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Molecular characterization and antimicrobial resistance profiling of *Salmonella enterica* subsp. *enterica* isolated from 'Selom' (*Oenanthe stolonifera*)

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Abstract: Fifty-nine isolates of *Salmonella enterica* subsp. *enterica* (*S. enterica*) isolated from indigenous vegetables, 'selom' (*Oenanthe stolonifera*) associated with 13 different serovars were obtained from Chemistry Department of Malaysia. The isolates encompass the common serovar, *Salmonella enterica* subsp. *enterica* serovar Weltevreden (*S*. Weltevreden) (39%) and *Salmonella enterica* subsp. *enterica* serovar Agona (*S*. Agona) (8.5%). Frequencies of the other 11 *Salmonella* serovars were ranged from 1.7% to 5.1%. All isolates were characterized by Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR), random amplified polymorphic DNA (RAPD), plasmid profiling and antimicrobial susceptibility testing. The results demonstrated ERIC-PCR, RAPD and composite analysis of both are suitable typing methods for *S. enterica* by demonstrating good discriminative ability and can be utilize as a rapid approach of comparing *S. enterica* isolates for epidemiological investigation. From this study, ERIC-PCR is exhibited lower discriminatory power when compare with RAPD. On the other hand, plasmid profiles yielded 32 profiles with molecular size ranging from 1129 bp to 17911 bp. Thirteen antimicrobial agents were included in this study and all isolates showed 100% (59/59) resistant to erythromycin and showed Multiple Antimicrobial Resistance (MAR) indexes ranging from 0.08 to 0.68. Dendrogram generated from antimicrobial resistance profiling exhibited poor discriminatory capability at serovar level. Although poultry still remain as the common reservoir for multi-drug resistant (MDR) *Salmonella*. The isolation of 13 *Salmonella* serovars from selom that showed high MDR in this study is alarming. These results supported the notion that indigenous vegetable (selom) are gaining more antimicrobial resistance and could be potential health hazards.

Keywords: *S. enterica*, ERIC-PCR, RAPD, plasmid profiling, multiple antimicrobial resistance, indigenous vegetable

Introduction

The widespread of *S. enterica* in humans and animals worldwide have always been a major public health concern (Yan *et al*., 2003; Yoke-Kqueen *et al*., 2008a). In recent years, the growing consumption of fresh vegetables has led to an increase in the number of outbreaks of food-borne disease linked to fresh produce (Anon, 2002). The common practice worldwide in agricultural irrigation with animal waste fertilizer or wastewater contributed to the main source of pathogen contamination (Madden, 1992; Thong *et al*., 2002). Therefore fresh produce have become one of the transmission mode for pathogens to causing human illness (Beuchat, 1996). In Malaysia, fresh 'selom' (*Oenanthe stolonifera*) are often gathered from natural growth and consumed raw or minimally processed. On the other hand, there is no microbiological monitoring for this type of vegetables (Noor Zaleha *et al*., 2003). *Salmonella* is one of the most common bacterial enteropathogens associated with fruits and vegetables (Thunberg *et al*., 2002). According to Center of Disease Control and Prevention (CDC), salmonellosis is responsible for 1.4 million illnesses and 500 deaths annually in develop country like USA (Mead *et al*., 1999). *S*. Weltevreden as the most common serovar type in this study has emerged as the most important cause of nontyphoidal salmonellosis in South East Asia and Western Pacific (WHO, 2005).

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In recent years, antimicrobial resistance in *Salmonella* has received considerable attention as the emergence of MDR *Salmonella* may result in treatment failure (Yan *et al*., 2003). Continuous monitoring revealed the isolation frequency of MDR *Salmonella* has been increased in the UK (Snow *et al*., 2007), USA (Frye and Fedorka-Cray, 2007), Malaysia (Yoke-Kqueen *et al*., 2008a) and elsewhere globally. Therefore, characterization of MDR *S. enterica* is important to gain better understanding on the epidemiology of the pathogenesis of multi-resistant bacteria as well as the movement of antimicrobial resistance genetic elements between bacterial populations (Yoke-Kqueen *et al*., 2008b).

The increased of hazards possessed by *S. enterica* towards human health globally have made the role of molecular typing and subtyping crucial in the surveillance of community food-borne outbreaks of salmonellosis. RAPD is a PCR-based detection method, where usually one short arbitrary (8-12 mer) primer is used to amplified sequences without the extent of homology of the DNA needing to be known (Welsh and McClelland 1990; William *et al*., 1990; Yoke-Kqueen *et al*., 2008b). RAPD fingerprinting produces a spectrum of amplified products based on characteristic of each template DNA due to arbitrary priming at multiple locations throughout the genome. The resulting fingerprints can be of epidemiological value and RAPD could be a powerful tool to assess genetic diversity in situations where traditional typing method like multilocus enzyme electrophoresis (MLEE) and ribotyping could not discriminate the different between isolates (Tenover *et al*., 1995). ERIC-PCR involves the use of oligonucleotides targeting short repetitive sequences dispersed throughout various bacterial genomes. ERIC sequences are dispersed throughout the genome of enterobacteriaceae in different orientations thus enable their location in bacterial genomes allows discrimination at genus and serovars level based on their electrophoretic amplification products (Appuhamy *et al*., 1998; Biendo *et al*., 2005; Yoke-Kqueen *et al*., 2008b). Plasmid profiling has been reported as an essential tool in providing important extra dimension to the flexibility of the organisms' response to change in its environment by encoding useful additional properties of the cell like antimicrobial resistance (Low *et* al., 1997; Rychlik *et al*., 2006), virulence (Rychlik *et al*., 2006) and also capability to produce protein like colicin and bacteriocin. Furthermore plasmid profiling has been widely applied to study different serovars of *Salmonella* organisms, including *S*. Typhi (Wain and Kidgell, 2004), *S*. Derby (Ling *et al*., 2001), *S*. Enteritidis (Song and Suh, 2006) and many other serovars (Chu *et al*., 2001; Nayak *et al*., 2004).

