite removed from a bat, harbored a *Bartonella* sp. (96%) that was closely related to an unnamed *Bartonella* found in rodents. Reeves et al. (2007) detected a part of the *groEL* gene from one of eight pools of tropical rat mites (*Ornithonyssus bacoti*) collected from *R. rattus* in Egypt by sequence analysis and demonstrated it to be an unique sequence with 81% similarity to a *Bartonella* species. In addition to mites associated with rodents, evidence of *Bartonella* DNA has been reported from mites of bats (Chiroptera, *Steatonyssus* sp.) and house dust mites (*Dermatophagoides farinae* and *D. pteronyssinus*) (Valerio et al. 2005, Reeves et al. 2006).

To the best of our knowledge, this is the first report of the presence of *Bartonella* DNA in the chigger mite. Durden et al. (2004) reported that none of the examined chigger mites collected from gray squirrels was positive for *Bartonella* by PCR targeting of *gltA* gene. Our study is also the first report showing the detection of *B. tamiae* DNA in chigger mites. *B. tamiae* is a novel *Bartonella* species that was isolated from patients in Thailand (Kosoy et al. 2008). Strains Th239, Th307, and Th339 were isolated from three separate patients. Although all the patients reported exposures to rats, the role of the rats in the transmission of *B. tamiae* has not been proved (Kosoy et al. 2008). Interestingly, the DNA sequence of *gltA* which we identified in mites infesting rodents were highly similar to those of *B. tamiae* isolated from Thai patients. These findings suggest a possible role of the chigger mites in the transmission of *B. tamiae* to humans.

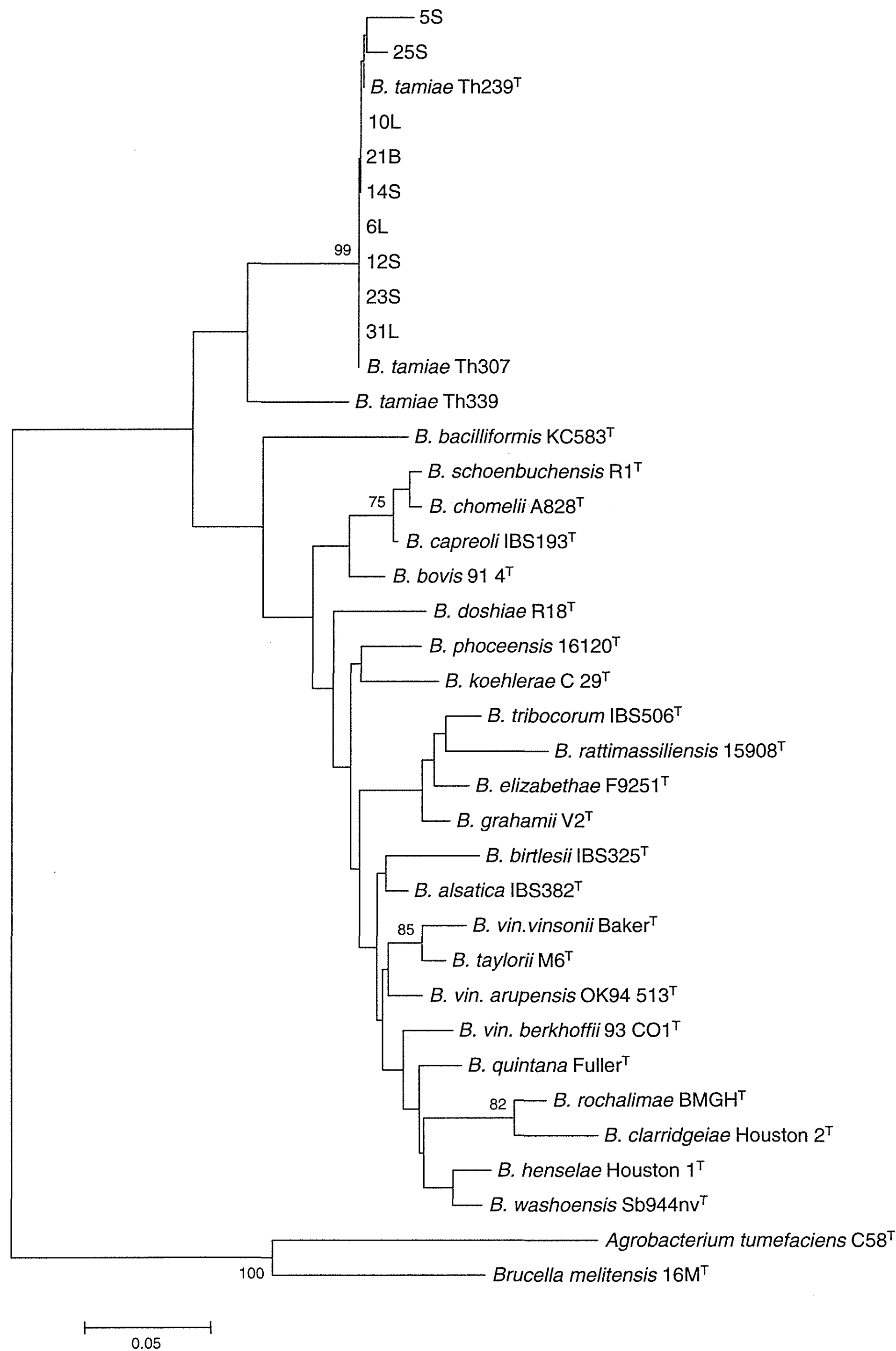

FIG. 2. Phylogenetic analysis of the sequences of *gltA* from *Bartonella* detected in ectoparasites collected from Thai rodents. The phylogenetic tree was constructed by the neighbor-joining method; bootstrap values were estimated with 1000 replicates. Only bootstrap replicates >70% are noted. The nine samples that were positive for *gltA* were classified into the same cluster. Sample ID represents sample no., followed by the genus of ectoparasite (L for *Leptotrombidium*, S for *Schoengastia*, and B for *Blankarrtia*). The sequences from *Brucella melitensis* 16 M^T and *Agrobacterium tumefaciens* C58^T were used as out-group bacteria.

