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Research Note

Genetic Relatedness of *Salmonella* Serovars Isolated from Catfish (*Clarias gariepinus*) and Tilapia (*Tilapia mossambica*) Obtained from Wet Markets and Ponds in Penang, Malaysia

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ABSTRACT

A total of 43 *Salmonella enterica* isolates belonging to different serovars (*Salmonella* Albany, *Salmonella* Agona, *Salmonella* Corvallis, *Salmonella* Stanley, *Salmonella* Typhimurium, *Salmonella* Mikawasima, and *Salmonella* Bovismorbificans) were isolated from catfish (*Clarias gariepinus*) and tilapia (*Tilapia mossambica*) obtained from nine wet markets and eight ponds in Penang, Malaysia. Thirteen, 19, and 11 isolates were isolated from 9 of 32 catfish, 14 of 32 tilapia, and 11 of 44 water samples, respectively. Fish reared in ponds were fed chicken offal, spoiled eggs, and commercial fish feed. The genetic relatedness of these *Salmonella* isolates was determined by random amplified polymorphic DNA PCR (RAPD-PCR) using primer OPC2, repetitive extragenic palindromic PCR (REP-PCR), and pulsed-field gel electrophoresis (PFGE). Composite analysis of the RAPD-PCR, REP-PCR, and PFGE results showed that the *Salmonella* serovars could be differentiated into six clusters and 15 singletons. RAPD-PCR differentiated the *Salmonella* isolates into 11 clusters and 10 singletons, while REP-PCR differentiated them into 4 clusters and 1 singleton. PFGE differentiated the *Salmonella* isolates into seven clusters and seven singletons. The close genetic relationship of *Salmonella* isolates from catfish or tilapia obtained from different ponds, irrespective of the type of feed given, may be caused by several factors, such as the quality of the water, density of fish, and size of ponds.

Key words: Catfish; Genetic diversity; *Salmonella* serovars; Tilapia

Salmonella spp. are gram-negative, rod-shaped bacteria that cause salmonellosis *(1)*. In humans, typhoidal *Salmonella* strains are responsible for enteric fever, while nontyphoidal *Salmonella* strains are the causative agents for acute gastroenteritis *(1, 18)*. The symptoms of salmonellosis are fever, diarrhea, and cramping, with the incubation period generally between 6 and 72 h *(18)*. Outbreaks of salmonellosis due to fish consumption have been reported in several countries *(3)*. Various hazards associated with cultured fish usually originate from the environment or human or animal activities. Dissemination of *Salmonella* may occur within cultured fish or aquaculture environments and, in a more complex situation, from cultured fish to the aquaculture environment or vice versa.

Molecular typing tools are useful in determining the possible routes of transmission, source, and traceability of the pathogens. Various typing methods based on phenotypic and genotypic analyses have been used to differentiate between or to determine similarities among *Salmonella* strains *(14, 28, 36)*. As reported in previous studies, random amplified polymorphic DNA PCR (RAPD-PCR) *(2, 6)*, repetitive extragenic palindromic PCR (REP-PCR) *(2, 11)*, and pulsed-field gel electrophoresis (PFGE) *(14, 30, 34)* are widely used in determining the genetic relatedness of *Salmonella* isolates in order to trace the dissemination of *Salmonella* serovars in the food chain. Generally speaking, PFGE, which differentiates the isolates based on the restriction sites within the genomes of bacteria, is considered the "gold standard" for typing *Salmonella* because of its high reproducibility and discriminatory power *(23)*. RAPD-PCR and REP-PCR, which target the random and repetitive DNA elements of *Salmonella* serovars, respectively, are widely used, as these two techniques are considerably cheaper and less laborious than PFGE. However, both RAPD-PCR and REP-PCR lack reproducibility, and thus, molecular typing using combined methodologies is increasing in popularity and utility *(26)*.

In the Asia-Pacific region, cultured fish are fed either commercial or homemade feed (fresh feed material or farm feed material) *(15)*. Homemade feed is used to reduce cost and usually consists of chicken viscera and by-products produced during poultry processing, kitchen refuse, and other by-products of food industries *(15, 16)*. Homemade feed can serve as a potential source for foodborne pathogens,

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especially *Salmonella* bacteria *(9, 24)*, which could then be transmitted to cultured fish *(22)* and, in turn, to humans.