The aims of this study were: (i) To determine the antimicrobial susceptibility of all isolates; (ii) To characterize all isolates using antibiograms, plasmid profiling, RAPD and ERIC-PCR; (iii) To compare single and composite data analysis of RAPD and ERIC-PCR in deciphering the relatedness among different *Salmonella* serovars.

Materials and Methods

Bacterial isolates

Fifty-nine isolates of *S. enterica* as shown in Table 1 were successfully recovered from 'selom' (*Oenanthe stolonifera*). Isolates were obtained from Chemistry Department of Malaysia. The Chemistry Department of Malaysia is a national reference laboratory for microbiology food testing and it comprises of four main divisions, namely Forensic, Environmental Health, Research and Quality Assurance, Industrial and Classification Trade Tariff, Development and Information Technology. All bacterial strains were serotype with respect to three antigenic sites, the flagella (H), Capsular (Vi) and somatic (O) by Veterinary Research Institute (Ipoh, Malaysia).

Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested according to CLSI guidelines (Clinical and Laboratory Standards Institute, 1999 and 2002) for the disk diffusion method (Bauer *et al*., 1966) using antimicrobial disks from Oxoid, Basingstoke, U.K. All isolates were tested for their susceptibility to ampicilin (10 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cefuroxime (30 µg), cephalothin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamicin (10 µg), nalidixic acid (30 µg), streptomycin (10 µg), tetracycline (30 µg) and trimethoprim-sulphamethoxazole (25 µg).

Single colony of *Salmonella* serovars pure cultures were grown overnight in Luria Bertani (LB) at 37°C until a 0.5 McFarland turbidity standard was attained. A volume of 0.1 ml of the culture was then swabbed onto dry Mueller Hinton agar (Difco, Michigan, USA) using a sterile cotton swab. Four antimicrobial disks were dispensed onto each plate sufficiently separated from each other so as to avoid overlapping of inhibition zones in mm. The plates were incubated at 37^{0}C for 16 to 18 hours. The inhibition zones were measured and scored as sensitive, intermediate and resistant according to the CLSI guidelines. *Escherichia coli* ATCC 25922 was used as a reference strain for antimicrobial

Table1. Antibiograms, multiple antimicrobial resistance (MAR) indices and plasmid profiles of 59 *S. enterica* isolates from Selom (*Oenanthe Stolonifera*)