One tick of the genus *Haemaphysalis* was positive for DNA specific for the sequence group of ITS307/339. Recently, Billeter et al. (2008b) also reported the presence of *B. tamiae*-like sequences in two *A. americanum* by using the same primer pairs that were used in our study. Thus, ticks as well as chigger mites might play a role in the transmission of *B. tamiae*.

We identified two distinct sequence groups, ITS307/339 and ITS239, which show high sequence similarity with the isolates from the patients. The 16 sequences belonging to the

TABLE 3. DETECTION OF *BARTONELLA* DNA INTERGENIC SPACER (ITS) FROM MITES AND TICKS INFESTING WILD RODENTS IN THAILAND: COMPARISON BY RODENT HOST

Host rodents	Number tested	*Number positive (%) for* Bartonella tamiae *sequence group*		
		I-239	*I-307/339*	*Total*
Rattus rattus	29	6 (20.7)	13 (44.8)	19 (65.5)
Rattus argentiventer	2	1 (50.0)	0 (0)	1 (50)
Bandicota indica	5	4 (80.0)	1 (20.0)	5 (100)
Bandicota saviley	3	2 (66.7)	1 (33.3)	3 (100)
Mus cervicolor	2	1 (50.0)	1[a] (50.0)	2[a] (100)

[a]This is a tick pool.

sequence group ITS307/339 and 14 sequences belonging to the sequence group ITS239 demonstrated only 0-4 and 1-4 single-nucleotide polymorphisms (SNPs) among the sequences for each group, respectively. Only one SNP was found between the sequences of *B. tamiae* strains Th307/339 and that from the tick examined in this study. In comparison, the DNA that was detected in the *A. americanum* tick collected in Virginia was distinguishable from three cultures of *B. tamiae* isolated from Thai patients by the presence of several deletions and SNPs (Hill-02-28, Hill-02-66; Fig. 1). These results suggest the possibility that indigenous *B. tamiae*-like agents are distributed in geographically different areas.

To date, there have been no reports showing the isolation of *B. tamiae* or detection of *B. tamiae*-specific DNA from any animals, including rats, in Thailand (Castle et al. 2004). One of possible explanations is that chigger mites themselves serve as the principal natural reservoirs of *B. tamiae* and do not require involvement of the rodents for the transmission cycle. Because chigger mites generally feed only on a single host in their larval stage, they do not have an opportunity to transmit acquired pathogens during a subsequent feeding (Mullen and Durden 2002). There are three possible ways by which these mites may therefore play a role in the transmission of *Bartonella*. First, the mite may transmit *B. tamiae* transovarially and/or transstadially, thus resulting in the preservation of the organisms among the chigger mites, similar to the parasitic system of *O. tsutsugamushi* and *Leptotrombidium* mites. Second, the organisms may persist in the body of the rodents outside of their blood. Because chigger mites feed on body fluids and partially digested tissues rather than blood, some *Bartonella* species could potentially be acquired by chiggers from the former two sources. Third, the organisms may exist in the blood but are not cultivable on agar medium. Also, it is possible that more prevalent *Bartonella* species in the blood of rodents might have prevented the detection of *B. tamiae*. Additional investigations are needed to clarify the source of infection and the mode of transmission of *B. tamiae* infection in Thailand.

TABLE 4. DETECTION OF *BARTONELLA* DNA INTERGENIC SPACER (ITS) FROM MITES AND TICKS INFESTING WILD RODENTS IN THAILAND: COMPARISON BY GEOGRAPHIC REGION

Regions	Number tested	*Number positive (%) for* Bartonella tamiae *sequence group*		
		I-239	*I-307/339*	*Total*
Northern	7	4 (57.1)	3[a] (42.9)	7[a] (100)
Northeastern	8	0 (0)	6 (75.0)	6 (75.0)
Eastern	1	0 (0)	0 (0)	0 (0)
Central	3	0 (0)	2 (66.7)	2 (66.7)
Southern	22	10 (45.5)	5 (22.7)	15 (68.2)

[a]This includes a tick pool.

When comparing the PCR results of the two target regions used here for the detection of *B. tamiae* DNA, ITS was clearly more sensitive than *gltA*. Among the 34 positive samples, 25 were detected by the ITS target only and 4 were positive for *gltA*; only 5 were positive for both targets (ITS and *gltA*). The *gltA* primers used for the detection of *B. tamiae* were specifically designed for this study because preliminary results demonstrated that the application of the primers proposed by Norman et al. (1995) was not effective. Our results suggest that ITS primers are the most effective tool for screening ectoparasites for the presence of *B. tamiae*. Application of the same primers allowed Billeter et al. (2008b) to detect *B. tamiae* DNA in ticks. It would therefore be an important assignment to design a primer pair that amplifies all *Bartonella* species including *B. tamiae* more specifically.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Michael Y. Kosoy
Division of Vector-Borne Infectious Diseases
Centers for Disease Control and Prevention
3150 Rampart Road
Foothills Research Campus
Fort Collins, CO 80521

E-mail: mck3@cdc.gov

Original article

Isolation of *Salmonella* from Flies in the Mekong Delta, Vietnam

LY Thi Lien Khai[1], TRAN Thi Thuy Duyen[1], NGUYEN Van Hao[1], TRAN Thi Phan[1], Taketoshi IWATA[2], Takahide TANIGUCHI[2], Ha Thanh TOAN[3] and Hideki HAYASHIDANI[2*]

[1]Department of Veterinary Medicine, College of Agriculture and Applied Biology, and [3]Biotechnology Research and Development Institute, Can Tho University, Can Tho, Can Tho, 3/2 St., Can Tho, Vietnam,
[2]Division of Animal Life Science, Institute of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan
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Summary

