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Molecular typing among poultry isolates of *Salmonella agona* by PCR-based techniques and pulsed field gel electrophoresis (PFGE)

(Penjenisan molekul antara pencilan ayam *Salmonella agona* dengan menggunakan teknik berasaskan PCR dan PFGE)

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Key words: *Salmonella agona*, PCR-ribotyping, enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), random amplified polymorphic DNA-PCR (RAPD-PCR), pulsed field gel electrophoresis (PFGE)

Abstrak

Sembilan DNA genom *Salmonella agona* yang telah dipencilkan daripada sumber ayam telah dicirikan dengan tiga variasi teknik yang berasaskan PCR (PCR-ribotaip, ERIC-PCR dan RAPD-PCR) dan PFGE. Pencilan ini berasal dari tiga negeri di Semenanjung Malaysia (Pulau Pinang, Negeri Sembilan dan Selangor). Keputusan yang diperoleh daripada PCR-ribotaip, ERIC-PCR, RAPD-PCR dan PFGE untuk membezakan sembilan isolat telah dibandingkan. Pencilan telah dipisahkan kepada tiga jenis genom yang berbeza apabila menggunakan PCR-ribotaip, dan sembilan serta lapan jenis genom masing-masing apabila menggunakan ERIC-PCR dan PFGE.

RAPD-PCR profil menggunakan tiga 10-mer oligonukleotida mendapati aras aturan kepelbagaian DNA yang tinggi antara pencilan *S. agona* yang telah dikaji. Gabungan tiga teknik yang berasaskan PCR dan teknik PFGE dengan menggunakan sekatan enzim *Xba*I, telah dapat mengenal pasti sembilan jenis genom *S. agona*. Keputusan ini menunjukkan kedua-kedua teknik tersebut sesuai untuk penjenisan molekular terhadap pencilan yang dikaji.

Abstract

Nine genomic DNA of *Salmonella agona* isolated from poultry sources were characterised by three variations of PCR-based techniques [polymerase chain reaction (PCR)-ribotyping, enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR), and random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR)] and pulsed field gel electrophoresis (PFGE). These isolates originated from three different states of Peninsular Malaysia (Pulau Pinang, Negeri Sembilan and Selangor). The results of PCR-ribotyping, ERIC-PCR, RADP-PCR and PFGE to differentiate between nine isolates of *S. agona* were compared. The isolates were separated into three different genome types by PCR-ribotyping, nine and eight genome types by ERIC-PCR and PFGE, respectively.

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The RAPD-PCR profiles using three 10-mer oligonucleotides revealed a high level of DNA sequence diversity within the *S. agona* isolates tested. Using a combination of three variations of PCR-based techniques and PFGE with restriction enzyme *Xba*I, nine genome types of *S. agona* were identified. These results demonstrate that both techniques are suitable tools for molecular typing of the isolates examined.

Introduction

Salmonellae are the etiological agents of different diseases collectively referred to as salmonellosis. More than 2 200 serovars of *Salmonella* have been identified and every serovar has a tendency to cause illness (John 1998). Salmonellosis can be categorised into typhoidal or non-typhoidal. In human, there are four syndromes of salmonellosis: enteric fevers (typhoidal-like disease), gastroenteritis (food poisoning), bacteremia with or without gastroenteritis and asymthomatic carrier states.

Salmonella agona infections can cause a non-typhoidal salmonellosis with diarrheal illness (Fisher et al. 1994). Its incidence was related to imported contaminated fish meal and cereal to poultry and pigs. Many outbreak cases in human were reported in United Kingdom, Israel, the United States and Canada (Fisher et al. 1994; Synnott et al. 1998).

Outbreaks of salmonellosis in human related with *S. agona* have not been reported in Malaysia. However, a survey to estimate the prevalence of *Salmonella* serovars from broilers at retail outlets, processing plants and farms, indicates that this food product is a potential vector for *Salmonella* in Malaysia (Rusul et al. 1996). In this study, *S. agona* represented 1.7% of total isolates while other reports have shown that the major source of *Salmonella* was also from chicken carcasses (64.3%) and *S. agona* ranks the fourth most frequent serovar (Chye and Mohd. 1994).