Serovars	Isolates code	Antibiograms	MAR index	Plasmid Size (bp) 13450	Plasmid profile
Agona	A1, A2	E	0.08	5882, 7142, 10785, 13450	4.6
	A3	E	0.08	8831	1.1
	A4, A5	E	0.08	5882, 7142, 8831, 10785	4.1
Brunei	B1	AmpCipCroCtxCxmENaSTe	0.69	8831, 10785	2.1
	B2	AmpCipCroCtxCxmENaSTe	0.69	8831, 10785, 13450, 17911	4.4
	B3	AmpCnCroCtxCxmENaSTe	0.69	4918, 7142, 8831, 10785	4.3
Bovismorbificans	Bm1	E	0.08	1389, 8831, 10785	3.5
	Bm2	E	0.08	1129, 10785	2.6
	Bm3	E	0.08	1389, 8831	2.2
Matopeni	Mo1, Mo3	E	0.08	1129, 8831, 10785	3.3
	Mo2	E	0.08	8831, 10785	2.1
Stanley	Sn1	E	0.08	1129, 8831, 10785	3.3
	Sn2, Sn3	E	0.08	1389, 5882, 10785	3.6
Paratyphi B	PB1	E	0.08	8831	1.1
	PB2	E	0.08	8831, 10785	2.1
	PB3	E	0.08	1389, 8831, 10785	3.5
Richmond	Rd1	CnENaS	0.31	10785, 13450	2.5
	Rd2	CipCnENaSSxtTe	0.54	1389	1.3
	Rd3	ES	0.15	1842	1.2
Seftenberg	Sb2	ETe	0.15	4277, 7142, 8831, 10785	4.5
	Sb3	E	0.08	4918, 7142, 8831, 10785	4.3
Sada	Sa1	ESxtTe	0.23	1842, 8831, 10785, 13450, 17911	5.2
	Sa2,Sa3	ESxtTe	0.23	1389, 5882, 8831	3.4
Newport	N1	CroETe	0.23	8831, 10785, 13450	3.1
	N2	ESTe	0.23	1389, 2103, 2397, 2637, 3472, 4277, 5882, 8831, 13450	9.1
	N3	ETe	0.15	1389, 2103, 2637, 3472, 5882, 8831, 13450	7.1
Mbandaka	Mk1	CipCroCxmESSxtTe	0.54	1389, 8831, 10785	3.5
	Mk2	CipCroCxmEKfSTe	0.54	1389, 8831, 10785	3.5
	Mk3	CroCxmESTe	0.38	1389, 8831, 10785	3.5
Albany	Ay3	AmpCCroCxmEKfNaSxtTe	0.69	1389, 8831, 10785	3.5
Typhimurium	Ty3	CESSxtTe	0.38	2103, 2637, 3472, 8831	4.10
Weltevreden	W1,W5	E	0.08	3472, 5882, 7142, 8831	4.2
	W2,W16,W18	E	0.08	8831, 10785	2.1
	W3	AmpENaSSxtTe	0.46	2103, 8831, 13450, 17911	4.7
	W4	E	0.08	2103, 8831	2.3
	W6, W13, W19	E	0.08	8831	1.1
	W7	E	0.08	2103, 8831, 13450, 17911	4.7
	W8	AmpCtxES	0.31	3472, 5882, 7142	3.8
	W9,W14	E	0.08	8831, 13450	2.4
	W10,W11	E	0.08	3472, 5882, 7142, 8831, 10785	5.1
	W12	E	0.08	1842, 8831, 10785, 17911	4.9
	W15	CnE	0.15	2637, 5882, 7142, 8831, 10785, 17911	6.1
	W17	CnEKfSxt	0.31	8831, 10785	2.1
	W20	EKf	0.15	1129, 1842, 8831, 10785	4.8
	W21	CipE	0.15	2103, 8831, 13450, 17911	4.7
	W22	EKf	0.15	8831, 13450, 17911	3.7
	W23	E	0.08	8831, 10785, 17911	3.2

Antimicrobial Agent: Amp, Ampicillin; C, Chloramphenicol; Cn, Gentamycin; Cip, Ciprofloxacin; Cro, Ceftriaxone; Ctx, Cefotaxime; Cxm, Cefuroxime; E, Erythromycin; Kf, Cephalothin; Na, Nalidixic Acid; S, Streptomycin; Sxt, Trimethoprim-sulphamethoxazole; Te, Tetracycline.

disc control. The recorded resistance profiles were subjected to Bionumerics ver 5.1 (Applied Maths, Kortrijk, Belgium) analysis.

Multiple antimicrobial resistances indexing of isolates

The MAR index is defined as a/b whereby 'a' represents the number of antibiotics to which a particular isolates was resistant and 'b' the total number of antibiotics tested (Krumperman, 1983).

Plasmid profiling

Overnight culture of *S. enterica* in Luria Bertani (Difco, USA) broth at 37^{0}C were harvested and cells' pellets collected were subjected to conventional plasmid DNA extraction methods as described by Sambrook *et al*. (1989). The plasmids were resolved by electrophoresis in 1% agarose gels and visualized under gel documentation system (Alpha Imager, Alpha Innotech, USA).

DNA extraction

Genomic DNAs of the bacterial isolates were extracted and purified using RBC Genomic DNA extraction kit (Realbiotech, Taiwan) according to the manufacturer's instructions.

RAPD-PCR

Three arbitrary primers (Operon, Germany) namely OPAR2 (5'-CACCTGCTGA-3'), OPAR17 (5'-CCACCACGAC-3') and OPAR19 (5'-CTGATCGCGG-3') were selected for the RAPD analysis. PCR reactions for the RAPD assays were performed in 25 µl volumes containing 20 ng of genomic DNA, 2.5 µl 10x PCR buffer, 0.5 µl 10 mM dNTPs, 1.5 µl 25 mM $MgCl_2$, 1 unit *Taq* polymerase (Finzymes, Finland) and 5 pmol primer. The amplifications were carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with cycling parameters of 4 min at 94°C for pre-denaturation, 45 cycles each of 1 min at 94°C for denaturation, 1 min at 36°C for annealing, 2 min at 72°C for extension, and a final extension at 72°C for 8 min. The post-PCR process includes electrophoresis process using 1.5% agarose gel (Sigma, St Louis, MO, USA) to resolved amplified products, staining with ethidium bromide (0.5 µg ml^{-1}) and viewing by gel documentation system (Alpha Imager, Alpha Innotech, U.S.A). The RAPD assays were repeated three times to determine the reproducibility of the banding patterns generated. The RAPD profiles were compared on the basis of the presence or absence of each DNA band and a data matrix was constructed. Strain diversity was calculated using the Jaccard's coefficient and UPGMA (Unweighted Pair-Group Arithmetic Average Clustering) cluster analysis in Bionumerics ver 5.1 (Applied Maths, Kortrijk, Belgium)

ERIC-PCR

The primers used for ERIC-PCR were ERIC-1 (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') as described by Versalovic *et al*. (1991). ERIC-PCR amplification reactions consisted of 25 µl volumes as in RAPD section. The cycling parameters were 4 min at 94°C for pre-denaturation, 35 cycles each of 45 s at 94°C for denaturation, 1 min at 52°C for annealing, 8 min at 65°C for extension and a final extension at 65°C for 10 min. The post-PCR processes were conducted in the same manner as mentioned in RAPD section. The ERIC-PCR was repeated three times to determine the reproducibility of the banding patterns generated.