Detailed information on salmonellosis due to consumption of catfish and tilapia in Malaysia is lacking, as most food poisoning cases are not reported to the authorities. Nevertheless, the National Public Health Laboratory of Malaysia reported that the five most prevalent nontyphoidal *Salmonella* serovars were *Salmonella* Enteritidis, *Salmonella* Weltevreden, *Salmonella* Corvallis, *Salmonella* Typhimurium, and *Salmonella* Tshiongwe *(32)*. In Malaysia, freshwater fish are reared using pond culture, ex-mining pools, freshwater cages, cement tanks, canvas tanks, and freshwater pen culture systems. Most of the cultured freshwater fish are reared using pond culture systems (59.5%) and ex-mining pools (25%). Catfish (58.1%) and tilapia (41.3%) are reared in earthen ponds and ex-mining pools, respectively *(13)*.

In this study, REP-PCR, RAPD-PCR, and PFGE analyses were used to determine the genetic relatedness of various *Salmonella* serovars isolated from catfish (*Clarias gariepinus*) and tilapia (*Tilapia mossambica)* obtained from nine wet markets and eight ponds in Penang, Malaysia. Another aim of this study was to determine whether the three typing methods can be used to elucidate the sources of different *Salmonella* serovars. Composite analysis of the REP-PCR, RAPD-PCR, and PFGE results was performed to enhance the discriminative power.

MATERIALS AND METHODS

Samples. Forty-three isolates belonging to different *Salmonella* serovars (*Salmonella* Albany, *Salmonella* Agona, *Salmonella* Corvallis, *Salmonella* Stanley, *Salmonella* Typhimurium, *Salmonella* Mikawasima, and *Salmonella* Bovis-morbificans) were isolated from catfish, tilapia, and water samples obtained from nine local wet markets and eight ponds (Table 1). These isolates were identified by standard microbiological methods as previously described *(8)*.

Preparation of genomic DNA for DNA fingerprinting. A single colony of pure *Salmonella* culture was inoculated into 5 ml of tryptic soya broth (Merck KGaA, Darmstadt, Germany) and incubated in a Lab Companion S1-300 orbital shaker (Jeio Tech, Seoul, South Korea) with constant shaking at 200 rpm (at 37 ± 1°C) for 16 to 18 h. The overnight culture (3 ml) was centrifuged (Eppendorf 5415R, Dublin, Ireland) at 1,000 × *g* for 5 min. Cell pellets were allowed to dry by inverting the centrifuge tubes onto paper towel and were subsequently subjected to genomic DNA extraction using the Genomic Purification kit (Promega, Madison, WI), following the manufacturer's instructions *(4)*.

Genotyping of *Salmonella* isolates using RAPD-PCR. Three random primers, namely, OPC2 (5′-GTGAGGCGTC-3′), OPC5 (5′-GATGACCGCC-3′), and OPG13 (5′-CTCTCCGCCA-3′), were selected for RAPD-PCR after screening 40 random primers. The PCR reaction mixture (25 μl) consisted of 2.5 μl of 10× PCR buffer (Sigma Aldrich, St. Louis, MO), 1 μl of 10 mM deoxynucleoside triphosphate (dNTP) mixture (Promega), 0.5 μl *Taq* DNA polymerase (5 U/μl) (Promega), 5.6 μl of 25 mM $MgCl_2$ (Promega), 1.6 μl of 5 mM random primer, 100 ng of genomic DNA, and sterile nuclease-free double-distilled water (Promega). Amplification was performed using the TProfessional Standard Gradient 96 Thermocycler (Biometra, Göttingen, Germany). PCR was performed as previously described by Szczuka and Kaznowski *(29)*, with modifications of the denaturation, annealing, elongation, and final extension conditions. This consisted of four initial denaturation cycles (94°C for 3 min, 34°C for 3 min, and 72°C for 3 min), followed by 35 cycles at 94°C for 30 s, 34°C for 1 min, 72°C for 2 min, and a final extension cycle (94°C for 30 s, 34°C for 1 min, and 72°C for 30 min). Five microliters of the PCR product was mixed with 1 μl of EZ-Vision One DNA Dye (Amresco, Solon, OH) and separated in a 1.5% (wt/vol) agarose gel (Promega) using a horizontal gel electrophoresis system (GES Elite 300, Wealtec, Taipei, Taiwan) at 100 V for 135 min in 0.5× Tris-acetate-EDTA buffer (Promega). The DNA bands were visualized using the Molecular Imager (Gel Doc XR+ System, Bio-Rad, Hercules, CA). The approximate size of each DNA band was determined by comparison with a 1-kb DNA ladder (Promega).

