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

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Original article

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

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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].

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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.*

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 1 ml 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.

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 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)

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

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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。

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Original article

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

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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.

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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.

Yen, et al.[36], in a study of non-typhoid *Salmonella* bacteremia cases in Taiwan, showed that all the studied children had fever, 85 % of them had gastrointestinal symptoms (diarrhea and/or nausea/vomiting) and 55% had bloody or mucoid stools. Lee, et al.[14] reported that symptoms such as gross bloody or mucoid stool, and fever were more common in the pediatric group, indicating that enterocolitis is a more common mode of salmonellosis in children in Taiwan. Likewise, Newcomb, et al.[22] also showed that patients admitted to medical centers in Germany had at least one of the following symptoms : diarrhea, fever, nausea, abdominal pain and vomiting. In the present study, symptoms such as fever, abdominal pain, nausea, vomiting, mucoid and bloody stools were commonly observed in patients having diarrhea, associated with *Salmonella* infection. In those patients, fever (55.6%) and mucoid in stool (57.8%) were often found, followed by vomiting, abdominal pain, watery, mucoid and bloody stools.

E. coli O157 : H7 is one of the greatest concerned pathogens today in developed countries as the cause of foodborne illness[20]. However, we could not detect *E. coli* O157 : H7 in diarrheic patients even in bloody stools as the same previously reported in Vietnam[3,21,22,24] as well as in Thailand[8] and in Korea[10] although this pathogen has been isolated in a strain (O157 : NM) from 111 diarrheic children in Hanoi[2] as well as in diarrheic children in China[7]. This result hopefully indicates the limited circulation of *E. coli* O157 : H7 in diarrheic children in the Mekong Delta, Vietnam although this pathogen was isolated from cattle in this area[18].

Therefore, occurrence of gastroenteritis associated with *Salmonella* in children and the presence of multi-drug resistance constitute an important public health problem. Further studies should be made to clarify the etiology of diarrhea in children and to control the proliferation of drug resistant pathogens in the Mekong Delta, Vietnam.

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原　著

ベトナム・メコンデルタで急性下痢症を呈した子供における サルモネラと腸管出血性大腸菌 O157 の分布

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要旨

2001年8月から2002年7月ならびに2004年6月から12月の間に，急性下痢を呈し，Can Tho 市，Dong Thap および Soc Trang 県の病院に来院した922人の子供の糞便からサルモネラおよび腸管出血性大腸菌 O157 の分離を行った。サルモネラは922人中45人（4.9%）から分離された。45人の患者から分離されたサルモネラ47菌株は，9血清型に型別され，London, Bareilly, Agona および Typhimurium の順に多かった。分離されたサルモネラ47菌株のうち，12菌株（25.5%）が薬剤耐性を示し，11菌株（23.4%）は多剤耐性菌であった。これらのことからベトナム・メコンデルタにおいて，サルモネラは子供の下痢症の重要な原因菌になっているものと思われる。なお，腸管出血性大腸菌 O157 は分離されなかった。

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FULL PAPER *Public Health*

Seroepidemiological Survey of Pathogenic *Yersinia* in Breeding Squirrel Monkeys in Japan

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ABSTRACT. To investigate the prevalence of antibodies to pathogenic *Yersinia* in breeding squirrel monkeys, the serum samples of 252 squirrel monkeys from 9 zoological gardens in Japan were tested by ELISA using plasmid-encoded *Yersinia* outer membrane protein (Yops) as the antigen. The cutoff value was calculated by using the serum samples of the squirrel monkeys from Suriname, where no prevalence of pathogenic *Yersinia* have been reported. According to the cutoff value, 164 of 252 (65.1%) squirrel monkeys were considered positive against pathogenic *Yersinia*. These positive monkeys belonged to 8 of the 9 zoological gardens, and the percentage of the seropositive monkeys ranged from 22.2 to 89.4%. Furthermore, in one zoological garden, the positive rate of the squirrel monkeys which were over 1 year old (95.7%) was significantly higher than those which were under 1 year old (23.3%). These results suggested that pathogenic *Yersinia* is highly prevalent among breeding monkeys in Japan.

KEY WORDS: ELISA, squirrel monkey, *Yersinia*, Yops.