Phylogenetic data analysis

Clonal relatedness of different *Salmonella* serovar were determined by RAPD and ERIC-PCR fingerprinting using BioNumerics ver 5.1 software (Applied Maths, Kortrijk, Belgium). Normalization steps were included in the analysis to ensure adequate gel-to-gel banding pattern comparison. A process of "band scoring" identifies bands in each lane that combine to make the fingerprint based on the threshold of stringency and optimization settings. The positions of the markers run in both RAPD and ERIC-PCR were normalized from lane-to-lane and gel-to-gel variation. Subsequent processes were performed using the unweighted pair group method with arithmetic averages (UPGMA) and cluster analysis with Jaccard coefficient. Finally, dendrogram had been generated.

Calculation of discrimination indices

The discriminatory index (*D* value) was calculated for each genotyping method using Simpson's index of diversity (Hunter and Gaston, 1988). Calculating the discrimination value *D* can compare the discriminatory powers of different typing methods.

Results and Discussion

Antimicrobial desistance profiling

All *S. enterica* isolates from 'selom' were resistant to Erythromycin (Table 1). This result is in agreement with other researchers in Malaysia (Thong *et al*., 2002; Yoke-Kqueen *et al*., 2008a). The high resistances towards this macrolide occur because erythromycin molecules are too large to pass through the cell