Genotyping of *Salmonella* isolates using REP-PCR. REP-PCR was performed using an 18-mer primer (5′-GCGCCGI CATGCGGCATT-3′) *(32)*. The PCR mixture (25 μl) consisted of 2.5 μl of 10× PCR buffer (Sigma Aldrich), 0.5 μl of 10 mM dNTP mixture (Promega), 0.6 μl of *Taq* DNA polymerase (5 U/μl) (Promega), 1.5 μl of 25 mM $MgCl_2$ (Promega), 10 μl of 1 μM REP-PCR primer, 100 ng of genomic DNA, and sterile nuclease-free double-distilled water (Promega). Amplification was performed using the TProfessional Standard Gradient 96 Thermocycler (Biometra) with the following conditions: 2 cycles of 94°C for 5 min, 33°C for 5 min, and 68°C for 5 min, followed by 30 cycles of 94°C for 1 min, 45°C for 1 min, and 68°C for 2 min and a final extension at 68°C for 16 min *(32)*.

Genotyping of *Salmonella* isolates using PFGE. Genomic DNA for PFGE analysis was prepared in agarose plugs according to a previously described protocol *(31)*. Briefly, colonies of an overnight cell culture were suspended in cell suspension buffer (100 mM Tris and 100 mM EDTA, pH 8.0) and the cell density was adjusted to 0.8 to 0.9 by using a Dade Microscan turbidity meter (Baxter Diagnostics, Inc., Deerfield, IL). Portions of the standardized cell suspension were mixed with equal volumes of 1% SeaKem gold agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME) to form plugs. The plugs were then subjected to lysis in cell lysis buffer (50 mM Tris, 50 mM EDTA [pH 8.0], 1% sarcosyl, and 1 mg/ml proteinase K) and incubated in a water bath at 54 ± 1°C for 2 to 4 h. The plugs were then washed thoroughly in sterile deionized water and Tris-EDTA buffer. A slice of plug was subjected to overnight digestion using 10 U of restriction enzyme *Xba*I (Promega) at 37°C. The digested DNA fragments were subjected to PFGE in a 1% (wt/vol) agarose gel (Agarose Type 1, Sigma Aldrich) using the CHEF MAPPER (Bio-Rad, Berkeley, CA). PFGE was performed for 24 h at 14°C and 6 V/cm with an initial pulse time of 2.16 s and final pulse time of 63.8 s. *Xba*I-digested *Salmonella* Braenderup (H9812) was used as the standard for DNA size determination. The gel image was captured by using the Gel Doc XR imaging system (Bio-Rad) after staining with GelRed.

The DNA fingerprints produced by RAPD-PCR, REP-PCR, and PFGE were analyzed by using BioNumerics version 6.0 software (version II; Applied Maths, Kortrijk, Belgium). The variability of the strains was determined based on the Dice coefficient of similarity (*F*) and the unweighted pair group with arithmetic mean algorithm. The discriminatory index (*D*) was calculated using Simpson's index of diversity, as follows:

TABLE 1. Salmonella *serovars, sampling locations, and types of feed*

Location	Type of feed	Sample type (type of fish)[a]	*Salmonella* serovar	Isolate
Pond A1	Chicken offal	FCR (catfish)	Albany	AlbCpo1-R1
		Water	Albany	AlbCpo1-W2
		Intestines (catfish)	Stanley	StCpo1-I1
		Water	Stanley	StCpo1-W1
		Intestines (catfish)	Agona	AgCpo1-I3
		FCR (catfish)	Agona	AgCpo1-R3
		Water	Agona	AgCpo1-W3
Pond A2	Chicken offal	Intestines (catfish)	Albany	AlbCpo2-I3
		water	Albany	AlbCpo2-W2
Pond B1	Commercial fish feed	FCR (catfish)	Albany	AlbCpo3-R2
		Intestines (catfish)	Albany	AlbCpo3-I3
		FCR (catfish)	Albany	AlbCpo3-R3
		Water	Albany	AlbCpo3-W1
Pond B2	Commercial fish feed	FCR (catfish)	Albany	AlbCpo4-R3
Pond C1	Spoiled egg	FCR (tilapia)	Corvallis	CorTpo1-R3
		Intestines (tilapia)	Corvallis	CorTpo1-I3
		FCR (tilapia)	Corvallis	CorTpo1-R1
		Intestines (tilapia)	Corvallis	CorTpo1-I1
		Intestines (tilapia)	Corvallis	CorTpo1-I2
		Water	Corvallis	CorTpo1-W3
		Water	Corvallis	CorTpo1-W1
Pond C2	Spoiled egg	Intestines (tilapia)	Corvallis	CorTpo2-I2
		Intestines (tilapia)	Corvallis	CorTpo2-I1
		FCR (tilapia)	Corvallis	CorTpo2-R1
		Intestines (tilapia)	Corvallis	CorTpo2-I3
		FCR (tilapia)	Typhimurium	TyTpo2-R3
Pond D1	Commercial fish feed	FCR (tilapia)	Corvallis	CorTpo3-R2
		Water	Bovis-morbificans	BoTpo3-W3
		FCR (tilapia)	Bovis-morbificans	BoTpo3-R3
Pond D2	Commercial fish feed	FCR (tilapia)	Corvallis	CorTpo4-R2
		FCR (tilapia)	Mikawasima	MiTpo4-R1
		Intestines (tilapia)	Mikawasima	MiTpo4-I1
		FCR (tilapia)	Mikawasima	MiTpo4-R3
Bukit Mertajam wet market	NA[b]	FCR (catfish)	Albany	AlbCM4-R3
		Water	Corvallis	CorCM4-W3
		Intestines (catfish)	Corvallis	CorCM4-I4
Gelugor wet market	NA	Water	Corvallis	CorCM1-W2
Bayan Baru wet market	NA	Water	Albany	AlbCM2-W4
		FCR (tilapia)	Corvallis	CorTM1-R2
		Intestines (catfish)	Typhimurium	TyCM2-I1
		FCR (tilapia)	Agona	AgTM1-R3
		Intestines (catfish)	Albany	AlbCM2-I2
Hypermarket S1	NA	Intestines (tilapia)	Corvallis	CorTM3-I4

[a] FCR, fish carcass rinsate.
[b] NA, not applicable.

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j - 1).$$

where N is the total number of strains in the sample population, n_j is the number of strains falling into the jth type, and s is the total number of types described. A D-value of 1.0 would indicate that a typing method was able to distinguish each member of a strain population from all other members of that population. A D-value of 0.5 would indicate that if one strain was chosen at random from a strain population, then there would be a 50% probability that the next strain chosen at random would be indistinguishable from the first *(20)*.

RESULTS AND DISCUSSION

The genetic relatedness of 43 *Salmonella* strains belonging to seven different serovars was determined by analyzing the results of RAPD-PCR (Fig. 1), REP-PCR (Fig. 2), and PFGE (Fig. 3). RAPD-PCR was performed using multiple primers, and as the PCR products obtained using OPC2 gave the highest D-value, the data obtained with OPC2 are presented. In this study, RAPD-PCR using OPC2 produced better and more discriminative typing results for *Salmonella* isolates than did PFGE and REP-PCR. Forty-three *Salmonella* isolates were grouped into 11 clusters (C21 to 29, C210, and C211) and 10 singletons (Fig. 1). REP-

