JARQ 37 (4), 237 – 241 (2003) http://www.jircas.affrc.go.jp

Isolation of *Salmonella* Strains from the Aquatic Environment and Comparison with those of Animal Origin in Tan Phu Thanh Village, Mekong Delta, Vietnam

Tran Thi PHAN[1], Ly Thi Lien KHAI[1], Chau Ba LOC[1], Hideki HAYASHIDANI[2], Toshiya SAMESHIMA[3], Takeshi WATANABE[4], Toshiaki TANIGUCHI[4], Hideki KOBAYASHI[5], Hiroya ITO[6] and Masato AKIBA[6]*

[1] Department of Veterinary Medicine, Can Tho University (Can Tho City, Can Tho, Vietnam)
[2] Department of Veterinary Medicine, Tokyo University of Agriculture and Technology (Fuchu, Tokyo 183–8538, Japan)
[3] Department of Safety Research, National Institute of Animal Health (Tsukuba, Ibaraki 305–0856, Japan)
[4] Crop Production and Environment Division, Japan International Research Center for Agricultural Sciences (Tsukuba, Ibaraki 305–8686, Japan)
[5] Department of Epidemiology, National Institute of Animal Health (Tsukuba, Ibaraki 305–0856, Japan)
[6] Kyushu Research Station, National Institute of Animal Health (Kagoshima, Kagoshima 891–0105, Japan)

Abstract

This study was conducted in Tan Phu Thanh Village in the Mekong Delta, Vietnam in order to determine the prevalence of *Salmonella* spp. in the water system, and to reveal the exchange of *Salmonella* spp. occurring between the water system and livestock in this village. A total of 142 water samples were collected from 40 farms in this village from February to March, 2001. Thirty (21.1%) samples out of the 142 water samples analyzed were positive for *Salmonella* spp. in culture, based on biochemical tests. A total of 19 different serovars were observed among the 36 isolates from the positive water samples. Serovars Derby (n = 6), O3,10: r :– (n = 4), Anatum (n = 3), Bardo (n = 3), Javiana (n = 3), London (n = 2), Bovismorbificans (n = 2), and Dessau (n = 2) were isolated from more than one water sample. Serovars Derby and Javiana had been observed among the strains isolated from swine in Tan Phu Thanh Village in 2000 (Phan et al., in preparation). Consequently, we attempted to compare these strains of water and animal origins by pulsed-field gel electrophoresis. The difference in the number of bands between the isolates from water and swine was less than 4 in part of the *S.* Derby and Javiana isolates. These data provide indirect evidence for an exchange of *Salmonella* between the water system and swine population in this area. Moreover, 3 (6.1%) out of the 49 samples of stored water used for animal watering were positive for *Salmonella* spp. including the serovar Javiana. Sterilization of stored water is essential to prevent the spread of infection in this area.

Discipline: Agricultural environment
Additional key words: serovar, pulsed-field gel electrophoresis, swine

Introduction

The Mekong Delta is located in the southern part of the Indochina peninsula, and is a wet alluvial fan consisting of fertile soil derived from sediments deposited in the bed of the Mekong River. The dominant crop in this area is rice which is adapted to soils with a high water content. Agriculture in the Mekong Delta is characterized by a recycling-oriented system combining rice cropping, animal husbandry, and aquaculture. This management system is called the "VAC Farming System" or "VAC Integrated Farming System"[7]. Indica rice which is mainly grown in this area, is a long-grain rice easily bro-

*Corresponding author: fax +81–99–268–3088; e-mail akiba@affrc.go.jp
Received 1 July 2003; accepted 8 September 2003.

ken in the course of milling. The large amounts of cracked rice and rice bran thus produced are used as swine feed. Swine manure and waste water are directly discharged into on-site ponds and are used to grow plankton as bait for fish. These ponds are thus utilized for aquaculture. Most of these ponds are connected to a subsidiary stream of the Mekong River through canals, and the water content is exchanged naturally under the influence of tides.