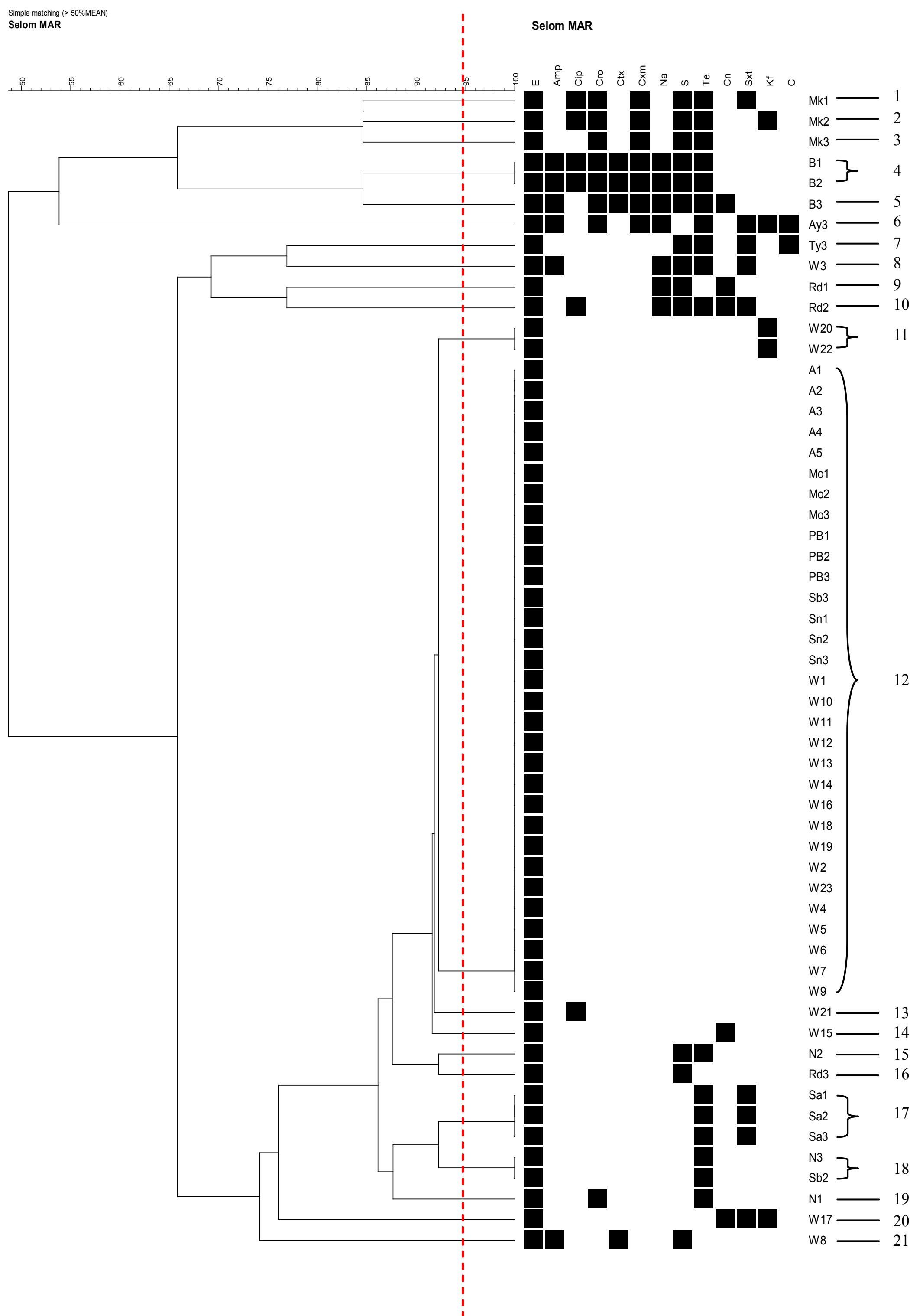

Figure 1. Dendrogram derived from Antibiograms using Bionumerics ver 5.1. Total of 5 clusters (4, 11, 12, 17, 18) and 16 single isolates (1, 2, 3, 5, 6, 7, 8, 9, 10, 13, 14, 15, 16, 19, 20, 21) were generated at similarity level of 95%

wall outer membrane of the bacterium. Thus, all *S. enterica* were resistant to this antimicrobial agent. The order of resistant profile obtained in this study includes tetracycline (27%), streptomycin (20%), ceftriaxone (14%), trimethoprim-sulphamethoxazole (14%), cefuroxime (12%), nalidixic acid (12%), ciprofloxacin (10%), ampicilin (10%), cephalothin (9%), gentamycin (9%), cefotaxime (7%) and chloramphenicol (3%) (Table 1).

The high resistance of isolates towards tetracycline was alarming as the result obtained in this study is in agreement with various researchers (Chai *et al*., 2008; Yoke-Kqueen *et al*., 2008a) which related the resistances to the frequent use of this common antimicrobial agent for animal production. This could be an indication for the lateral transference of resistance genes between *Salmonella* isolates from poultry-related sources to vegetable sources. Furthermore, 14% and 10% of *Salmonella* isolates resistant to third generation cephalosporins (ceftriaxone) and fluoroquinolones (ciprofloxacin) poses a particular concern as these compounds is the drugs of choice in the treatment of invasive forms of salmonellosis (Rossi *et al*., 1995; Fey *et al*., 2000; Gordon, 2000; White *et al*., 2001). On the other hand, low resistance to chloramphenicol (3%) observation in this study was best supported by claims reported by other researchers (Ryder *et al*., 1975; Kambal, 1996) that suggested the restricted use of chloramphenicol in certain countries due to the fear of its serious side effects indirectly increase the efficiency of the antibiotic. Resistance profile of *S*. Newport in this study was in agreement with Yan *et al*. (2003) which stated that *S*. Newport were highly resistance (100%) towards tetracycline, streptomycin and ceftriaxone. Similarly, *S*. Typhimurium resistance profile in this study were in concordance with Yang *et al*. (2002) that reports high resistance profile of streptomycin and tetracycline with the incidence of MAR index of 100%.

Moreover, results indicated that MDR isolates tend to exhibit resistant towards antimicrobial agent in the same class or group, i.e., majority of isolates (78%) resistant to ceftriaxone were also resistant to either cefotaxime or cefuroxime or both. These three agents belong to the same category, namely cephalosporin. These results were supported by other researchers, who stated that resistance towards antimicrobial agents is usually not limited to a single drug, but can be also included other structurally related compounds of the same class (Roberts, 1996; Schwarz and Noble, 1999).

This study demonstrated 59% (35/59) of selom isolates were mono-resistant to erythromycin, while 41% (24/59) were multi-resistant. MDR profiles obtained in this study were in agreement with researchers' worldwide (Ruiz, 1987; Melloul and Hassani, 1999; Thong *et al*., 2002) which reported that the presence and emergence of multi-resistant profiles from raw vegetables could be the cause of public health problems globally. This could have significant health implication since multi-drug resistance hinder the possible therapeutic treatments. A total of 59 *S. enterica* isolates from selom produced 21 antimicrobial resistance profiles with MAR index ranged from 0.08 to 0.69. Results obtained in this study revealed that three isolates of *S*. Brunei (B1, B2, B3) and one isolates of *S*. Albany (Ay3) exhibited resistances up to nine antimicrobial agents with MAR index of 0.69 (Table 1).

When comparing to the previous reports by Thong *et al*. (2002) from Malaysia and Aarestrup *et al*. (2003) from Thailand, isolates from this current study (Malaysia) exhibited higher frequency of MDR (41%). The emergence of MDR profile in *S. enterica* isolated from indigenous vegetables like selom possibly due to the increase exposure of natural reservoirs to large amount of antimicrobial agents (Aarestrup *et al*., 2003; Yoke-Kqueen *et al*., 2008a). The evolution of MDR *S. enterica* isolates is an important issue concerning to public health when there is an association of MDR *S. enterica* with vegetables that are normally consumed in raw. The increased of MDR patterns in *S. enterica* isolated from vegetables are in concordance with reports published by various researchers globally (Altekruse, 1997; Guo *et al*., 2000).