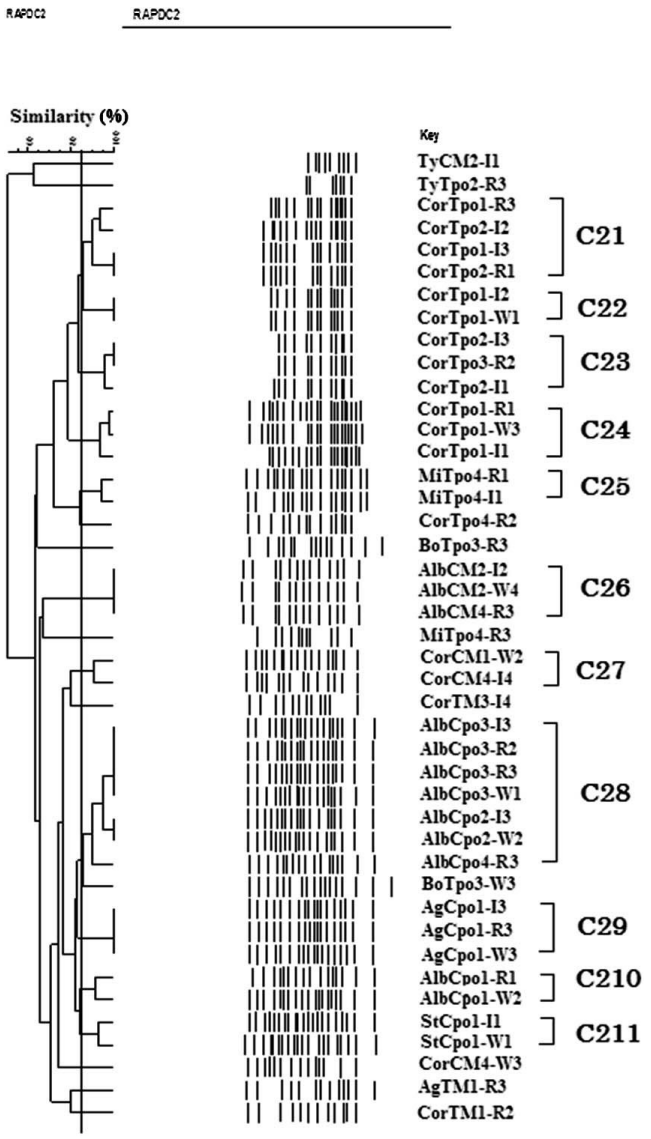

FIGURE 1. *Dendrogram of* Salmonella *serovars in catfish, tilapia, and water samples constructed using RAPD-PCR (primer OPC2) results. Codes indicate* Salmonella *serovars as follows: Ag, Agona; Alb, Albany; Bo, Bovis-morbificans; Cor, Corvallis; Mi, Mikawasima; Ty, Typhimurium; St, Stanley.*

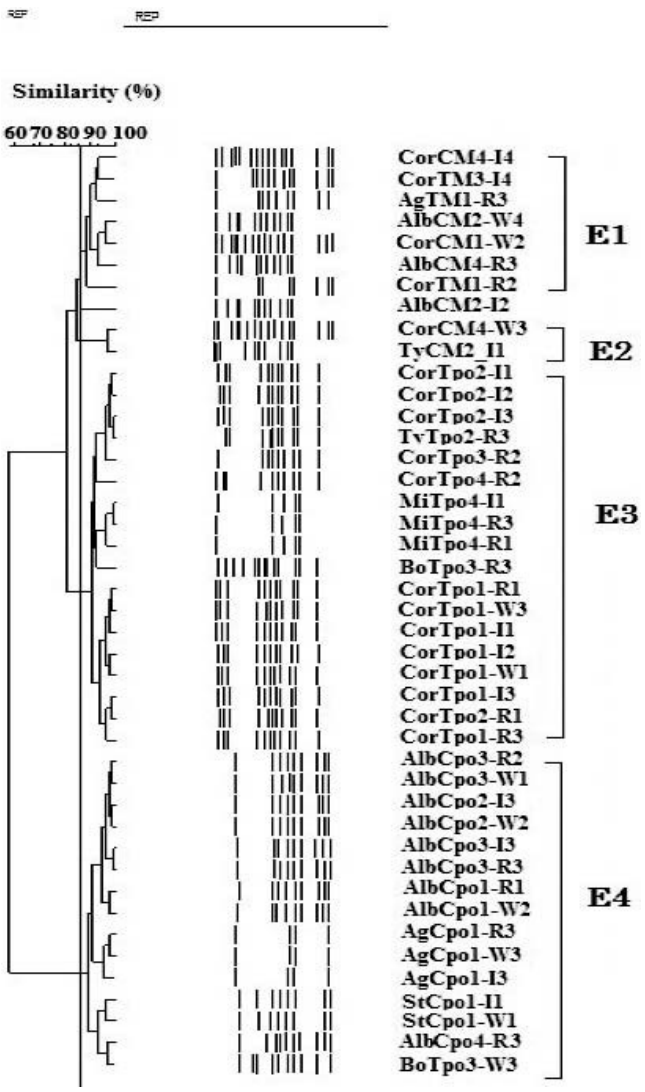

FIGURE 2. *Dendrogram of* Salmonella *serovars in catfish, tilapia, and water samples constructed using REP-PCR results. Codes indicate* Salmonella *serovars as follows: Ag, Agona; Alb, Albany; Bo, Bovis-morbificans; Cor, Corvallis; Mi, Mikawasima; Ty, Typhimurium; St, Stanley.*

PCR differentiated the strains into four clusters (E1 to E4) and one singleton (Fig. 2). PFGE differentiated the *Salmonella* strains into seven clusters (F1 to F7) and seven singletons (Fig. 3). The composite analysis of the results of RAPD-PCR, REP-PCR, and PFGE enhanced the discriminative power, as all 43 *Salmonella* serovars were differentiated into six clusters (I to VI) and 15 singletons (Fig. 4). *Salmonella* Corvallis ($n = 18$) isolates were differentiated into two clusters (I and II), which contained six isolates each, and six singletons.