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Yersiniosis is an infection with pathogenic *Yersinia*, which is comprised of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis*. These pathogens cause gastrointestinal symptoms including enteritis, diarrhea and mesenteric lymphadenitis, and sometimes septicemia in humans and animals [3, 18]. Monkey species are especially sensitive to yersiniosis, and many fatal cases in breeding monkeys have been reported throughout the world [2, 8, 17, 23, 27]. In Japan, *Y. pseudotuberculosis* in particular frequently causes fatal infection in breeding monkeys [11, 14, 15, 30]. The highest number of dead monkeys by *Y. pseudotuberculosis* infection in Japan has occurred among the squirrel monkey (*Saimiri* spp.) [14, 30]. The habitat of the squirrel monkey is South and Central America, but many zoological gardens in Japan have been breeding monkeys imported from those regions. Many authors have published clinical and/or pathological reports of fatal infection with pathogenic *Yersinia* in breeding monkeys, including squirrel monkeys, but detailed information on the epidemiology of yersiniosis in breeding monkeys has not yet been obtained.

The pathogenicity of pathogenic strains of *Yersinia* depends on the presence of a 70-kb virulence plasmid termed "pYV". This plasmid is essential for virulence and is used to differentiate pathogenic from nonpathogenic *Yersinia*. To establish infection and subvert host defenses, pathogenic *Yersinia* require a type III secretion system which translocates virulence factors, called Yops (*Yersinia* outer membrane proteins), into host cells [6, 25]. Some researchers have reported that enzyme-linked immunosorbent assay (ELISA) and immunoblot assays using Yops as antigen are a specific and sensitive method for detecting pathogenic *Yersinia* infection [22, 26, 28]. To determine the prevalence of pathogenic *Yersinia* infection in breeding monkeys, we conducted a seroepidemiological study in squirrel monkeys in Japan by ELISA using semi-purification Yops as antigen.

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MATERIALS AND METHODS

Serum samples: Two hundreds fifty-two serum samples were collected from 9 zoological gardens (A–I) in Japan, and tested by ELISA for antibodies to Yops. In addition, 91 serum samples which were collected from Suriname immediately after importation were used as negative control. The serum samples were stored at –20°C until use, and inactivated at 56°C for 30 min before use.

Yops preparation: Yops were prepared according to the method of Heesemann *et al.* [12]. *Y. pseudotuberculosis* serovar 4b isolated from a dead squirrel monkey was precultured in BHI broth (Becton, Dickinson and Company, Franklin Lakes, NJ, U.S.A.) at 25°C with shaking (110 rpm) overnight. This preculture was then diluted 1:20 with fresh BHI broth and incubated with shaking (110 rpm) at 37°C for 90 min. Filter-sterilized EGTA (Sigma, St. Louis, MO, U.S.A.) was added to the medium to final concentration of 2.5 mM, and incubation was continued for 90 min at 37°C. The bacterial cells were then removed by centrifugation (7,000 × *g* at 4°C for 20 min), and clarified culture supernatant was filter-sterilized. The proteins were precipitated from this culture supernatant by the addition of solid ammonium sulfate (40 g/100 m*l* of supernatant). The precipitated proteins were dissolved in distilled water, and dialyzed with

Spectra/PoraCE Membrane MWCO:10,000 (Spectrum® Laboratories Inc., Rancho Dominguez, CA, U.S.A.). The retained volume was lyophilized and stored at –30°C until use.

SDS-PAGE: Yops were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The method used for SDS-PAGE was essentially the one described by Laemmli [16]. Briefly, Yops was suspended in Laemmli sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.), boiled for 3 min, and then subjected to a 12.0% polyacrylamide gel. After that, the gel was stained with silver nitrate.

ELISA: ELISA was carried out in flat-bottom 96-well microtiter plates (MaxiSorp; Nunc, Roskilde, Denmark). The plates were coated with 250 μg of Yops antigen/m*l* (50 μl/well) in phosphate buffer saline (pH 7.2) and incubated overnight at 4°C. The wells were then blocked with Diluent/Blocking Concentrate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, U.S.A.) at 25°C for 15 min. In each of the three wells assigned for the individual serum sample, the wells were loaded with the sample (1:40 dilution in Wash solution; KPL) and incubated at 37°C for 1 hr. The plates were washed three times with Wash solution and incubated with peroxidase-conjugated Protein G (1:1,000 dilution in Diluent/Blocking Concentrate; Invitrogen Co., Carlsbad, CA, U.S.A.) at 25°C for 1 hr. After being washed five times, the plates were incubated with substrate ABTS (KPL) for 20 min at 25°C, and the optical density (OD) was measured at 405 nm by a MTP-120 microplate reader (Corona Electric Co. Ltd., Ibaraki, Japan).

