Tropical Biomedicine 32(4): 573–586 (2015)

Antibiograms, Resistance Genes, Class I Integrons and PFGE profiles of Zoonotic *Salmonella* in Malaysia

Abatcha, M.G.[1,2], Zakaria, Z.[1*], Gurmeet, K.D.[3] and Thong, K.T.[4]
[1]Department of Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[2]Food Technology Division, School of Industrial Technology, Universiti Sains Malaysia, Minden 11800, Penang, Malaysia
[3]Department of Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[4]Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur 50603, Malaysia
[*]Correspondence: Zunita Zakaria, Department of Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia, email: Zunita@vet.upm.edu.my
Received 25 January 2014; received in revised form 26 September 2014; accepted 29 September 2014

Abstract. *Salmonella* infections occur worldwide, in both developed and developing countries, and a major contributor to morbidity and economic costs. A total of 32 *Salmonella* isolates isolated from dogs (n=15/162), cats (n=1/126) and snakes (n=16/42) in the Klang valley, Peninsular Malaysia during 2012-2013, were used in this study and 6 serovars were identified. The isolates were then characterized for their susceptibility to commonly used antimicrobial agents using the standard disk diffusion method. The presence of relevant resistance genes and class 1 integrons were investigated by using PCR. Pulsed-field gel electrophoresis (PFGE) was carried out to determine the genetic diversity of these *Salmonella* strains. Higher resistance rates were observed for tetracycline (40.6%), nalidixic acid (21.9%), sulphamethazole-trimethoprim (18.7%), ampicillin (18.7%) followed by chloramphenicol (15.6%), streptomycin (6.25%), enrofloxacin (12.5%), cephalexin (6.25%), cephalothin (6.25%) and amoxicillin-clavulanic acid (3.12%). Nine percent (3/32) presented a single type of resistance, 6% (2/32) showed resistance to two classes of antimicrobials and 34% (11/32) were multidrug-resistant (MDR) (resistant to 3 or more antimicrobials). Analysis of the carriage of resistance genes in the isolates revealed that seven (*bla*TEM-1, *str*A, *str*B, *sul*II, *dfrh*I, *tet*A, *and cml*A) out of 10 resistance genes were present. Classes 1 integrons were present in 68.75% (11/16) of the resistance strains. PFGE analysis showed that the strains were very diverse and certain PFGE pattern clusters correlated well with antimicrobial resistance phenotypes. In conclusion, high rates of multidrug resistance were found among the dogs *Salmonella* strains.

INTRODUCTION

Salmonellosis is major zoonotic disease with a worldwide distribution. The increasing antimicrobial resistance in non-typhoidal *Salmonella* is a major public health concern. Antibiotic strains have emerged, presumably due to the extensive use of antimicrobial agents both in humans and animals (Tennant *et al*., 2010). In veterinary medicine, antibiotics are used in therapeutics, disease prevention and as supplement in feed additives (Soto *et al*., 1999). Animals infected with multidrug resistant (MDR) *Salmonella* are a major risk for public health, as resistance genes located on mobile genetic elements can be transferred to other bacteria of clinical importance (Miko *et al*., 2005; Van *et al*., 2007). The mechanisms of resistance to antimicrobial agents are triggered by many factors, such as changes in the bacterial cell wall permeability, enzymatic drug modifications and energy-dependent removal of antimicrobials via membrane-bound efux pumps (Chen *et al*., 2004). Usually the *genes* to be *transferred* lie on mobile genetic

elements such as plasmids, transposons and integrons, which are able to disseminate antibiotic resistance genes by vertical or horizontal transfer (Caratoli, 2001; Rowe-Magnus & Mazel, 2002). Class 1 integrons are the most prevalent in multi-resistant gram negative bacteria and predominate in MDR *Salmonella* (Khemtong & Chuanchuen, 2008). The *Salmonella* genomic island 1 (SGI1) harbors an antibiotic resistance gene cluster and was previously identified in various *Salmonella enterica* serovars including Typhimurium and Agona (Boyd *et al*., 2000).

Phenotypic typing methods such as biotyping, serotyping and antibiotic susceptibility testing, have been widely used and are less discriminative (Yan *et al.*, 2004; Shabnam & Thong, 2010). Genotypic techniques such as multi-locus variable tandem repeats (MLVA), amplified fragment length polymorphism (AFLP), and pulsed-field gel electrophoresis (PFGE), offer higher discrimination of serovars (Kotetishvili *et al*., 2002; Yan *et al*., 2004). PFGE has been considered as the "gold standard" for the molecular subtyping of *Salmonella* strains (Kotetishvili *et al*., 2002; Zheng *et al*., 2007). This technique has a considerable discriminatory power, typeability and reproducibility for subtyping *Salmonella* strains and would be useful for differentiating *Salmonella* serovars (Harbottle *et al*., 2006).

In Malaysia, there is a paucity of data on *Salmonella* in animals and this study was carried out to better discriminate serovars of the strains isolated from dogs, cats and snakes through a combination of methods. The detection and analysis of antibiotic resistance genes and integrons were carried out by using PCR and DNA sequencing, and the genetic relatedness was determined by PFGE.