In this study, *Salmonella* Corvallis isolates in cluster F3 (PFGE) were further differentiated by RAPD-PCR into two clusters (C21 and C24). Similarly, *Salmonella* Albany isolates belonging to cluster F1 (PFGE) were differentiated into two clusters (C28 and C210) by RAPD-PCR. A similar observation was made for *Salmonella* Agona isolates (Fig. 1 and 3). These results are in agreement with those of other researchers, who reported that PFGE was inadequate to differentiate genetically monomorphic isolates *(5, 10, 33)*.

RAPD-PCR is able to demonstrate genetic relatedness between isolates of the same serovar and produce serovar-specific clusters. *Salmonella* Corvallis was grouped into five different clusters (C21 to C24 and C27), and *Salmonella* Albany was clustered into three clusters (C26, C28, and C210). *Salmonella* Agona, *Salmonella* Mikawasima, and *Salmonella* Stanley were grouped into C29, C25, and C211, respectively. Similarly, PFGE grouped members of the same serovars into one cluster except for *Salmonella* Corvallis isolates, which were differentiated into two clusters (F3 and F4). REP-PCR, however, did not differentiate *Salmonella* strains according to serovar, as each cluster had more than two different serovars in the same cluster.

Another finding of this study that should be highlighted is the genetic relatedness of *Salmonella* isolates from catfish (*C. gariepinus*) and tilapia (*T. mossambica*) obtained from