Cutoff value: The OD values of 91 monkeys from Suriname, where no presence of pathogenic *Yersinia* have been reported [9], were considered to be a negative control. The cutoff value was calculated as the mean OD of the negative sera plus 3 standard deviations (SD). The Yops antibodies were considered positive when the OD value was higher than the cutoff value. The OD values of the 91 monkeys from Suriname ranged between 0.023 and 0.112, and the mean was 0.050 (Fig. 2). The SD was calculated to be 0.021 from those results. Therefore, the cutoff value was calculated to be 0.113.

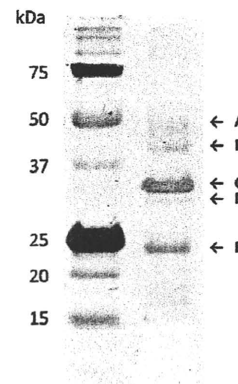

Fig. 1. SDS-PAGE followed by silver staining of the Yops. Left lane shows the prestained broad range protein molecular mass markers (Bio-Rad), and right lane shows Yops used as the antigen for ELISA.

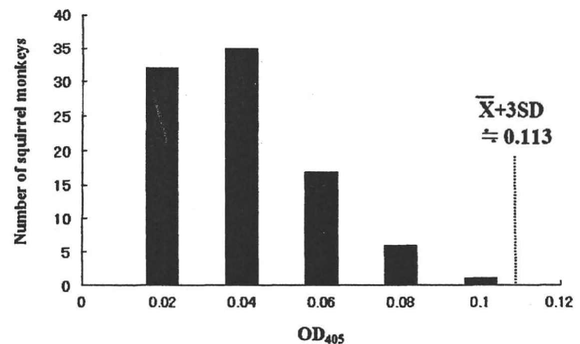

Fig. 2. Antibody titers to Yops of 91 squirrel monkeys just after imported from Suriname. The vertical dashed line represents the cutoff value, which was calculated as 3 standard deviations (SD) from the mean of this group.

RESULTS

SDS-PAGE analysis of Yops: The silver stained Yops showed 5 bands, and low background (Fig. 1). Designated bands (A-E) were considered to be YopH (51.0 kDa), YopB (41.8 kDa), YopD (33.3 kDa), YopN (32.6 kDa) and YopE (22.9 kDa), respectively [6, 21].

Prevalence of IgG antibodies to Yops in squirrel monkeys in Japan: Among the 252 squirrel monkeys tested, 164 (65.1%) showed an OD higher than the cutoff value, 0.113, and were therefore considered positive (Fig. 2). These positive monkeys belonged to 8 of the 9 zoological gardens, and the percentage of the seropositive monkeys ranged from 22.2 to 89.4% (Table 1).

Prevalence of serum antibody to Yops by age in squirrel monkeys of institution H: All squirrel monkeys in institution H were individually recognized by electronic microchips, so that information about them, including the age, was controlled. To investigate the relationship between the age and prevalence of pathogenic Yersinia, the prevalence of antibody to Yops in institution H was arranged by age (Table 2). The positive rate of the monkeys that were over 1 year old (95.7%) was significantly higher than that under 1 year old (23.3%) ($P<0.05$).

DISCUSSION

The present study demonstrated that pathogenic *Yersinia* is highly prevalent among breeding monkeys in Japan. Yops used as an antigen of ELISA are encoded in pYV, which is harbored in pathogenic strains of *Yersinia*. Regardless of the species and serovars of *Yersinia*, it is known that pathogenic *Yersinia* infection elicits specific antibody response to Yops in humans and animals [5, 13, 19]. Therefore, the squirrel monkeys considered Yops positive in the

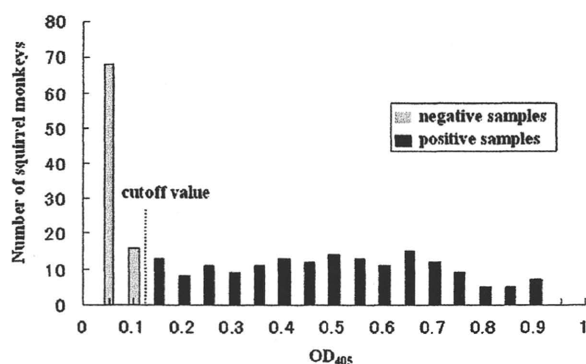

Fig. 3. Analysis of sera obtained from 252 breeding squirrel monkeys in Japan. The vertical dashed line represents the cutoff value, which was calculated as 0.113.