Salmonella are ubiquitous intestinal bacteria, that cause typhoid and paratyphoid fever in humans and animals[3]. Many kinds of vertebrates including farm animals are potential hosts of these bacteria. *Salmonella* infections in livestock cause problems related to both productivity in the animal industry and public health. Asymptomatic infections of *Salmonella* in farm animals lead to contamination of farm products including meat, milk, and eggs, and consumption of these contaminated products causes Salmonellosis in humans[3]. *Salmonella* infections that are not only foodborne but also waterborne are a cause for concern in terms of public health, especially in developing countries where wastewater is directly discharged into lakes, ponds, and rivers. Contaminated surface water is a potential source of waterborne infections[5].

Tan Phu Thanh Village is located in the Chau Thanh A district of Can Tho province in the Mekong Delta. The total area of this village is 2,528 ha. According to the statistical data collected by the hamlet and village officers, 5,750 pigs, 56,000 chickens, and 40,700 ducks were raised in this village in 1999[8]. In a previous study conducted in 2000, we had investigated the prevalence of *Salmonella* among the domestic animals in 6 provinces of the Mekong Delta, including Tan Phu Thanh Village, and isolated *Salmonella* strains from 5.2% of the pigs, 7.9% of the chickens, and 8.9% of the ducks (Phan, T. T. et al., in preparation). Because animal excreta are directly discharged into ponds or canals, contamination of water systems by *Salmonella* spp. is assumed to occur. Moreover, water from rivers and canals is utilized for cleaning and as drinking water in stockbreeding, making it one of the most important sources of *Salmonella* infection in domestic animals in this area.

The present study was conducted in Tan Phu Thanh Village in order to determine the prevalence of *Salmonella* in the water system, and to highlight the exchange of *Salmonella* spp. occurring between the water system and livestock in this village.

Materials and methods

The Ba Lang River is a subsidiary stream of the Mekong River and flows through the Tan Phu Thanh Village from north to south. The 40 farms located along this river and its connecting canals within the village were targeted for sample collection. Thirty-two (80%) of the 40 farms raised pigs, 18 (45%) raised poultry including ducks and chickens, and 21 (52.5%) combined animal husbandry with aquaculture. Water samples were collected from the surface of the river, canals, ponds, and from pots containing stored water from February to March 2001. The stored water in pots originated from wells or the river, and was used for animal watering. Well water was obtained by using pumps. A total of 142 water samples were collected for isolation of *Salmonella*.

One hundred mL of each water sample was collected at the site in a sterile 250 mL culture bottle containing Enterobacteriaceae Enrichment Mannitol (EEM) broth powder (Eiken Chemical, Tokyo, Japan). The volume of the powder was appropriate for the preparation of 100 mL media as indicated by the manufacturer. The culture bottles were brought back to the laboratory and placed in a 37ºC incubator within 4 h of collection. After preenrichment in EEM broth media at 37ºC for 18 h, 10 mL of each culture was added to 100 mL of Hajna Tetrathionate Broth media (Eiken Chemical) for selective enrichment and incubated at 37ºC for 24 h. Immunomagnetic separation (Dynabeads [anti-Salmonella]; Dynal, Oslo, Norway) was then carried out using 1 mL of each enrichment culture according to the manufacturer's instructions. The beads were spread on deoxycholate hydrogen sulfide lactose (DHL) agar plates (Eiken Chemical) and incubated at 37ºC for 18 h. Three morphologically suspicious colonies from each DHL plate were picked up for biochemical analyses. The isolates were identified by their inability to ferment lactose and sucrose, and their ability to ferment glucose and to produce H_2S by using triple sugar iron (TSI) agar (Eiken Chemical). The isolates were also tested for their ability to produce lysine decarboxylase, and were tested for positive motility using lysine indol motility (LIM) medium (Eiken Chemical). The colonies thus biochemically identified as *Salmonella* spp. were serotyped using commercial O and H antisera (Denka Seiken, Tokyo, Japan, and Difco Laboratories, Detroit, MI, USA) in order to determine the antigenic formula. Polyvalent *Salmonella* O and H antisera were used to obtain a preliminary diagnosis, and the definitive antigenic formula was then determined using monovalent antisera.