The clinical importance of *S. agona* in gastrointestinal diseases was not as important as *S. typhimurium* and *S. enteritidis* which most commonly implicate poultry products. However in recent years, there has been a gradual increase in the incidence of *Salmonella* outbreaks worldwide due to *S. agona*. In Malaysia, the data obtained shows that non-human sources may act as reservoirs of *S. agona* and that in the early phase of an epidemic due to clone *Salmonella*, may play an important role as sources of human infection.

It is well accepted that precise determination of *Salmonella* clones is an important part in the investigation of human epidemics of salmonellosis, and the application of DNA-based technique is one way of examining *Salmonella* that may be held to elucidate the epidemiology of salmonellosis. In this study, three variations of PCR-based techniques and pulsed field gel electrophoresis were used to analyse the DNA diversity of *S. agona* isolated from poultry sources.

Materials and methods

Bacterial strains

The poultry isolates of *S. agona* used in this study were washed carcasses of poultry meat obtained from wet markets in three states of Peninsular Malaysia (Pulau Pinang, Negeri Sembilan and Selangor). The laboratory methods used to isolate the *Salmonella* serovar were as described by Rusul et al. (1996).

Bacterial growth and DNA preparation

All strains were grown in Lauria-Bertani (LB) broth at 37 °C with shaking at 200 rpm overnight. Prior to amplification by PCR-ribotyping, RAPD-PCR and ERIC-PCR, total genomic DNA of the *S. agona* strains were extracted by the conventional phenol-chloroform-isoamyl method as desribed by Sambrook et al. (1989).

PCR-ribotyping

The 16S-23S rDNA was amplified with primers: 5'-TTGTACACACCGCCCGTCA-3' (F) and 5'-GGTACTTAGATGTTTCAGTTC-3' (R) (Lagatolla et al. 1996). Amplifications were performed in total reaction volumes of 100 µL and contained 2.5 µM of each prime, 250 µM each of NTP and 0.5 U of *Taq* polymerase (Promega, U.S.A.) in the presence of the buffer with the polymerase (final concentration 2.5 mM $MgCl_2$). Amplification conditions comprised 35 cycles of 1 min at 94 ºC, 1 min at 55 ºC and 2 min at 72 ºC. Followed by a final elongation step for 5 min at 72 ºC. Amplified products (15 mL) were separated by electrophoresis in agarose 1.2% gels in 1X TBE (0.1 M Tris, 0.1 M boric acid, 0.2 mM EDTA), stained with ethidium bromide and photographed under UV transillumination. The 1.0 kb DNA ladder (Promega, USA) was used as DNA size marker.

RAPD-PCR

A randomly designed 10-mer oligonucleotide set, designated as GEN1 to GEN10 was obtained from the Genosys Biotechnologies Inc. (Texas, USA). The GEN1, GEN6 and GEN9 were chosen for RAPD-PCR analysis because they yielded clear patterns. PCR-mixes were conducted in a final volume of 25 µL containing variable volumes genomic DNA as a template. The mixture: 1X PCR containing 20.0 mM Tris-HCl (pH 8.4), 50.0 mM KCl, 2.0 mM $MgCl_2$, 100 mM (each) deoxynucleotide triphosphate (dNTP), 0.5 µM primer, and 2.5 U of *Taq* polymerase (Promega). For all PCR experiments, negative controls containing sterile deionised water instead of DNA as template were always included.

RAPD was carried out using a thermal cycler (Perkin Elmer 2400). The cycling parameters were 1 min at 92 °C, 1 min at 36 °C and 2 min at 72 °C for a total of 45 cycles, with a final cycle extending amplification conditions to 72 °C for 5 min. Amplified products (15 mL) were separated by elecrophoresis in agarose 1.2% gels in 1X TBE, stained with ethidium bromide and photographed under UV transillumination. The 1.0 kb DNA ladder (Promega, USA) was used as DNA size marker.

ERIC-PCR

The primer used were ERIC1R (5'-CACTTAGGGGTCCTCGAATGTA-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') as described by Versalovic et al. (1991). PCR amplification reactions consisted of 25 µL volume containing 10 ng of genomic DNA, 2.5 µL 10x buffer, 2.5 unit *Taq* DNA polymerase, 5 µM each of the forward and reverse primers, 2 mM $MgCl_2$, and 1 mM each of dCTP, dGTP, dATP and dTTP. Amplification was done using a Perkin Almer 2400 thermocycler (Perkin-Elmer, Norwalk, USA) as follows: 92 °C for 45 s, 52 °C for 1 min, 70 °C for 20 min and a final elongation step at 70 °C for 20 min at the end of 35 cycles. The amplification products were fractionated by electrophoresis through 1.2% agarose gel in 1X TBE buffer (0.1 M Tris, 0.1 M boric acid, 0.2 mM EDTA), detected by staining with ethidium bromide and photographed under UV transillumination. The 1.0 kb DNA ladder (Promega, USA) was used as DNA size marker.