From July 2004 to April 2005, total of 494 flies, including 128 houseflies (*Musca domestica*) and 366 blowflies (*Genus Calliphora*), were captured at 6 pig farms, at a slaughterhouse and at 2 wet markets in the Mekong Delta, Vietnam to know the role of flies as a transmitter of *Salmonella*. *Salmonella* were isolated from 38 (7.7%) of the 494 flies samples. The isolation rates of *Salmonella* from flies captured at a slaughterhouse (15.5%) were significantly higher than that in pig farms (4.5%) and in wet markets (6.5%). The isolation rates of *Salmonella* from blowflies (9.8%) were higher than those houseflies (1.6%). From those 38 *Salmonella*-positive fly samples, 41 *Salmonella* strains were isolated and 14 serovars were identified. The predominant serovars were *S.* Typhimurium, *S.* Panama, *S.* Newport, *S.* Derby, *S.* Bareilly, *S.* Lexington and *S.* Anatum. Of the 41 *Salmonella* isolates examined, 38 (92.7%) showed resistance to at least one or following antibiotics ; SM, OTC, KM, NA, ABPC, SMX, CP, and GM. No isolates showed resistance to CEZ, CTRX and CPFX. Among the resistant isolates, 22 isolates were multi-drug resistance. Flies seem to play an important role, as an epidemiological link between humans and domestic animals in the Mekong Delta, Vietnam.

Keywords : housefly, blowfly, *Salmonella*, isolation, Vietnam

Introduction

Human *Salmonella* infection is endemic in developing tropical countries[24]. *Salmonella* is well established as one of the most important causes of food borne illness worldwide and transmission is usually by the fecal-oral route[2]. It is transmitted by food and water contaminated by animal excreta and human feces containing this organism. Flies are also recognized as contributing epidemiological factor in the spread of foodborne pathogens. They can transmit contagious diseases, such as *Salmonella,* to livestock and humans[1,4,11,14,17,26]. The housefly is most frequently encountered around pig pens, and can be considered as critical disease conveyor[10]. The housefly is also categorized by Food and Drug Administration (FDA) as an important contributing factor in the dissemination of various infectious foodborne diseases such as cholera, shigellosis and salmonellosis[15] and as well as blowfly[6,7].

However, no report has been published regarding the role of houseflies and blowflies as a transmitter of *Salmonella* in the Mekong Delta, southern Vietnam. In the present study, we isolated *Salmonella* from flies in the Mekong Delta, Vietnam to know the role of fly as a carrier of *Salmonella.*

Materials and methods

Sample collection :

From July 2004 to April 2005, 494 fly samples were captured by sterilized hand net at 6 pig farms, 1 slaughterhouse and 2 wet markets in Can Tho city located in the Mekong Delta, Vietnam. The slaughterhouse is located near the center of Can Tho city, and the domestic animals slaughtered there were originated mainly from farms around the city. Flies captured were immediately frozen by dry ice and were immediately transported to Can Tho University for examination. Fly species were identified morphologically. The captured flies were identified as housefly (*Musca domestica*), and blowfly which belonging to the genus *Calliphora.*

*Corresponding author :
Hideki HAYASHIDANI
Tel : +81-42-367-5775 ; Fax : +81-42-367-5775
E-mail : eisei@cc.taut.ac.jp

Isolation and identification of *Salmonella*

In the laboratory, each fly was put aseptically into a tube containing 9 ml of buffered peptone water (BPW, Oxoid, Hampshire, UK) for pre-enrichment and incubated at 37℃ for 24 h. Then 1ml of the pre-enrichment broth in each tube was transferred to 9 ml of Hajna Tetrathionate broth (Eiken) and further incubated at 37℃ for 24 h. One loopful of each enrichment tube was inoculated onto a plate of brilliant green agar (BGA, Oxoid, UK) supplemented with 20 μg novobiocin/ml and mannitol lysine crystal violet brilliant green agar (MLCB, Nissui). The plates were inoculated at 37℃ for 24 h and three suspicious colonies morphologically similar to those of *Salmonella* from each plate were subcultured for biochemical examination. Biochemical characteristics were examined using triple-sugar iron agar (Eiken), VP medium (Eiken) and lysine indole motility medium (Eiken). When typical *Salmonella* reactions were seen, additional biochemical tests were performed as described by Farmer[3]. Serotyping of *Salmonella* isolates was accomplished with commercial O and H antisera (Denka, Seiken, Japan) according to the method of Popoff and Le Minor[18].

Antimicrobial susceptibility testing :

Salmonella isolates from fly samples were examined for susceptibility to 11 different antimicrobial agents by agar dilution method according to the National Committee for Clinical Laboratory Standards procedure M7-A5[12]. The antimicrobial agents were ampicillin (ABPC), streptomycin (SM), kanamycin (KM), gentamycin (GM), oxytetracycline (OTC), chloramphenicol (CP), cefazolin (CEZ), ceftriaxone (CTRX), sulfamethoxazole (SMX), nalidixic acid (NA), and ciprofloxacin (CPFX). *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as a control strains according to NCCLS.

Statistical analysis :

Fisher's exact test with statistical significance set at the 95% confidence level ($P<0.05$) was used for statistical comparisons of prevalence of *Salmonella*.