Table 1. Prevalence of serum antibody to Yops in squirrel monkeys from 9 institutions in Japan

Region	Institution	No. of positive samples/ No. of samples tested (%)
Kanto	A	9 /23 (39.1)
	B	12 /15 (80.0)
	C	0 /10 (0.0)
	D	2 /9 (22.2)
Kinki	E	6 /8 (75.0)
	F	6 /23 (26.1)
Shikoku	G	11 /14 (78.6)
Kyusyu	H	76 /103 (73.8)
	I	42 /47 (89.4)
Total		164 /252 (65.1)

Table 2. Prevalence of serum antibody to Yops by age in squirrel monkeys of institution H

Age (years)	No. of positive samples / No. of samples tested (%)	Average OD value
<1	7/30 (23.3)	0.084
1 – 2	13/15 (86.7)	0.300
2 – 3	18/18 (100.0)	0.420
3 – 4	3/4 (75.0)	0.389
4 – 5	10/10 (100.0)	0.494
5 – 6	7/7 (100.0)	0.559
>6	16/16 (100.0)	0.420
Unknown	2/3 (66.7)	0.261
Total	76/103 (73.8)	0.315

present study must have been infected by pathogenic *Yersinia* in the past. However, squirrel monkeys that do not have any immunity to yersiniosis, such as infant monkeys, seem to die at a high rate when infected with *Y. pseudotuberculosis* considering past studies [14, 30].

Pathogenic *Yersinia* can be divided into low pathogenic strains, which induce a mild intestinal infection in humans, and highly pathogenic strains, which cause severe systemic infection in humans [4, 10]. Whether pathogenic *Yersinia* causes limited gastroenteritis or systemic infection in humans correlate with the presence of a high-pathogenicity island (HPI), encoding an iron uptake system represented by its siderophore yersiniabactin [4] or *Y. pseudotuberculosis*-derived mitogen (YPM), which is a superantigenic toxin [1]. It is known that the presence of YPM is limited to the Far East (Japan, Korea and Far-Eastern Russia) [10], and in Japan, *Y. pseudotuberculosis* harboring YPM were isolated from almost all the fatal cases of breeding monkeys [14]. On the other hand, *Y. enterocolitica*, in particular serotype O3, O5,27, and O9 which are frequent causative agents of yersiniosis and do not harbor HPI, usually cause mild intestinal infection in humans [4, 24]. Maruyama reported that 10 Crab-eating Macaques (*Macaca fascicularis*) infected with *Y. enterocolitica* serotype O3 in experimental infection did not show any noteworthy clinical symptoms, except 3 which showed water diarrhea [20]. Each zoological garden keeps many squirrel monkeys, so even if the squirrel monkeys infected with these low pathogenic strains show the symptoms of yersiniosis, for example mild diarrhea, it is possible that those are passed over, or are not diagnosed as yersiniosis. These results suggested that the squirrel monkeys showing antibodies to Yops have been inapparently or mildly infected with low pathogenic strains of *Yersinia*, not highly pathogenic strains of *Yersinia* like YPM producing *Y. pseudotuberculosis*.

The zoological gardens which we investigated kept a number of squirrel monkeys, but did not collect sufficient information on each individual for our research purposes. However, institute H, which is located in the Kyusyu region and keeps the highest number of squirrel monkeys in Japan, individually recognizes all monkeys by electronic microchips. The microchips were implanted into all squirrel monkeys born in the years from 1997 to 2003, so the prevalence of serum antibody to Yops was arranged by age in Institute H. Almost all of the squirrel monkeys which were over 1 year old were positive, while the positive rate of those under 1 year old was only 23.3% (Table 2). These results suggest that the majority of breeding squirrel monkeys in Japan were probably infected by pathogenic *Yersinia* within one year of birth. As described above, in the present study, many squirrel monkeys that have never shown clinical signs of yersiniosis had the antibody to Yops. It is likely that inapparent infections of low pathogenic *Yersinia* frequently occur in breeding squirrel monkeys in Japan.