Chromosomal analysis by PFGE

The PFGE technique of contour-clamped homogenous electric field (CHEF) electrophoresis (Chu et al. 1986) was used for genomic typing of *S. agona* strains. Genomic DNA was digested in agarose plug with *Xba*I (Promega, USA) as recommended by the manufacturer. The resulting fragments were resolved by CHEF-PFGE by using a CHEF-DRIII apparatus (Bio-Rad Laboratories, Richmond, CA, USA) in gels of 1.0% agarose in 0.5X TBE (0.1 M Tris, 0.1 M boric acid, 0.2 mM EDTA) at 6 V/cm for 20 h at 14 °C with ramped pulsed time of 0.1–30 s. Lambda concatamers (Promega,

USA) were used as DNA size standards. After electrophoresis, gels were stained in ethidium bromide and photographed.

Results

PCR-ribotyping

Electrophoretic analysis of the amplified products disclosed 4 bands ranging from 0.7 to 2.0 kb in each sample. Three PCR-ribotypes were consistently observed in repeated analysis *(Table 1)*. Some of the bands appeared weak, consisting probably of secondary products ranging from less than 0.5–1.0 kb. *Plate 1* shows the electrophoretic patterns of PCR-ribotypes of *S. agona* isolates.

Table 1. Molecular typing of *Salmonella agona*

Strains	PCR-ribotypes	ERIC-PCR	PFGE	RAPD-PCR profiles of different primers			Genome types
				GEN1 (A)	GEN6 (B)	GEN9 (C)	
SA1	R1	E1	X1	A1	B1	C1	1
SA2	R2	E2	X2	A2	B2	C2	2
SA3	R1	E3	X3	A3	nd	C3	3
SA4	R3	E4	X4	A4	B3	C4	4
SA5	R3	E5	X5	A5	B4	C5	5
SA6	R2	E6	X6	A6	B5	C6	6
SA7	R3	E7	X7	A7	B6	C7	7
SA8	R2	E8	X7	A8	B7	C8	8
SA9	R3	E9	X8	A9	B8	C9	9

A = primer pattern for GEN1, B = primer pattern for GEN6, C = primer pattern for GEN9,
R = pattern for PCR-ribotyping, E = pattern for ERIC-PCR, X = based on PFGE pattern by *Xba*I.
nd = not detected

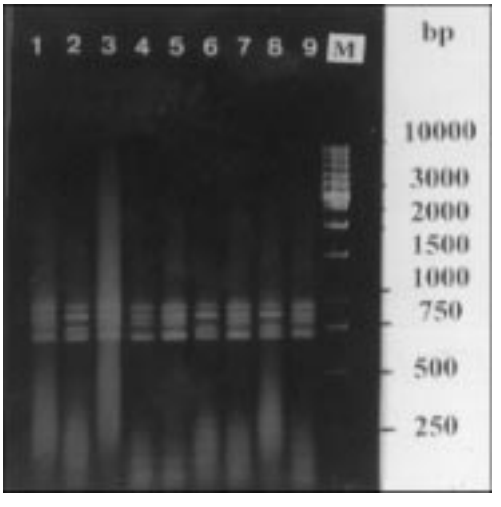

Plate 1. The electrophoretic patterns of PCR-ribotypes of ***Salmonella agona*** *isolates electrophoresed on 1.2% agarose gel.*
Lane 1 = SA1, 2 = SA2, 3 = SA3, 4 = SA4, 5 = SA5, 6 = SA6, 7 = SA7, 8 = SA8, 9 = SA9.
M = Molecular weight sizes (base pairs, bp) are indicated by numbers on the right