Results

Salmonella was isolated from 38 (7.7%) of 494 flies captured in the present study. It was isolated from 9 of 199 (4.5%) flies at pig farms, 17 of 110 (15.5%) at a slaughterhouse, and 12 of 185 (6.5%) at Markets. The isolation rate of *Salmonella* from slaughterhouse was significantly higher than that from farms ($p<0.01$) and markets ($p<0.05$). It was isolated from 2 of 128 (1.6%) houseflies and 36 of 366 (9.8%) blowflies. The isolation rate of *Salmonella* from blowflies were significantly higher than that from houseflies ($p<0.01$) (Table 1). Of the 38 *Salmonella*-positive fly samples, 41 *Salmonella* strains were isolated and 14 serovars were identified. Of the 38 *Salmonella*-positive fly samples, each of 2 blowflies and 1 housefly harbored 2 *Salmonella* serovars. The predominant serovars from flies were *S.* Typhimurium (29.3%), *S.* Panama (9.8%), *S.* Newport (9.8%), *S.* Derby (7.3%), *S.* Bareilly (7.3%), *S.* Lexington (7.3%), and *S.* Anatum (7.3%). The other isolates were *S.* London, *S.* Braenderup, *S.*Javiana, *S.*Senftenberg, *S.*Lagos, *S.*Irumu and *S.* Warnow, but only one strain of each were isolated. Two strains were untypeable (Table 2). Of the 41 *Salmonella* isolates examined, 38 (92.7%) showed antimicrobial resistance against one or more antimicrobial agents. Thirty-five isolates (85.4%) were resistant to SM, 24 (58.5%) to OTC, 19 (46.3%) to KM, 19 (46.3%) to NA, 2 (4.9%) to ABPC, 2 (4.9%) to SMX, 1 (2.4%) to CP and 1 (2.4%) to GM. None of the isolates showed resistance to CEZ, CTRX, and CPFX (Table 3). Twenty-two (56.1%) of the 41 *Salmonella* isolates from flies showed multi-drug resistance (Table 4).

Discussion

In the present study, *Salmonella* was isolated from flies in the Mekong Delta, Vietnam at a high rate (7.7%). Also, it might be noted that among the predominant *Salmonella* serovars, that is, *S.* Typhimurium (29.3%), *S.* Panama (9.8%), *S.* Newport (9.8%), *S.* Derby (7.3%), *S.* Bareilly (7.3%), *S.* Lexington (7.3%) and *S.* Anatum (7.3%) isolated from the flies samples. Among those serovars, *S.* Typhimurium, *S.* Derby, *S.* Lexington, and *S.* Anatum was previous isolated from domestic animals, and were also the predominant serovars in foods including pork, beef, duck meat, chicken meat and shrimps in the Mekong Delta[22,23]. Moreover, *S.* Bareilly, and *S.* Typhimurium was also the predominant serovars in human. Ogasawara, et al.[13] reported that *Salmonella* isolates originated from several sources such as domestic animals, and food in the Mekong Delta showed resistance to OTC, CP,

Table 1 Prevalence of *Salmonella* from flies by place in the Mekong Delta, Vietnam

Fly species	No. of *Salmonella* positive samples/ No. of samples examined (%)			
	Farm	Slaughterhouse	Market	Total
Housefly	0/ 38 (0.0)	2/ 56 (3.6)	0/ 34 (0.0)	2/128 (1.6)
Blowfly	9/161 (5.6)	15/ 54 (27.8)	12/151 (7.9)	36/366 (9.8)
Total	9/199 (4.5)	17/110 (15.5)[a,b]	12/185 (6.5)	38/494 (7.7)

[a] Slaughterhouse > Farm ($p<0.01$)
[b] Slaughterhouse > Market ($p<0.05$)

NA, SM, KM and ABPC. In the present study, *Salmonella* isolates from flies also showed resistance to OTC , CP, NA, SM, KM and ABPC.. These results indicate that *Salmonella* isolates from flies seem to be originated from the same sources of *Salmonella* strains isolated from domestic animals, foods and human in the Mekong Delta. Therefore, flies seem to play an important role as a transmitter of *Salmonella* between human and environment in the Mekong Delta.

Table 2 Serovars of *Salmonella* from flies in the Mekong Delta, Vietnam

Serovar	No. of isolate (%)
S. Typhimurium	12 (29.3)
S. Panama	4 (9.8)
S. Newport	4 (9.8)
S. Derby	3 (7.3)
S. Bareilly	3 (7.3)
S. Lexington	3 (7.3)
S. Anatum	3 (7.3)
S. London	1 (2.4)
S. Braenderup	1 (2.4)
S. Javiana	1 (2.4)
S. Senftenberg	1 (2.4)
S. Lagos	1 (2.4)
S. Irumu	1 (2.4)
S. Warnow	1 (2.4)
Untypeable	2 (2.9)
Total	41 (100)

In the present study, *Salmonella* isolation rate from blowflies (9.8%) was higher than that from houseflies (1.6%). Furthermore, the blowflies at slaughterhouse (27.8%) harbored *Salmonella* at a high rate rather than those in the farms (5.6%) and in markets (7.9%). Greenberg, et al.[6] reported that the most obvious source of *Salmonella* for flies was carrion derived from livestock, and the blowflies which were found exclusively on carrion, had high infection rates and the greatest diversity of *Salmonella* types at slaughterhouse in Mexico. Therefore, the reason that blowflies of this study harbored *Salmonella* at a high rate seems to be due to a behavior of blowflies which thrive in higher density in carrion.