On the other hand, dendrogram as shown in Figure 1 revealed poor serovar discriminatory capability with 5 clusters (4, 11, 12, 17, 18) and 16 single isolates (1, 2, 3, 5, 6, 7, 8, 9, 10, 13, 14, 15, 16, 19, 20, 21) generated at the similarity level of 95% with the *D* value of 0.7247. The findings indicate that most of the serovars are clustered together at the lower level of similarity because all of them from the same species. Besides that, the results suggested that antimicrobial resistance profiling have lower typability capacity when comparing with other molecular typing approach used in this study in regards to serovar segregation and discrimination.

Plasmid profiling

Plasmids of *S. enterica* is varies in sizes that range from 2 to more than 200 kb (Rychlik *et al*., 2006). Despite limited knowledge on their function, their presence is frequently used for strain or serovar differentiation in epidemiological studies (Chu *et al*., 2001; Ling *et al*., 2001; Wain and Kidgell, 2004; Song and Suh, 2006). Fifty-nine *S. enterica* isolates belonging to 14 different serovars were analyzed

for the presence of plasmids. A total of 59 isolates harbored 1 to 15 different plasmids ranging from 1129 bp to 17911 bp. In this study, plasmid profiling has demonstrated a good discriminatory capability in term of serovars clustering i.e. 32 profiles based on the number and pattern of the plasmids present (Table 1). The largest profile clustered was profile 2.1 which consists of 7 isolates (12%) with two plasmids size of 8831 bp and 10785 bp. In this particular profile, 4, 1, 1, 1 isolates were from *S. Weltevreden, S. Brunei, S. Matopeni* and *S. Paratyphi B* respectively. On the other hand, 27 profiles (84%) comprises of one serovar in each profile. These results have demonstrated a good discriminatory capacity by plasmid profiling in deciphering different *Salmonella* at serovar level which in concordance with reports published by other researchers worldwide (Ling *et al*., 2001; Nayak *et al*., 2004; Wain and Kidgell, 2004; Song and Suh, 2006).

Other than serovar differentiation, plasmid profiling often used as a complement assay to antimicrobial resistance profiling as plasmids in *Salmonella* have been reported to control medically important properties such as virulence factor and resistance to antimicrobial agents (Rychlik *et al*., 2006). The antimicrobial resistance genes are often located within transposons which transpose from plasmids to chromosome, and vice versa. Therefore, plasmids are important for storage of genetic information and dissemination of genetic information including the antimicrobial resistance (Rychlik *et al*., 2006).

In this study, *S. Brunei* isolates (B1 and B2) exhibited resistant to nine antimicrobial agents (AmpCipCroCtxCxmENaSTe) but possessed only single plasmid as shown in Table 5.1. However, *S.* Newport isolates (N2) that possessed nine plasmids yielded resistant to three antimicrobial agents (ESTe). These findings indicated that it is rather difficult to determine the association between antimicrobial resistances with the possession of plasmids especially in this study. This is because MDR could even occur in the absence of plasmid or transposon (Cheah *et al*., 2006). Furthermore, drug resistance may not be conferred by plasmid and these unique *Salmonella* virulence traits could be acquired through horizontal gene transfer and integration into bacteria chromosome (Bäumler *et al*., 1997). Rychlik *et al*. (2000), who had detected low molecular weight plasmids among *S. Enteritidis* was also unable to conclude any possible role of molecular weight plasmids in the transmission of antimicrobial resistance.

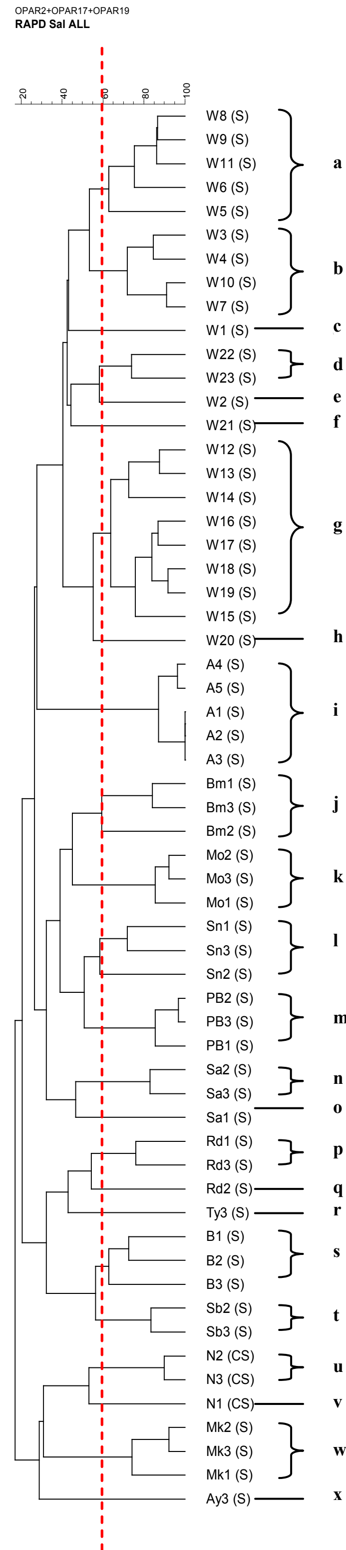

Figure 2. Dendrogram derived from RAPD banding profiles using Bionumerics ver 5.1. Total of 15 clusters (a, b, d, g, i, j, k, l, m, n, p, s, t, u, x) and nine single isolates (c, e, f, h, o, q, r, v, w) were generated at similarity level of 60%