Pulsed-field gel electrophoresis (PFGE) was performed by clamped homogeneous electric field electrophoresis using a CHEFF DR II apparatus (Bio-Rad Laboratories, Hercules, CA, USA) in order to compare the characteristics of the *Salmonella* strains. Genomic

DNA of each isolate was prepared by a previously described method[1]. Restriction endonuclease digestion of each sample plug was carried out using 30 U of *Xba* I (Takara Shuzo Co., Ltd, Kyoto, Japan) at 37ºC for 6 h. PFGE was performed in 1% agarose gels in twofold-diluted TBE buffer at 10ºC at 6 V/cm. The pulse time was increased from 5 to 50 s for 26 h. Lambda ladders (Bio-Rad Laboratories) were used as size markers. The PFGE profiles were scanned and analyzed using BioNumerics software (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Pairwise comparisons were made between all strains in terms of the Dice similarity coefficient[4] with the position tolerance set at 0.5%. Cluster analysis was performed using the unweighted pair arithmetic average algorithm, and a dendrogram was prepared**.**

Results and discussion

Thirty (21.1%) out of the 142 water samples collected from Tan Phu Thanh Village were positive for *Salmonella* spp. in culture based on biochemical tests. Table 1 shows the classification of the water samples and number of positive samples. Approximately 40% of the pond, canal, and river samples were positive. Meanwhile, *Salmonella* spp. were not detected in 23 well water samples. Pollution of groundwater seems therefore not to have occurred in this area. Though we recovered *Salmonella enterica* subsp. IV (Table 2) from stored water of well origin, the source of this contamination may not be the well water itself. However, the fact that 3 samples from water stored in pots were positive for *Salmonella* spp. indicates that stored water is one of the possible sources of *Salmonella* infection in farm animals. According to the manufacturer's instructions, EEM broth should be dissolved in distilled water and autoclaved before addition of samples. We added 100 mL of sample

Table 1. Classification of *Salmonella*-positive water samples

Classification	No. of samples	Positive (%)
Well	23	0
Stored water of well origin[a)]	38	1 (2.6)
Stored water of river origin[a)]	11	2 (18.2)
Pond	55	21 (38.2)
Canal	8	3 (37.5)
River	7	3 (42.9)
Total	142	30 (21.1)

a):Drinking water for animals.

Table 2. Distribution of *Salmonella* serovars isolated from water samples

Serovar	Samples					Total
	Water stored in pots		Pond	Canal	River	
	Well origin	River origin				
Derby			4	2		6
O3,10: r: –		1	3			4
Anatum			3			3
Bardo			1	1	1	3
Javiana		1	1	1		3
London			2			2
Bovismorbificans			2			2
Dessau			2			2
Paratyphi B					1	1
Schleissheim			1			1
Lexington			1			1
Mbandaka			1			1
Enteritidis			1			1
Stanley			1			1
Typhimurium			1			1
Tennessee					1	1
O3,10: UT			1			1
Subsp. III b			1			1
Subsp. IV	1					1