In the present study, 92.7% of *Salmonella* isolates showed antimicrobial resistance against one or more antimicrobial agents, and 23 (56.1%) of the 41 *Salmonella* isolates showed multi-drug resistance (Table 4). *S.* Typhimurium DT 104, which showed resistance to up to 9 antimicrobial agents, has emerged as a global health problem in human and animal medicine during the last dec

Table 3 Antimicrobial resistance of *Salmonella* from flies in the Mekong Delta, Vietnam

Serovars	No. of isolates examined	No. of resistant isolates[a] (%)											No of resistant[b] (%)
		ABPC	OTC	CP	NA	SM	KM	GM	CEZ	CTRX	CPFX	SMX	
S. Typhimurium	12		8		7	11	7						12 (100.0)
S. Panama	4	1	3		3	4	3	1				1	4 (100.0)
S. Newport	4	1	4	1	2	3	2						4 (100.0)
S. Derby	3					3							3 (100.0)
S. Bareilly	3					2							2 (66.7)
S. Lexington	3					2							2 (66.7)
S. Anatum	3		3		2	3	1					1	3 (100.0)
S. London	1		1		1	1	1						1 (100.0)
S. Braenderup	1		1		1	1	1						1 (100.0)
S. Javiana	1					1							1 (100.0)
S. Senftenberg	1		1		1		1						1 (100.0)
S. Lagos	1		1		1	1	1						1 (100.0)
S. Irumu	1					1							1 (100.0)
S. Warnow	1		1		1	1	1						1 (100.0)
Untypeable	2		1			1	1						1 (50.0)
Total	41	2 (4.9)	24 (58.5)	1 (2.4)	19 (46.3)	35 (85.4)	19 (46.3)	1 (2.4)	0 (0.0)	0 (0.0)	0 (0.0)	2 (4.9)	38 (92.7)

[a] ABPC : Ampicillin, SM : Streptomycin, KM : Kanamycin, GM : Gentamycin, OTC : Oxytetrecycline, CP : Chloramphenicol, CEZ : Cefazolin, CTRX : Ceftriaxone, NA : Nalidixic acid, CPFX : Ciprofloxacin, SMX : Sulfamethoxazole

[b] Isolates resistant to at least one antimicrobial agent

Table 4 Antimicrobial resistance patterns of *Salmonella* isolates from flies in the Mekong Delta, Vietnam

No. of antimicrobial agents	Resistance pattern[a]	No. of resistant isolates	Serovars (No.of isolates)
1	SM	14	Lexington (2), Bareilly (2), Irumu (1), Typhimurium (4), Derby (3), Panama (1), Javiana (1)
	OTC	2	Typhimurium (1), Newport (1)
2	OTC + SM	1	Anatum (1)
3	OTC + SM + NA	1	Anatum (1)
	OTC + KM + NA	2	Warnow (1), Senftenberg (1)
4	OTC + NA + SM + KM	15	Braenderup (1), Panama (2), Typhimurium (7), Anatum (1) London (1), Newport (2), Lagos (1),
	ABPC + OTC + CP + SM	1	Newport (1)
5	OTC + NA + SM + KM + SMX	1	Anatum (1)
7	ABPC + OTC + NA + SM + KM + GM + SMX	1	Panama (1)
Total		38	

[a] ABPC : Ampicillin, OTC : Oxytetrecycline, SM : Streptomycin, KM : Kanamycin, GM : Gentamycin, NA : Nalidixic acid, , SMX : Sulfamethoxazole, CP : Chloramphenicol,

ade[5,8,19,20,21]. However, *S.* Typhimurium DT 104 strain was not isolated from any fly samples in the present study. Ogasawara et al.[13] also reported that *S.* Typhimurium DT 104 was not isolated from animals, meats and foods in the Mekong Delta. However, Vo, et al.[24] isolated *S.* Typhimurium DT 104 from pig and human in Vietnam at a low rate although it was still unclear whether these isolates were multi-drug resistant strains or not. Since *S.* Typhimurium DT 104 have been isolated from neighbor countries such as Thailand[16] and Taiwan[27], further continuous survey should be needed to clarify the presence of *S.* Typhimurium DT 104 in Vietnam.

It can be concluded that the flies are carrying viable *Salmonella* associated with domestic animals, foods and environments. It is possible that flies may function as an epidemiological link between humans and domestic animals in the Mekong Delta, Vietnam. Therefore, flies control should be pay more attention to reduce the risk from pathogenic bacteria such as *Salmonella* in the Mekong Delta.

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原 著

ベトナム・メコンデルタのハエからのサルモネラの分離

LY Thi Lien-Khai[1]・TRAN Thi Thuy Duyen[1]・NGUYEN Van Hao[1]・TRAN Thi Phan[1]・
岩田剛敏[2]・谷口隆秀[2]・Ha Thanh TOAN[3]・林谷秀樹[2]*

[1]Department of Veterinary Medicine, College of Agriculture and Applied Biology, and
[3]Biotechnology Research and Development Institute, Can Tho University
[2]東京農工大学大学院農学研究院動物生命科学部門

要旨

2004年7月から2005年4月までの間に，ハエのサルモネラの媒介者としての役割を知るために，ベトナム・メコンデルタの養豚場6ケ所，と畜場1ケ所および市場2ケ所でイエバエ128匹とクロバエ366匹の計494匹のハエを捕獲した。サルモネラは494匹中38匹（7.7%）から分離された。と畜場（15.5%）で捕獲したハエからのサルモネラの分離率は，養豚場（4.5%）や市場（6.5%）のものに比べ有意に高かった。また，クロバエ（9.8%）からの分離率はイエバエ（1.6%）に比べ有意に高かった。38匹のハエから分離されたサルモネラ41菌株は，14血清型に型別され，Typhimurium, Panama, Newport, Derby, Bareilly, Lexington および Anatum の次に多かった。分離されたサルモネラ41株中38株（92.7%）が SM, OTC, KM, NA, ABPC, SMX, CP および GM のいずれか1つかそれ以上に耐性を示したが，CEZ, CTRX および CPFX に耐性を示した株は認められなかった。ハエはベトナム・メコンデルタにおいて，人と家畜との間で疫学的に重要な役割を果たしているものと思われる。

連絡先：林谷秀樹
東京農工大学大学院農学研究院動物生命科学部門
〒183-8509 東京都府中市幸町3-5-8
Tel/Fax：042-367-5775
E-mail：eisei@cc.tuat.ac.jp

Original article

Prevalence of *Salmonella* and *Escherichia coli* O157 from Acute Diarrheic Children in the Mekong Delta, Vietnam