RAPD fingerprinting

RAPD assay have been described as a simple, rapid and less expensive method when comparing to other genomic typing methods (Fadl *et al*., 1995; Chansiripornchai *et al*., 2000; Yan *et al*., 2003). A total of 20 randomly designed 10-mer primers with 70% G+C content (Operon, Germany), designated as OPAR1 to 20 were screened and only 3 primers were chosen i.e. OPAR2, 17 and 19 as they provide reproducible and discriminatory banding patterns. Dendrogram generated demonstrated high discriminatory capability by producing serovars specific clusters that could discriminate all 14 serovars in this study. From 59 isolates, 15 clusters (a, b, d, g, i, j, k, l, m, n, p, s, t, u, x) and nine single isolates (c, e, f, h, o, q, r, v, w) were generated at the similarity level of 60% (Figure 2) with the *D* value of 0.9550. All 15 clusters comprise of isolates from single serovar. The bands obtained using selected arbitrary primer ranged from 270 bp to 10000 bp.

RAPD fingerprinting has shown as an effective serovar based clustering. Furthermore, RAPD rely on amplification profiles without DNA restriction to discriminate bacteria isolates which enable it to serve as a unique role when a rapid preliminary result is desired (Chansiripornchai *et al*., 2000). Results obtained in this study are in agreement with other researchers (Houf *et al*., 2002; Yan *et al*., 2003; Lim *et al*., 2005; Yoke-Kqueen *et al*., 2008b) that RAPD is a technique with high discrimination power, good reproducibility, simplicity and rapidity that enable the monitoring of emerging pathogen possible.

ERIC-PCR fingerprinting

Versalovic *et al*. (1991) has described the presence of repetitive sequences of DNA in some eubacteria (ERIC). In this study, ERIC-PCR of *Salmonella* produced clear resolvable bands. Furthermore, Van Lith *et al*. (1994) have showed that ERIC-PCR can be use as serovar-based typing of *Salmonella.*

In view of the fact that ERIC-PCR assay can accomplish excellent results in the previous study, therefore a pair of ERIC-PCR primer was used to characterize 59 *S. enterica* isolates from 14 different serovars in this study. Findings from this study indicate that ERIC-PCR could generate satisfactory discriminatory outcome by producing serovars specific clusters that could discriminate all 14 different serovars. A total of 12 serovar specific clusters (I, II, III, IV, V, VI, VII, IX, XI, XIII, XIV, XV) and five single isolates (VIII, X, XII, XVI, XVII) were generated at the 60% similarity level with the *D* value of 0.8452 (Figure 3). The bands obtained were ranged in the size between 100 bp to 5000 bp.

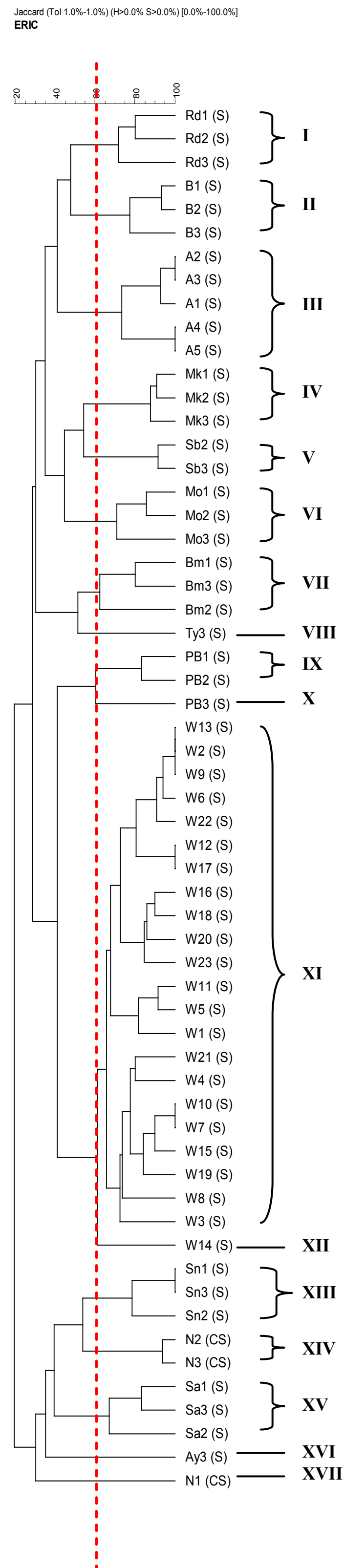

Figure 3. Dendrogram derived from ERIC-PCR analysis using Bionumerics ver 5.1. Total of 12 clusters (I, II, III, IV, V, VI, VII, IX, XI, XIII, XIV, XV) and five single isolates (VIII, X, XII, XVI, XVII) were generated at similarity level of 60%

Generally, the distribution of ERIC sequences in the genomes of *S. enterica* isolates were sufficient to be used for genetic discrimination of different *Salmonella* serovars (Lim *et al*., 2005; Yoke-Kqueen *et al*., 2008b). Thus, allowing ERIC-PCR assay to be used as the identification of inter-strain genotypic diversity and potentially to differentiate pathovars (Versalovic *et al*., 1991; Versalovic *et al*., 1994; Yoke-Kqueen *et al*., 2008b).