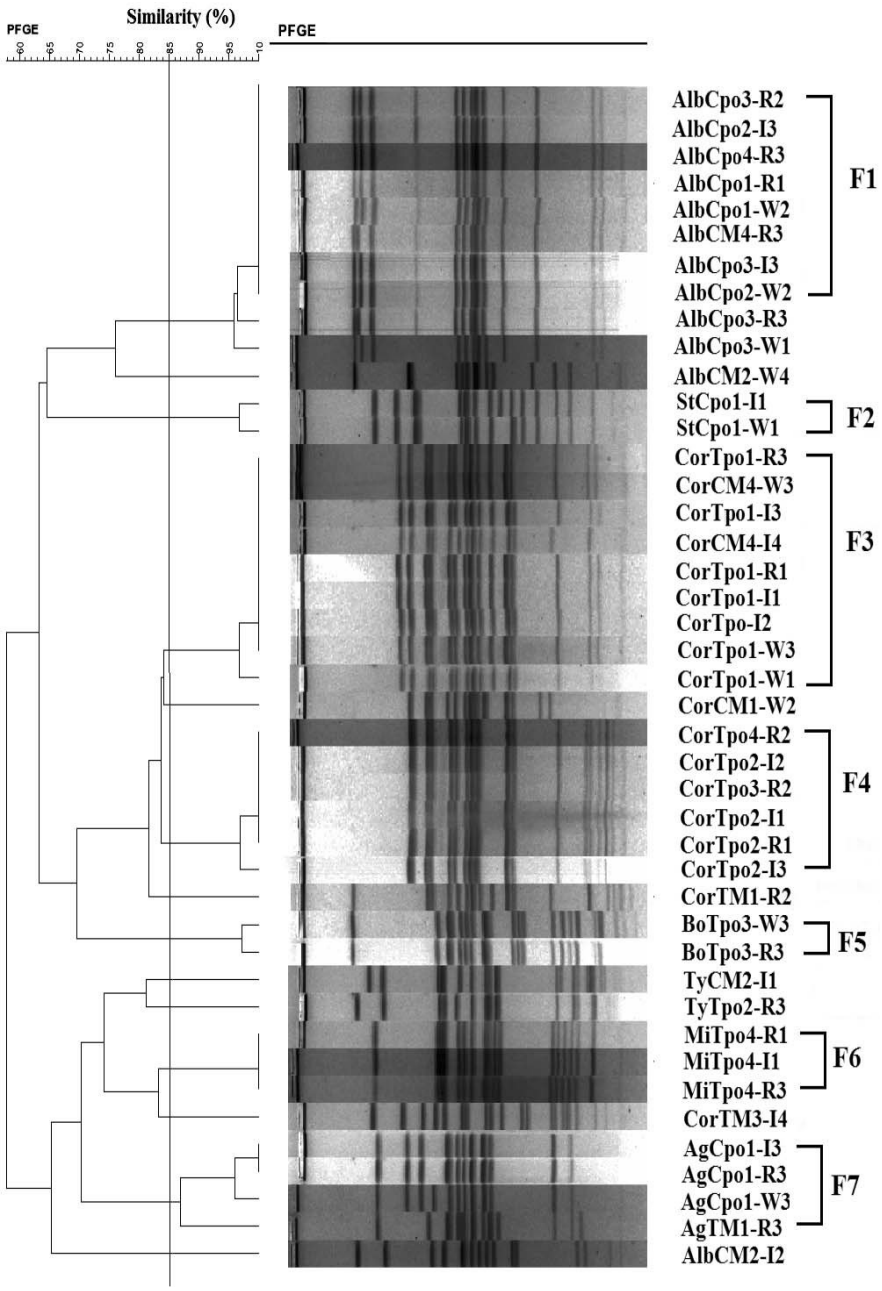

FIGURE 3. *Dendrogram of* Salmonella *serovars in catfish, tilapia, and water samples constructed using PFGE results. Codes indicate* Salmonella *serovars as follows: Ag, Agona; Alb, Albany; Bo, Bovis-morbificans; Cor, Corvallis; Mi, Mikawasima; Ty, Typhimurium; St, Stanley.*

ponds (where fish were fed chicken offal, spoiled eggs, or commercial fish feed) and those from wet markets. The three typing methods used in this study differentiated these *Salmonella* serovars according to source (location of pond) or the type of feed used. The dendrograms in Figures 1 and 2 show that RAPD-PCR and REP-PCR each grouped three isolates of *Salmonella* Corvallis from tilapia obtained from ponds C1 and C2 into one cluster (C21 using RAPD-PCR and E3 using REP-PCR). The common feature for these two ponds was the use of spoiled eggs as feed, even though the ponds were approximately 10 km from each other. The results suggested that the *Salmonella* Corvallis isolates might have originated from spoiled eggs. This homemade feed originated from the same source, and the ponds are managed by the same company. The presence of *Salmonella* Enteritidis on the shells of eggs and in their contents *(19),* migration of *Salmonella* Enteritidis from the shells of eggs into the albumen and yolk *(7, 12),* and the predicted growth of *Salmonella* Enteritidis in eggs *(17)* have been reported extensively.

Besides the finding described above, the close genetic relatedness of *Salmonella* isolates from fish (catfish or tilapia) and water samples should be highlighted. The dendrogram in Figure 4 shows that most of the strains of *Salmonella* Corvallis, *Salmonella* Albany, *Salmonella* Agona, and *Salmonella* Stanley, originating from fish and water samples, were clustered together, suggesting that cross-contamination may have occurred between fish and the aquatic environment in ponds, irrespective of the type of feed used. These findings are in agreement with those reported by Amagliani et al. *(3),* who observed that *Salmonella* could enter the aquatic environment through poor sanitation. Iwamoto et al. *(21)* reported that polluted water promotes *Salmonella* colonization of fish and, thus,