LY Thi Lien-Khai[1], DUONG Thi Thanh Thao[1], NGUYEN Thu Tam[1], TRAN Thi Phan[1], TRAN Thi Thuy Duyen[1], Aya NAKADAI[2], Taketoshi IWATA[2], Takahide TANIGUCHI[2], HA Thanh Toan[3] and Hideki HAYASHIDANI[2*]

[1]Department of Veterinary Medicine, College of Agriculture and Applied Biology, and [5]Biotechnology Research and Development Institute, Can Tho University, Can Tho, 3/2 St., Can Tho City, Vietnam,
[2]Division of Animal Life Science, Institute of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509,
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Summary

From August 2001 to July 2002 and between June and December 2004, fecal samples from 922 children with acute diarrhea admitted to hospitals at Can Tho City, Dong Thap and Soc Trang provinces were examined for the presence of *Salmonella* and *Escherichia coli* O157. *Salmonella* was isolated from 45 (4.9%) of 922 diarrheic children. From the 45 patients, 47 *Salmonella* strains were isolated and 9 serovars were identified. The predominant serovars were *S.* London, *S.* Bareilly, *S.* Agona and *S.* Typhimurium. Of the 47 *Salmonella* isolates examined, 12 (25.5%) of isolates showed resistance to one or more antibiotics. Of 12 resistance isolates, 11 (23.4%) showed multi-drug resistance. *Salmonella* seems to be an important diarrhea-causing pathogen in children in the Mekong Delta, Vietnam. *E. coli* O157 was not isolated from any children's fecal samples with or without bloody stools.

Keywords : *Salmonella*, *E. coli* O157, child, isolation, Vietnam

Introduction

Diarrheic disease continues to be a global health problem, particularly among young children in developing and developed countries. *Salmonella* and *Escherichia coli* (*E. coli*) O157 are recognized worldwide as the most important foodborne pathogens[29]. A few reports about the isolation of *Salmonella* and *E. coli* O157 from human have been published in Northern Vietnam[2,3,24,25,26]. However, no report about those pathogens from human has been published in the Mekong Delta, Southern Vietnam.

The aim of the present study was to determine the prevalence of *Salmonella* and *E. coli* O157 from acute diarrheic patients and their antimicrobial susceptibility in the Mekong Delta, Vietnam.

*Corresponding author :
Hideki HAYASHIDANI
Tel : +81-42-367-5775 ; Fax : +81-42-367-5775
E-mail : eisei@cc.taut.ac.jp

Materials and methods

Sample collection

From August 2001 to July 2002 and between June and December 2004, a total of 922 human fecal samples were collected from acute diarrheic children (age from 2 months to 15 years old) in hospitals of Can Tho City, Dong Thap and Soc Trang Provinces. Acute diarrhea was defined as the passage of three or more liquid or loose stools in 24 hr in association with abdominal pain, nausea, vomiting, or fever. The information of patients about age, sex, features of stools, onset of illness, and other symptoms were collected together. All rectal swab samples were put into Carry-Blair transport medium (Nissui, Tokyo, Japan), and the samples of the feces placed in sterile plastic bags. All samples were cooled in an icebox and immediately transported to Can Tho University for examination.

Isolation and identification of *Salmonella*

In the laboratory, one gram of each fecal sample or a swab was put aseptically into a tube containing 3 ml of phosphate -buffered saline (PBS ; pH7.2). One ml of PBS suspension was inoculated into 9 ml of Enterobacteriaceae enrichment mannitol broth (EEM) (Eiken Chemical Co., Ltd., Tokyo, Japan) for pre-enrichment and

incubated at 37℃ for 24 hr. Then 1ml of the pre-enrichment broth in each tube was transferred to 9 ml of Hajna Tetrathionate broth (Eiken) and further incubated at 37℃ for 24 hr. One loopful of each enrichment tube was inoculated onto a plate of Brilliant Green agar (BGA, Oxoid, UK) and Mannitol Lysine Crystal Brilliant Green agar (MLCB, Nissui). The plates were incubated at 37℃ for 24 hr and three morphologically suspicious colonies from each plate were picked up and subcultured for biochemical examination. Biochemical characteristics were examined using triple-sugar iron agar (Eiken), VP medium (Eiken) and lysine indole motility medium (Eiken). When typical *Salmonella* reactions were seen, additional biochemical tests were performed as described by Barrow and Feltham[1]. Serotyping of *Salmonella* isolates was accomplished with commercial O and H antisera (Denka, Seiken, Tokyo, Japan) according to the method of Popoff and Le Minor[28].

Isolation and identification of *E. coli* O157 :

One ml of PBS suspension described above was inoculated into 9ml of modified *Escherichia coli* broth supplemented with novobiocin (mEC; Eiken Chemical Co., Ltd, Tokyo, Japan). The mEC broth was then incubated at 37℃ for 18 to 24 hr. After enrichment, 1ml of the culture was treated with immunomagnetic beads coated with anti-O157 antibody (Dynal beads anti-*E. coli* O157) (Dynal, Oslo, Norway) following the manufacturer's instructions, and then beads were inoculated onto sorbitol MacConkey agar containing cefixime and potassium tellurite (CT-SMAC) (Eiken). The plates were incubated at 37℃ for 24 hr, and 20 sorbitol non–fermented colorless colonies morphologically similar to *E. coli* O157 on each plate were subcultured with CT-SMAC and trypticase soy agar (BBL, Cockeysville, USA). Biochemical tests were performed as described by Barrow and Feltham[1]. If an isolate was identified as *E. coli,* serotyping was accomplished with commercial O antisera (Denka Seiken, Co. Ltd., Japan).