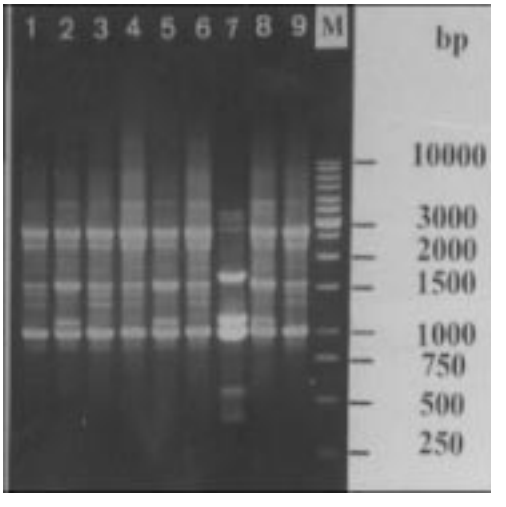

Plate 2. RAPD-PCR profiles of ***Salmonella agona*** *obtained with primer GEN1 electrophoresed on 1.2% agarose gel.*
Lane 1 = SA1, 2 = SA2, 3 = SA3, 4 = SA4, 5 = SA5, 6 = SA6, 7 = SA7, 8 = SA8, 9 = SA9.
M = Molecular weight sizes (base pairs, bp) are indicated by numbers on the right

RAPD-PCR fingerprintitng

A total of 10-mer of different oligonucleotide primers were used for RAPD-PCR analysis of a subset of three isolates to detect polymorphism within *S. agona*. The three primers produced a clear pattern and were used to analyse the whole set of nine *S. agona* strains. *Plates 2–4* show the RAPD profiles of *S. agona* obtained with primer GEN1, GEN6 and GEN9, respectively. The possible number of RAPD-profiles was estimated on the basis of changes in one or more clear bands or band sizes. Nine RAPD-profiles were apparent from primer GEN1, GEN6 and GEN9.

However, no band produced with isolates SA3 *(Plate 2)* with primer GEN6. The number of RAPD bands produced for a given primer ranged from 0 to 12, with molecular sizes ranging from 0.25 kb to 6.0 kb. The RAPD-profiles generated with the three primers were consistently observed in repeated analysis.

ERIC-PCR

In RAPD-PCR a single arbitrarily primer anneal at a low temperature (36 °C) at sites within 200 to 2000 base pair of each other in the correct orientation (5' to 3') on opposite strands of the target DNA, will amplify the sequence between the two sites. In contrast with ERIC-PCR, the primers were annealed at a higher temperature of 52 °C and amplification of DNA sequences by consensus PCR primers, located between successive repetitive elements.

The ERIC-PCR genomic fingerprinting of *S. agona* revealed nine distinct patterns *(Table 1). Plate 5* shows the ERIC-PCR patterns of *S. agona* isolates. The possible number of ERIC-PCR pattern was estimated on the basis of changes in one or more clear bands or band sizes. The ERIC-PCR patterns generated with the primer were consistently observed in repeated analysis.

Chromosomal DNA analysis by PFGE

Pulsed field gel electrophoresis analysis of *Xba*I digested DNA yielded 2 to 11

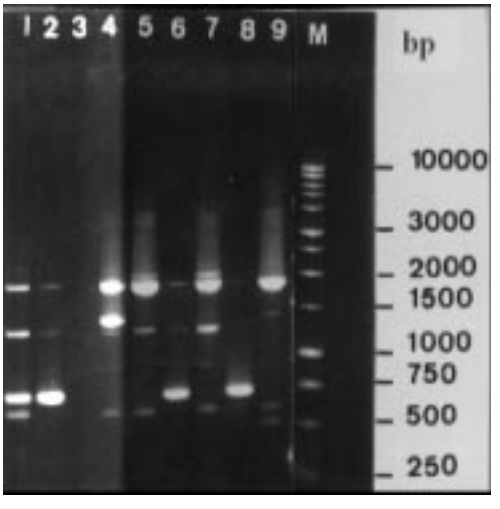

Plate 3. RAPD-PCR profiles of ***Salmonella agona*** *obtained with primer GEN6 electrophoresed on 1.2% agarose gel. Lane 1 = SA1, 2 = SA2, 3 = SA3, 4 = SA4, 5 = SA5, 6 = SA6, 7 = SA7, 8 = SA8, 9 = SA9. M = Molecular weight sizes (base pairs, bp) are indicated by numbers on the right*