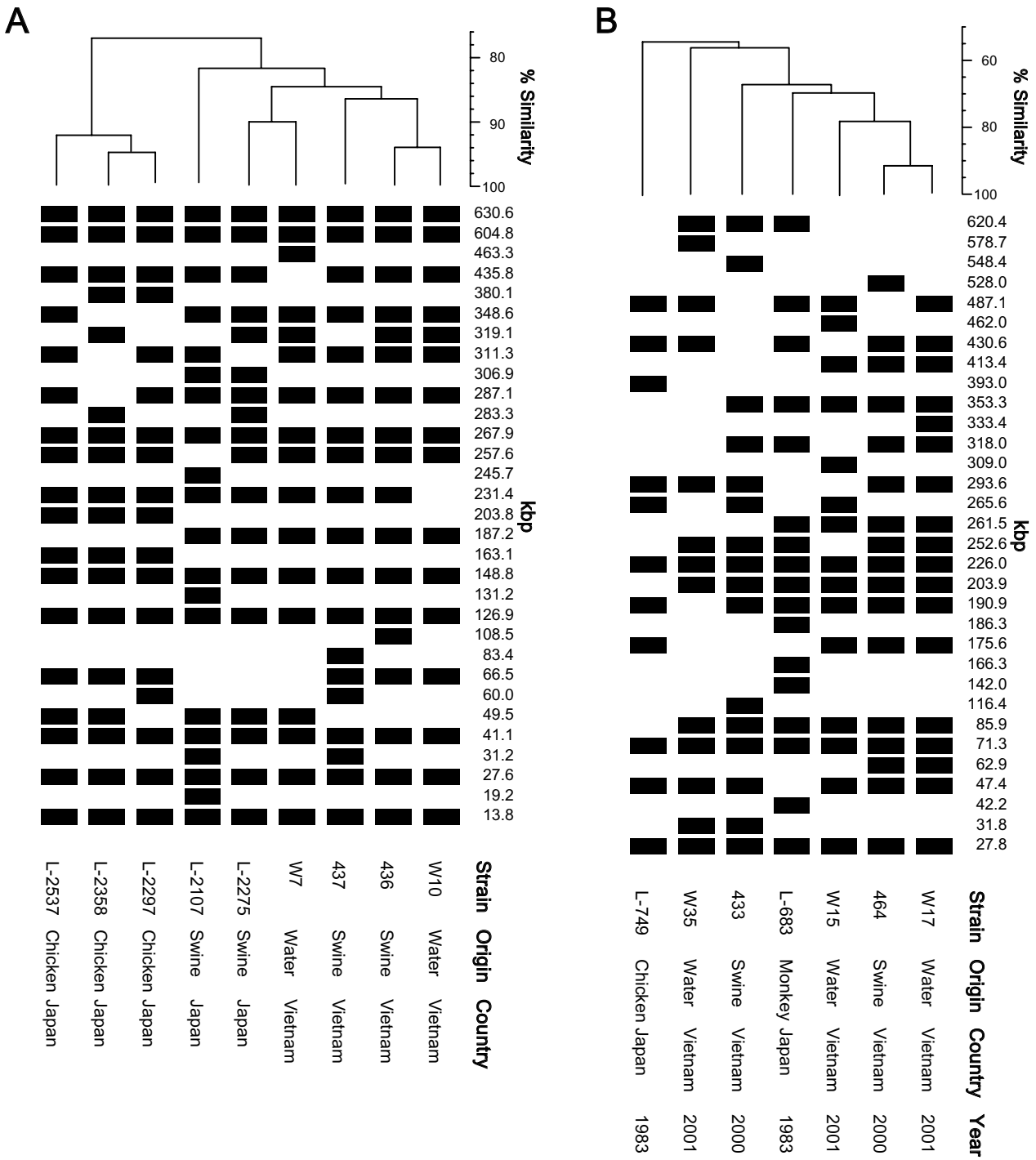

Fig. 1. Comparison by PFGE of *Salmonella* isolates obtained from the water system with those of swine origin

A: Dendrogram showing the similarity between *S.* Derby isolates and band pattern of *Xba* I cleavage fragments.

B: Dendrogram showing the similarity between *S.* Javiana isolates and band pattern of the *Xba* I cleavage fragments.

water into the EEM broth powder directly to increase the sample water volume in the enrichment broth throughout this study. We did not autoclave the EEM broth before incubation. The possibility that the positive results obtained could have been caused by *Salmonella* spp. from contaminated broth powder can not thus be ruled out in this case. However, *Salmonella* spp. were never isolated from sterilized distilled water used as negative control, indicating that all the *Salmonella* isolates were derived from sample water. On the other hand, it has not been determined whether this method generates false negative results or not.