Antimicrobial susceptibility testing

The bacteria isolated in the present study were examined for susceptibility to 10 different antimicrobial agents by the agar dilution method according to the National Committee for Clinical Laboratory Standards (NCCLS) procedure M7-A5[21]. The antimicrobial agents used were ampicillin (ABPC), streptomycin (SM), kanamycin (KM), gentamycin (GM), oxytetracycline (OTC), chloramphenicol (CP), cefazolin (CEZ), ceftriaxone (CTRX), nalidixic acid (NA), and ciprofloxacin (CPFX). Antimicrobial susceptibility was assessed following NCCLS procedure, but isolates showing intermediate susceptibility were classified as susceptible. *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as a control strains according to NCCLS.

Statistical analysis

Chi-Square tests with statistical significance set at the 95% confidence level ($p<0.05$) was used for statistical comparisons of prevalence. Fisher's exact test was used for comparison of proportions when at least 1 expected cell value was <5.

Results

Salmonella was isolated from 45 (4.9%) of 922 acute diarrheic children, and 47 strains were identified. *Salmonella* were isolated from 38 (5.0%) of 763 children under 3 years old, and 7 (4.4%) of 159 children over 3 years old. No significant difference of isolation rate was observed among 2 age groups. The *Salmonella* isolation rate from male (5.1%) and female (4.5%) was not significant difference (Table 1). Clinical manifestations in diarrheic children having S*almonella* infections were fever, abdominal pain, nausea, vomiting, watery diarrhea, mucoid or mucoid and blood in stools. Of these children, 55.6% had fever (>38℃), 57.8% had mucoid in stool, 15.6% had abdominal pain, 20.0% had nausea and or vomiting, 20.0% had diarrhea with loose and light mucoid in stool, 11.1% had watery diarrhea, 6.7% had mucoid and blood in stool (Table 2). Of the 47 *Salmonella* isolates, 9 serovars were identified. The predominant serovars were *S.* London (19.1%), *S.* Bareilly (17.0%), *S.* Agona (12.8%) and *S.* Typhimurium (10.6%), followed by *S.* Weltevreden (6.4%), *S.* Ohio (6.4%), *S.* Bovismobificans (4.3%), *S.* Newport (4.3%) and *S.* Paratyphi B (2.1%). Of the 45 *Salmonella*-positive children, 2 children had mixed infection, included 1 child was infected with *S.* Ohio and *S.* Bovismorbificans, and another infected with *S.* Newport and *S.* Agona. Antimicrobial resistance of *Salmonella* isolates was shown in Table 3.

Of the 47 *Salmonella* isolates examined, 12 (25.5%) showed antimicrobial resistance to one or more antibiotics. Ten isolates (21.3%) were resistant to ABPC, 9 (19.1%) to OTC, 8 (17.0%) to SM, 8 (17.0%) to KM, 6 (12.8%) to NA, 5(10.6%) to CEZ, 4

Table 1 Occurrence of *Salmonella* from diarrheic patients in the Mekong Delta by age or sex

Age (year)	No. of *Salmonella* positive patients /No. of patients examined (%)		
	Male	Female	Total
<3	26/465 (5.6%)	12/298 (4.0%)	38/763 (5.0%)
≥3	2/ 80 (2.5%)	5/ 79 (6.3%)	7/159 (4.4%)
Total	28/545 (5.1%)	17/377 (4.5%)	45/922 (4.9%)

(8.5%) to CP, 4 (8.5%) to GM, and 1 (2.1%) to CTRX. No isolates showed resistance to CPFX (Table 3). Of the 12 isolates, 11 (23.4%) were multi-drug resistance (Table 4). *E. coli* O157 : H7 was not isolated from any diarrheic children with or without bloody stools.

Table 2 Clinical and stool features among children with acute diarrhea caused by *Salmonella*

Symptoms[a]	No. of case showing signs /No. of case (%)
Percent of diarrhea cases with :	
Fever	25/45 (55.6%)
Nausea/ vomiting	9/45 (20.0%)
Abdominal pain	7/45 (15.6%)
Watery	5/45 (11.1%)
Mucoid in stool	26/45 (57.8%)
Mucoid and blood in stool	3/45 (6.7%)
Loose, light mucoid in stool	9/45 (20.0%)

[a] 1 patient had diarrhea with 'rose spots' in skin

Discussion

Lee and puthucheary[13] reported that *Salmonella* is one of the five common entero-pathogenic bacteria isolated from diarrheic children in Malaysia. Likewise, *Salmonella* was frequently isolated from children with diarrhea in Thailand[32]. In Vietnam, Bui, et al.[2] reported that *Salmonella* was isolated from 3.6% (4/111) of fecal samples from diarrheic children in Hanoi, Northern Vietnam. In the present study, *Salmonella* was isolated from 45 (4.9%) of 922 diarrheic children. Those results indicate that *Salmonella* may play an important role as a pathogen caused diarrhea in children in the Mekong Delta, Vietnam. This is the first report of the prevalence of *Salmonella* from human in the Mekong Delta, Southern Vietnam.

In the present study, the predominant *Salmonella* serovars from diarrheic children were *S.* London, *S.* Bareilly, *S.* Agona, and *S.* Typhimurium. Vo, et al.[34] reported that *S.* Typhimurium was the most common serovar among the 56 *Salmonella* isolates originated from human in Vietnam. Tran, et al.[30] reported that the predominant serovars of *Salmonella* isolated from domestic animals in the Mekong Delta were *S.* Javiana, *S.* Typhimurium and *S.* Weltevreden. Tran, et al.[31] also reported that retail meats and shrimps purchased from the wet markets in the Mekong Delta

Table 3 Antimicrobial resistance of *Salmonella* from diarrheic patients in the Mekong Delta, Vietnam