Composite analysis of RAPD and ERIC-PCR

Both RAPD and ERIC-PCR give relatively high *D* value of 0.9550 and 0.8452 in this study. According to Hunter and Gaston (1988), *D* value greater than 0.9 means good differentiation. The results showed that ERIC-PCR had significant lower discriminatory capability compared to RAPD with *D* value lower than 0.9. The reasons for this could be the genetic differences that may be detected by the typing method (Lim *et al*., 2005). Likewise, some of the genetic differences may be revealed by fingerprinting of target regions in total DNA when using RAPD. Other differences might occurred in repetitive consensus regions that would be detected by ERIC-PCR. Based on previous studies (Lim *et al*., 2005; Yoke-Kqueen *et al*., 2008b), the absence of correlations between ERIC and RAPD, enhanced the discriminatory power of composite analysis. Thus, composite analysis offers better discriminatory results than individual typing method.

Dendrogram in Figure 4 showed that the composite analysis of RAPD and ERIC-PCR had increased in discriminatory capacity as compared to RAPD or ERIC-PCR fingerprinting analysis alone. At the similarity level of 60%, there are 59 *S. enterica* isolates generated and group into 16 clusters (A, B, C, D, E, F, H, I, K, L, O, P, Q, R, T, V) and seven single isolates (G, J, M, N, S, U, W) with *D* value of 0.9556. All 16 clusters comprises of isolates from homogenous type of serovar. Therefore, our results are in agreement with others (Lim *et al*., 2005; Yoke-Kqueen *et al*., 2008b) that claimed the composite analysis of RAPD and ERIC-PCR fingerprinting offer better relationships establishment between serovars and deciphering different isolation sources of *S. enterica* serovars.

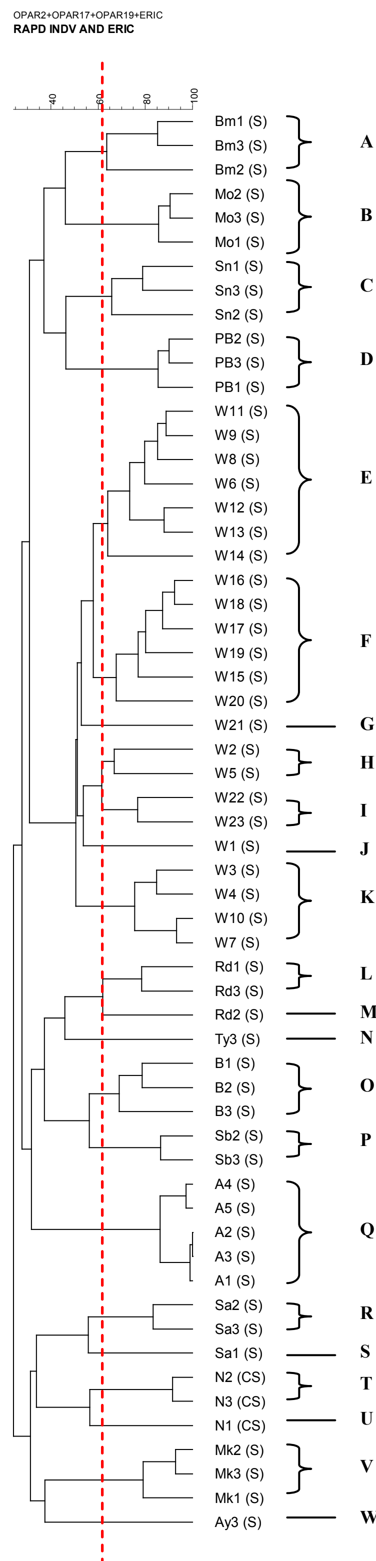

Figure 4. Dendrogram derived from composite analysis of RAPD and ERIC-PCR using Bionumerics ver 5.1. Total of 16 clusters (A, B, C, D, E, F, H, I, K, L, O, P, Q, R, T, V) and seven single isolates (G, J, M, N, S, U, W) were generated at similarity level of 60%

Conclusion

The main findings of this study were as follows: (i) *S. enterica* isolates recovered from 'selom' showed a gain in multi-drug-resistant properties. (ii) Plasmid profiles have good discriminatory capability with 84% of the profiles comprised of homogenous

serovar, but showed poor correlation between presence of plasmids and antimicrobial resistances. Antimicrobial resistance profiling had the lowest discriminatory capability while RAPD is the best single typing method with highest discriminatory capability as compared to other typing methods when used individually. (iii) Composite analysis of both RAPD and ERIC-PCR fingerprinting promises best discrimination of different *S. enterica* serovars with the highest *D* value, 0.9556. Generally this study provides important information regarding the dissemination of antimicrobial resistance properties and emphasized the need in exploring molecular typing methods in *Salmonella* epidemiology, surveillance and better infection control measures.

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