Two different serovars were recovered from 6 water samples and a total of 19 different serovars were observed among the tested colonies. We eventually obtained 36 isolates from 30 positive samples. Table 2 shows the distribution of the *Salmonella* serovars isolated from water samples. Serovars Derby, O3,10: r:–, Anatum, Bardo, Javiana, London, Bovismorbificans, and Dessau were isolated from more than one water sample. The remaining serovars were recovered from only one sample. Aquatic environments including rivers, ponds, and lakes can be polluted by various enteropathogenic bacteria. Baudart et al. isolated 544 strains of *Salmonella* from marine and fresh water sediments, fresh water, and waste water samples collected from a Mediterranean coastal watershed[2], among which, more than 40 different serovars were identified. In the present study, we identified 19 different serovars among 36 strains isolated from water samples, indicating that the water system in Tan Phu Thanh Village was polluted by various *Salmonella* serovars based on the type of waste water. The drainage of animal wastes and of human wastes into the river may contribute to this diversity. It is also likely that the water system in this area is contaminated with other enteropathogenic bacteria including diarrheagenic *Escherichia coli*[5]. More detailed investigations should be conducted in order to elucidate the total effect of waste water contamination on the water system in this area.

S. Derby and *S.* Javiana isolates were detected in 6 and 3 water samples, respectively (Table 2). These two serovars had also been observed among the strains isolated from swine in Tan Phu Thanh Village (Phan, T. T..et al., in preparation). Consequently, we attempted to compare these strains by PFGE. Four of 6 *S.* Derby strains from the water samples could not be typed by preliminary PFGE analysis, because of the degradation of their genomic DNAs. The phenomenon was reproducible, suggesting the existence of proteinase K-resistant nuclease in these strains. We used the remaining two isolates for comparison with two isolates of swine origin. The three *S.* Javiana isolates from the water samples were also compared with two isolates of swine origin. *S.* Derby and *S.* Javiana isolates obtained in Japan were used as reference for the analysis. Digestion of genomic DNA from *S.* Derby isolates with *Xba* I gave 16 to 19 fragments with sizes ranging between 13 and 630 kb (Fig. 1A). Strains W10, 436, and 437 formed a cluster at a cut off value of 82%. A difference of two bands (231 and 108 kb) was observed between strains W10 and 436. On the other hand, digestion of *S.* Javiana strains with *Xba* I gave 12 to 18 fragments with sizes ranging between 28 and 620 kb (Fig. 1B). Strains W17, 464, and W15 formed a cluster at a cut off value of 73%. A difference of three bands (528, 487, and 333 kb) was observed between strains W17 and 464. The presence of a difference of less than 4 bands on the PFGE profiles suggested the existence of a close relation between the isolates[6]. These data might provide indirect evidence for an exchange of strains between livestock and water systems in this area.

In the present study, we have shown that the water system in Tan Phu Thanh Village is polluted by various *Salmonella* serovars. We also recovered closely related *S.* Derby and *S.* Javiana isolates from water samples and pigs in this area. These data indicate that *Salmonella* strains are exchanged between the water system and farm animals. In fact, 3 (6.1%) out of 49 samples of water stored for animal watering purposes were positive for *Salmonella,* including the serovar Javiana (Table 2). At the very least, sterilization of the stored water is essential to prevent the spread of infection. In addition, adequate environmental hygiene guidance should be provided to the farmers in this area.

References

1. Akiba, M., Sameshima,T. & Nakazawa, M. (2000) Clonal turnover of enterohemorrhagic *Escherichia coli* O157:H7 in experimentally infected cattle. *FEMS Microbiol. Lett.,* **184**, 79–83.
2. Baudart, J. et al. (2000) Diversity of *Salmonella* strains isolated from the aquatic environment as determined by serotyping and amplification of the ribosomal DNA spacer regions. *Appl. Environ. Microbiol.,* **66**, 1544–1552.
3. Guthrie, R. K. (1992) Salmonella. CRC Press, USA, pp.220.
4. Nei, M. & Li, W. -H. (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA*, **76**, 5269–5273.
5. Ohno, A. et al. (1997) Enteropathogenic bacteria in the La Paz River of Bolivia. *Am. J. Trop. Med. Hyg.,* **57**, 438–444.
6. Tenover, F. C. et al. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol*., **33**, 2233–2239.
7. Tuyen, N. Q. et al. (2000) Farming systems of Tan Phu Thanh Village, Chau Thanh District, Can Tho Province in the recent years. *In* Proceedings of the 2000 annual workshop of JIRCAS Mekong Delta Project, JIRCAS, Tsukuba, Japan, 31–39.