Serovar	No. of isolates examined	No. of resistant isolates (%)[a]										No. of resistant (%)[b]
		ABPC	OTC	CP	NA	CEZ	SM	KM	GM	CTRX	CPFX	
S. London	9		1									1(11.1)
S. Bareilly	8											0(0.0)
S. Agona	6											0(0.0)
S. Typhimurium	5	4	5		1		4	5	4			5(100.0)
S. Weltevreden	3											0(0.0)
S. Ohio	3											0(0.0)
S. Bovismobificans	2											0(0.0)
S. Newport	2	1										0(0.0)
S. Paratyphi B	1				1							1(100.0)
O4 : UT[c]	3	2										0(0.0)
O8 : UT	2	1	1	2	2	2	2	2				2(100.0)
O3, 10 : UT	1	1	1	1	1	1	1					1(100.0)
O6, 14 : UT	1	1				1		1		1		1(100.0)
Untyped	1		1	1	1	1	1					1(100.0)
Total	47	10 (21.3)	9 (19.1)	4 (8.5)	6 (12.8)	5 (10.6)	8 (17.0)	8 (17.0)	4 (8.5)	1 (2.1)	0 (0.0)	12 (25.5)

[a] ABPC : ampicillin, OTC : oxytetracycline, CP : chloramphenicol, NA : nalidixic acid, CEZ : cefazolin, SM : streptomycin, KM : kanamycin, GM : gentamycin, CTRX : ceftriaxone, CPFX : ciprofloxacin,.

[b] Isolates resistant to at least one antimicrobial agent.

[c] UT : untypable

Table 4 Antimicrobial resistance patterns of *Salmonella* isolates from the patients in the Mekong Delta, Vietnam

No. of antimicrobial agents	Resistant patterns[a)]	No. of resistant isolates	Serovar[b)]
1	OTC	1	*S.* London(1)
2	ABPC + NA	1	*S.* Paratyphi B (1)
3	OTC + NA + KM	1	*S.* Typhimurium(1)
4	ABPC + CEZ + KM + CTRX	1	O6, 14 : UT(1)[c)]
5	ABPC + OTC + SM + KM + GM	4	*S.* Typhimurium(4)
6	ABPC + CP + NA + CEZ + SM + KM	1	O8 : UT(1)
6	ABPC + OTC + CP + NA + CEZ + SM	2	O3, 10 : UT(1), UT (1)
7	ABPC + OTC + CP + NA + CEZ + SM + KM	1	O8 : UT(1)
Total		12	

a) ABPC : ampicillin, OTC : oxytetracycline, SM : streptomycin, KM : kanamycin, CEZ : cefazolin, NA : nalidixic acid, CP : chloramphenicol, GM : gentamycin, CTRX : ceftriaxone.

b) ( ) : No. of isolates

c) UT : untypable

were contaminated by *Salmonella* at a high rate (33.8%) and the predominant serovars were *S.* Weltevreden, *S.* Derby, *S.* London, *S.* Dessau, and *S.* Lexington. Moreover, *S.* Typhimurium, *S.* London and *S.* Bareilly were isolated from flies captured at pig farms, slaughterhouse and wet markets in the Mekong Delta[18]. As *Salmonella* serovars isolated in this study were in common to those previous study, *Salmonella* infected to children in the Mekong Delta may be originated from animals, foods and environment. *S.* Enteritidis is the most common serovar isolated from human patients in Asian countries such as Japan[6,11,19], Malaysia[12,15], Singapore[16]. However, this serovar was not isolated in the present study. Furthermore, Tran, et al.[30] reported that *S.* Enteritidis was isolated from only 1 chicken sample collected in the Mekong Delta. Therefore, it seems to be the *S.* Enteritidis was not wildly distributed in the Mekong Delta, Vietnam.

Salmonella isolates from diarrheic children showed resistance to NA (12.8%). Wain, et al.[35] reported that NA resistant *S.* Typhi was first isolated in Vietnam in 1993. Ogasawara, et al.[27] reported that 17 (7.4%) of 230 *Salmonella* isolates originated from chicken meat, duck meat and chicken in the Mekong Delta showed resistance to NA. Fluoroquinolones are also a common choice of treatment for infections due to *Salmonella* spp. in human[5] and resistance to NA may impair fluoroquinolone therapy[4,29,35]. Therefore, the high level of NA resistance in this study is of special concern as it may lead to loss of therapeutic usefulness of fluoroquinolone[33]. Furthermore, *Salmonella* isolates also showed resistance to ABPC, OTC, SM, KM, CEZ, CP, GM and CTRX. The resistance to ABPC, SM, KM, CEZ and GM in this study was higher than those we found in animals and foods[27]. However, resistance to OTC, CP seems to be comparable to the resistant rate of those antimicrobials determined in the previous reported in animal and foods in the Mekong Delta[27]. It may be due to these antimicrobial agents are still wildly used in human therapy in the Mekong Delta because of low cost and ready availability[25]. Multi-drug resistance occurred in all *S.* Typhimurium isolates and *S.* Paratyphi B. Likewise, all *S.* Typhimurium isolated from flies were resistance to one or more antimicrobials agents tested[18]. The result indicates that *S.* Typhimurium showing multi-drug resistance in this area may be originated from the same source. Therefore, resistance to these antibiotics in diarrheic children infected with *Salmonella* may create problems for treatment. This study reckoned the emergence of quinolone, cephalosporin-resistance of *Salmonella*, especially multi-drug resistant in the Mekong Delta. Continuous monitoring of antimicrobial resistance in Vietnam should be done.

Kam[9] reported that, in Hong Kong, among 1983-1993, there was an increase of salmonellosis cases in >25 year old and a decrease in <1 year old, and also that, in children, males were more affected than females. In our study, though admittance of a higher number of patients under 3 years old were observed, *Salmonella* isolation rate was not significant difference between the two age groups examined. That difference, in the number of patient admitted, might be happened because diarrhea in very young children is usually the main reason which prompted parents to seek help from hospitals. Moreover, there were no difference between the incidence of salmonellosis in male (5.1%) and female (4.5%). Those results indicate that *Salmonella* infection in children in the Mekong Delta seems to be not affected by sex and age.