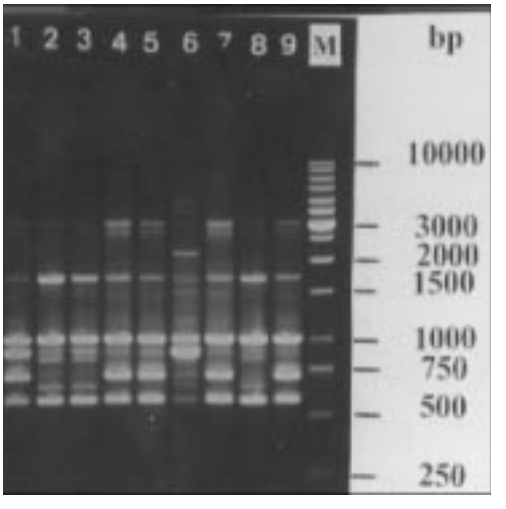

Plate 4. RAPD-PCR profiles of ***Salmonella agona*** *obtained with primer GEN9 electrophoresed on 1.2% agarose gel. Lane 1 = SA1, 2 = SA2, 3 = SA3, 4 = SA4, 5 = SA5, 6 = SA6, 7 = SA7, 8 = SA8, 9 = SA9. M = Molecular weight sizes (base pairs, bp) are indicated by numbers on the right*

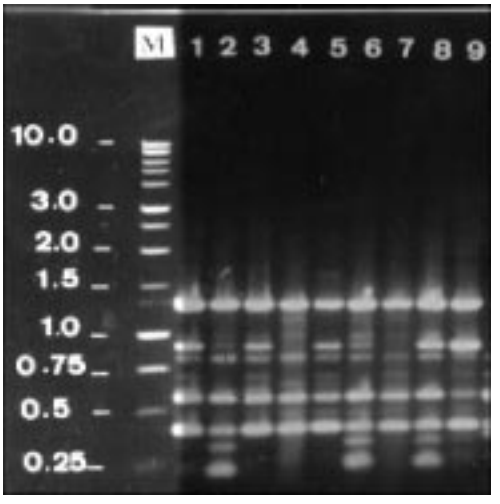

Plate 5. ERIC-PCR profiles of ***Salmonella agona*** *electrophoresed on 1.2% agarose gel.*
Lane 1 = SA1, 2 = SA2, 3 = SA3, 4 = SA4, 5 = SA5, 6 = SA6, 7 = SA7, 8 = SA8, 9 = SA9. M = Molecular weight sizes (base pairs, bp) are indicated by numbers on the right

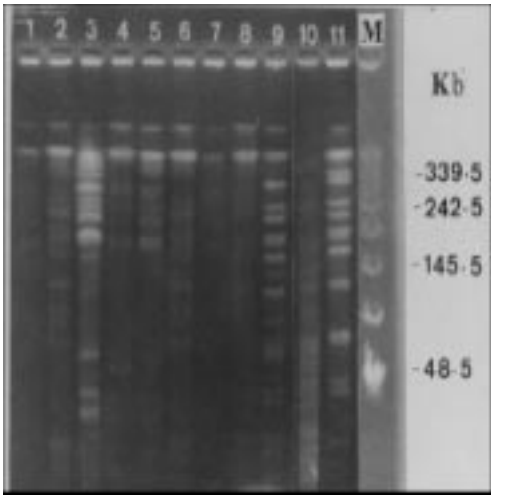

Plate 6. PFGE profiles of ***Salmonella agona*** *with restriction enzyme of XbaI, electrophoresed on 1.0% agarose gel.*
Lane 1 = SA1, 2 = SA2, 3 = SA3, 4 = SA4, 5 = SA5, 6 = SA6, 7 = SA7, 8 = SA8, 9 = SA9. M = Molecular weight sizes (kilobase pairs, kbp) are indicated by numbers on the right

separated DNA fragments ranging from 27 to 570 kbp (*Plate 6* and *Table 1*). The PFGE patterns obtained by digestions with *Xba*I differed eight isolates which consistently separated by repeated analysis. Only 100 kbp bands were scored. A strain was considered to be different if its PFGE patterns differed by one or more bands whose size was above this limit. In PFGE, digested plasmid migrates according to the sizes of fragments, while uncut plasmids are reported to present aberrant mobilities (Mathew et al. 1988). In this study, only bands that were larger than 100 kbp were scored. This size limit was chosen partly because the largest plasmid demonstrated is approximately 90 kbp (Son et al. 1998). Therefore, the presence of plasmids in DNA preparation for PFGE had no influence on the interpretation and stability of typing results.