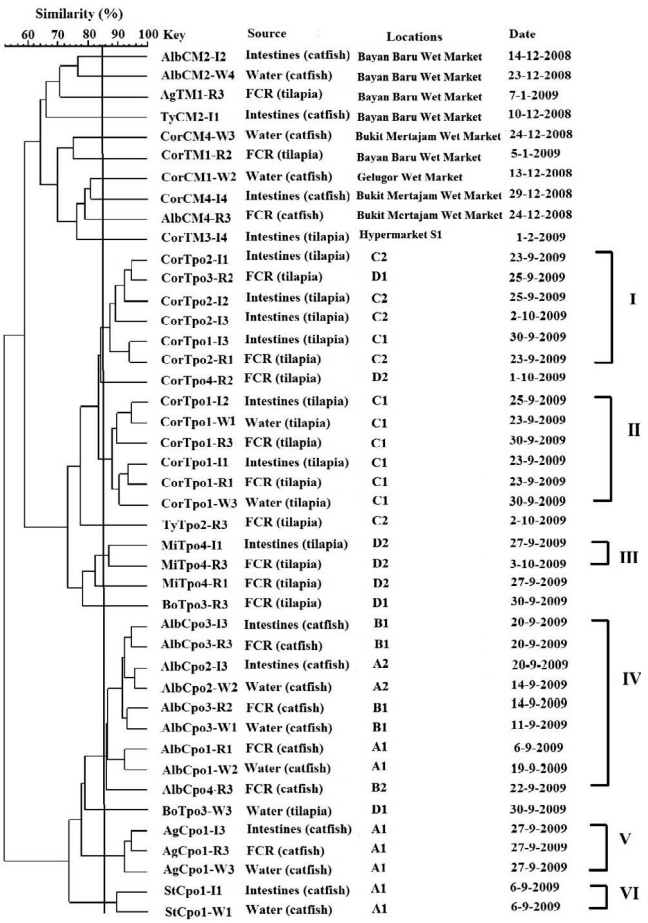

FIGURE 4. *Dendrogram of* Salmonella *serovars in catfish, tilapia, and water samples constructed using composite results from RAPD-PCR, REP-PCR, and PFGE. Codes indicate* Salmonella *serovars and sample sources as follows: Ag, Agona; Alb, Albany; Bo, Bovis-morbificans; Cor, Corvallis; Mi, Mikawasima; Ty, Typhimurium; St, Stanley; FCR, fish carcass rinsate; A1, A2, ponds with chicken offal feed system; C1, C2, ponds with spoiled egg feed system; B1, B2, D1, D2, ponds with commercial pellet feeding system; A1, A2, B1, B2, earthen ponds; C1, C2, D1, D2, ex-mining pools.*

becomes a potential agent of *Salmonella* infection in humans.

We also observed that two isolates of *Salmonella* Corvallis, from tilapia fed with spoiled egg and a water sample obtained from pond C1 were grouped together into clusters C22 (RAPD-PCR), E3 (REP-PCR), and F3 (PFGE), respectively. Three strains each of *Salmonella* Corvallis isolated from tilapia (fed with spoiled eggs) and from water samples from ponds C1 and C2 (where fish were fed with spoiled eggs) and D1 (fed with commercial feed) were grouped into clusters C24 and C23, respectively (RAPD-PCR), E3 for both (REP-PCR), and clusters F3 and F4, respectively (PFGE). Composite analysis clustered six strains of *Salmonella* Corvallis isolated from tilapia obtained from ponds C1, C2, and D1 in cluster I. A similar observation was noted for *Salmonella* serovars isolated from different sources. For instance, three strains of *Salmonella* Agona isolated from catfish and water samples obtained from pond A1 were clustered into cluster V. *Salmonella* Stanley isolates from catfish and water samples obtained from pond A1 were clustered into cluster VI. These results indicate that genetically different strains of *Salmonella* serovars may have originated either from the feed or from the ponds' environment. The presence of *Salmonella* in ponds depended on many factors, e.g., the stocking density, water temperature, size of the fish, organic matter content, and size of the ponds *(8, 25, 35)*.

Salmonella Typhimurium isolates from catfish obtained from Bayan Baru wet market and tilapia obtained from pond C2 (fed with spoiled eggs) were not genetically related, as they were isolated from different locations or sample types. No strains isolated from catfish, tilapia, and water samples obtained from wet markets were clustered by the composite diagram of RAPD-PCR, REP-PCR, and PFGE results, suggesting that the *Salmonella* isolates were genetically diverse. Based on the findings from wet markets, we are of the opinion that most probably catfish and tilapia sold in wet markets were purchased from suppliers who had purchased fish from ponds that were in different locations. In our study, RAPD-PCR was able to cluster isolates according to sources of isolation within a single serovar, and to a certain extent, it was able to explain the clusters formed based on epidemiological background information. These features have made RAPD-PCR fingerprinting a valuable epidemiological tool in this study. Moreover, our results are in agreement with those of others *(27, 36)*, who reported that RAPD-PCR is one of the most convenient, fast, and reliable techniques for discriminating intraspecific variations compared with many PCR-based and non–PCR-based techniques, such as PFGE, for *Salmonella*. The promising results obtained with primer OPC2 should be confirmed on a larger number of strains, but RAPD-PCR analysis seems as suitable as PFGE for comparison of *Salmonella* strains.

In conclusion, composite analysis of the results of RAPD-PCR, REP-PCR, and PFGE enhanced the discriminatory power compared with the results of RAPD-PCR, REP-PCR, and PFGE alone. Since different methods have different levels of discriminatory power, the application of more than one subtyping approach would provide a more accurate picture of the clonality of *Salmonella* strains. The genotyping data showed that the *Salmonella* strains isolated from fish were genetically diverse and heterogeneous. The genetic relatedness among *Salmonella* serovars suggests cross-contamination between *Salmonella* in fish and water or vice versa. Hence, surveillance programs are needed to monitor epidemiological information on the prevalence of pathogens in different aquatic environments in Malaysia.

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