The present study demonstrated that pathogenic *Yersinia* is highly prevalent among breeding monkeys in Japan. Pathogenic *Yersinia* is a causal agent of zoonotic disease, and we cannot deny the possibility of human infection from monkeys. Therefore, from the point of view of public health, it is important to develop preventive methods to prevent pathogenic *Yersinia* infection in monkeys. However, as described above, pathogenic *Yersinia* strains are widely distributed in wild animals and livestock, so it is possible

that pathogenic *Yersinia* is distributed around zoological gardens. Many zoological gardens maintain breeding monkeys not only in indoor cages, but also outdoor cages or enclosures to which wild animals have easy access, so it is difficult to prevent pathogenic *Yersinia* infection in breeding monkeys even with proper attention to facility maintenance and sanitation, as well as feed hygiene. Therefore, development of an effective vaccine is important for preventing pathogenic *Yersinia* infection in breeding monkeys.

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FULL PAPER *Public Health*

Early Mortality Following Intracerebral Infection with the Oshima Strain of Tick-Borne Encephalitis Virus in a Mouse Model

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ABSTRACT. Tick-borne encephalitis virus (TBEV) is a zoonotic agent that causes acute central nervous system (CNS) disease in humans. In this study, we examined the pathogenic process following intracerebral infection with the Oshima strain of TBEV in a mouse model. Intracerebral infection resulted in dose-dependent mortality, and all mice died following challenge with 10^2 PFU or more of the virus within 10 days. Acutely necrotic neurons and widespread inflammation were observed throughout the CNS. We therefore conclude that mortality following intracerebral infection results from a direct CNS pathology.

KEY WORDS: central nervous system, mouse, pathogenesis, Tick-borne disease, virus infection.

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Tick-borne encephalitis virus (TBEV), belonging to the genus *Flavivirus* in the family *Flaviviridae*, is a zoonotic agent of acute central nervous system (CNS) disease in humans [4, 12]. TBEV is transmitted by *Ixodes* tick species and rodents in nature and infects humans through the bite of an infected tick [12]. TBEV is geographically and genetically divided into three subtypes comprising the European, Siberian and far eastern subtypes [5, 8]. Our previous data showed that TBEV is also distributed throughout southern parts of Hokkaido, Japan [23–25].

In human cases, the neurological symptoms include fever, headache, meningitis, meningoencephalitis and meningoencephalomyelitis, the latter being observed in the most severe cases [4]. When death follows, it is usually within 5 to 7 days of the onset of neurological signs. The pathological findings in the brain in human cases are nonspecific, and lesions containing TBEV antigens are located in the brain stem, cerebrum, cerebellar cortex, pons, cerebellum, thalamus and motor neurons [4, 6, 7]. Thus, the clinical features are not unique to TBE, and laboratory diagnosis is required to distinguish it from other neurological disorders [1, 10, 14].

CNS pathology following TBEV infection is the consequence of viral infection of the corresponding cells and the resulting inflammatory responses in the CNS. Direct viral infection of neurons is considered to be the major cause of neurological disease because viral infections cause apoptosis or degeneration of neurons *in vivo* and *in vitro* [3, 11, 18, 21]. In addition, recent studies have demonstrated that immunopathological effects also contribute to the severity of CNS pathology [19, 27].

The laboratory mouse model is the system most commonly employed to study the CNS pathology of TBEV *in vivo* [2, 17, 22, 26]. The CNS pathology of TBEV consists of the two distinct features of neuroinvasiveness and neurovirulence, and death has been used as an index of pathogenesis [13, 15]. Thus, mortality following peripheral infection is considered to represent neuroinvasiveness, whereas mortality following direct intracerebral infection represents neurovirulence [13].

However, our previous studies in a mouse model found that peripheral infection with the Oshima strain of TBEV caused a dose-independent mortality [2, 9]. Furthermore, we showed that following peripheral infection mice died either early or late and that mortality resulted from a combination of CNS pathology, systemic stress and inflammatory responses [9]. Together, these results indicate that peripheral infection with TBEV does not represent neuroinvasiveness alone.

On the other hand, it is considered that the patterns and the mechanism of mortality following intracerebral infection differ from those of peripheral infection. Thus, in this study we investigated the pathogenic mechanisms that correlate with fatal infection following intracerebral infection with the Oshima strain of TBEV in a mouse model.

MATERIALS AND METHODS

Virus and cells: The stock virus of the Oshima strain of TBEV [23] was prepared in baby hamster kidney (BHK) cells after a few passages through suckling mouse brains. The BHK cells were maintained in Eagle's Minimal Essential Medium (EMEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 8% fetal calf serum (FCS). All

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