Discussion

In this study, the *S. agona* isolates were analysed by DNA-based techniques, the PCR-based methods namely PCR-ribotyping, RAPD-PCR and ERIC-PCR, and PFGE. The total genomic DNA from nine poultry isolates of *S. agona* provided templates for PCR-ribotyping. In the present study we investigated the possibility of differentiating *S. agona* isolates at the intraserovar level on the basis of polymorphism of 16S-23S rRNA intergenic spacer regions. Traditional ribotyping was obtained from the pattern of restriction fragments of chromosomal DNA which hybridised with probed for the operon encoding rRNA (*rrn*).

With appropriate choice of restriction endonuclease and probe for the species under investigation, this method has been shown to detect sufficient heterogeneity within many species to provide useful epidemiological information (Saunders et al. 1991; Bingen et al. 1994). The PCR-ribotyping which has been shown to discriminate between isolates within other species (Lagatolla et al. 1996), showed low potential in distinguishing *S. agona* isolates

in the present study. Only, three PCR-ribotyped observed in this serovar *(Table 1)*.

Therefore, in order to obtain a sufficient number of polymorphic bands which would permit reliable comparisons, 10 different 10-mer primers (Genosys Biotechnologie Inc. Texas, U.S.A) were used in RAPD-PCR amplification reaction. However, only three primers (GEN1 [5'-GTGCAATGAG-3'], GEN6 [5'-CGGATAACTG-3'] and GEN9 [5'-AGAAGCGATG-3']) produced different and informative banding patterns for all strains *(Plates 1–3)*.

The RAPD-PCR analysis effectively distinguished different *S. agona* isolates. The primers GEN1, GEN6 and GEN9 each detected nine, eight, and nine RAPD-PCR types among nine isolates, respectively (*Table 1*). However, in primer GEN6, isolate SA3 was consistently not typable in repeated experiment, this could be interpreted as the loss of specific sites for primer binding in the chromosomal DNAs of this isolate. The combination of three amplification patterns allow identification of nine RAPD-PCR types.

The same results were also observed in ERIC-PCR profiles, which nine ERIC-PCR types were obtained from the present study. These results were consistent with Burr et al. (1998) who reported the presence of a wide heterogeneity within *Salmonella* spp. isolates. In contrast to the great diversity of ERIC-PCR fingerprints in the present study, Millemen et al. (1996) identified just two patterns in 56 *Salmonella typhimurium* and 14 *Salmonella enteritidis* isolates from poultry.

The *Xba*I digest of the nine typable *S. agona* isolates and generated eight different PFGE patterns with two isolates (SA7 and SA8) possessed the same PFGE pattern *(Table 1)*. This method showed less extensive genetic diversity between typable *S. agona* isolates when compared to PCR-based techniques.

The techniques used in this study showed differences in capacity to differentiate between the nine *S. agona* isolates. In the present study, PCR-based techniques were shown to be a typing method with high degree discriminative power that was superior to PFGE. However, it is evident that PCR-based techniques and PFGE can complement each other, for instance isolates which possessed the same pattern (SA7 and SA8) in PFGE were resolved by ERIC-PCR and RAPD-PCR.

It has also been shown that the isolate (SA3) untypable by RAPD-PCR in primer GEN6 was resolved by other primers of GEN1 and GEN9. This was supported by the findings of Samore et al. (1996) who reported that isolates untypable by PFGE were typable by other techniques such as RAPD and restriction enzyme analysis. When the results of PCR-based techniques and PFGE with restriction enzyme *Xba*I were combined, all the *S. agona* isolates were shown to have individual genetic profiles. Thus, the isolates of this serovar examined exhibit high level of local geographical genetic variation. These results demonstrate the value of using more than one typing techniques in epidemiological investigation. In routine typing technique, PCR-based techniques can ease operation, simpler, more economical and many isolates could be analysed simultaneously. These characteristics were important for effective epidemiological surveillance, prevention and control of important bacterial infections. Whereas, PFGE is time consuming and labour-intensive.

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