MATERIALS AND METHOD

Salmonella isolates

A total of 32 non-repeat *Salmonella enterica* isolated from dogs (n=15/162), cat (n=1/126) and snakes (n=16/42) in the Klang Valley Area, Peninsular Malaysia during 2012–2013 were used in this study. All the isolates from dogs and cats (local and exotic breed) were isolated from rectal swabs, while that of snakes were from cloacal swabs (Reticulated python) in a non-repeated sampling. The isolates were previously identied by standard microbiological techniques (Abatcha *et al*., 2014) and then were submitted to the *Salmonella* Reference Centre at Veterinary Research Institute (VRI) Ipoh, Malaysia for serotyping. Serotyping was done using the Kauffmann-White classification scheme using a battery of somatic and flagellar antisera based on the protocol recommended by the World Organization for Animal Health (OIE Terrestial Manual, 2008). Six serovars were determined: *Salmonella* Corvallis (n=14), *Salmonella* Typhimurium (n=6), *S.* Mbandaka (n=6), *S.* Agona (n=1), *S.* Ruiru (n=1), *S.* Poona (n=1) and 3 were untypable strains *Salmonella enterica.*

Antibiotic susceptibility testing

All the 32 non-duplicate *Salmonella* isolates were examined for their antimicrobial susceptibility against a panel of 16 anti-microbials by using the agar disk diffusion method on Muller-Hinton agar with commercial antibiotic disks (Oxoid, Thermo Scientific, UK) as recommended by the Clinical and Laboratory Standard Institute (CLSI, 2010). The antimicrobials used included tetracycline (30 µl), streptomycin (25 µg), amoxicillin-clavulanic acid (30 µg), kanamycin (30 µg), ampicillin (10 µg), chloramphenicol (30 µg), sulphamethoxazole/trimethoprim (25 µg), gentamicin (10 µg), neomycin (10 µg), cephalexin (30 µg), cephalothin (30 µg), enrofloxacin (5 µg), ceftiofur (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg) and amikacin (30 µg). Interpretation of inhibition zones was according to CLSI (2010) guidelines. *Escherichia coli* (ATCC 25922) was used as the quality control strain for testing.

DNA template preparation for PCR

DNA template was prepared by using a suspension of a loopful of well-isolated colonies in 200 µL distilled water, boiled at 95°C for 10 min and snapped cold on ice for

5 min. The cell lysate was centrifuged at 13,000 rpm for 3 min and 5 µL of supernatant was used as the DNA template for PCR.

Polymerase chain reaction for detection of resistance genes and Class 1 integrons

Based on serotypes and antimicrobial resistance profiles, 14 resistant *Salmonella* isolates were selected for further characterization of antimicrobial resistance genes and class 1 integrons. PCR conditions to detect 10 antimicrobial resistance genes that confer resistance to six categories of antimicrobial agents, including the β-lactams, aminoglycosides, phenicols, tetracycline and sulfonamides using published oligonucleotide primers are listed in Table 1. All amplification for the PCR product was performed in 50 µl reaction volumes containing 5 µl DNA template, 25 µl Top taq master mix (Qiagen), 5 µl of 1x coral load (Qiagen), 1 µl each of forward and reverse primers and 13 µl of RNAse free water (Qiagen). The reaction was performed in thermal cycler (Eppendorf®, USA). The presence of class I integrons among the antibiotic resistance *Salmonella* isolates was determined by PCR using specific primers Int1F (5'-GGC-ATC-CAA-GCA-CAA-GC-3') and Int1R (5'-AAG-CAG-ACT-TGA-CTG-AT-3') as previously described (Levesque *et al.*, 1995, Benacer *et al.*, 2010, Thong and Modarressi, 2011). The cycling conditions consisted of an initial denaturation at 94ºC 10 min, 35 cycles each of 94ºC for 1 min, 55ºC for 1 min, and 72ºC for 5 min, and further extension at 72ºC for 5 min (Daly, *et al*., 2000). The amplified DNA products were analysed with electrophoresis on 1% agarose, then gels stained with ethidium bromide and visualized by UV illumination alpha imager (Innotech®).

Pulsed Field Gel Electrophoresis (PFGE) Analysis

Genomic DNA was prepared and embedded in agarose plugs as previously described (Thong *et al.*, 2002). DNA plugs were digested with 10 U of *Xba*I (Promega, Madison, Wis, USA) restriction enzyme overnight at 37ºC and electrophoresed on a CHEF MAPPER (Bio-Rad Laboratories, CA, USA) for 24 h at 6 Vcm^{-1}, 210º, with an initial pulse time of 2.2 s and final a pulse of 63.8 s at 14ºC. *Xba*I restricted-*Salmonella Braenderup* H9812 served as a DNA size marker. The DNA fragments on 1% w/v agarose gel and stained with ethidium bromide, destained and photographed under UV illumination (Gel DocTM XR Bio-Rad, CA, USA). DNA fragment patterns were visually assessed and distinct profiles were assigned an arbitrary pattern. Analysis of the restricted fragments was carried out using the BioNumerics Software (Applied Maths, Kortrijk, Belgium). A dendrogram based on the Dice coefficient was generated using the unweighted pair group with arithmetic mean (UPGMA) algorithm at 1.5% position tolerance.

RESULTS

Antimicrobial resistance of *Salmonella* isolates

Of the 32 *S. enterica* isolates, 14 were resistant to 1 – 6 categories of antimicrobial agents that include phenicols, tetracycline, β-lactams, sulfonamides, aminoglycosides and quinolones. Higher resistance rates were observed for tetracycline (40.6%), nalidixic acid (21.9%) sulphamethazole-trimethoprim (18.7%), ampicillin (18.7%), followed by chloramphenicol (15.6%), streptomycin (6.25%), enrofloxacin (12.5%), cephalexin (6.25%), cephalothin (6.25%) and amoxicillin-clavulanic acid (3.12%) (Table 2). Meanwhile, resistance to the first generation of cephalosporins (both cephalothin and cephalexin) was observed in *S.* Corvallis (n=2), *S.* Typhimurium (n=2) and *S.* Poona (n=2). Fifty percent of the isolates (16/32) were susceptible to all antimicrobials tested; 9% (3/32) presented a single type of resistance, 6% (2/32) showed resistance to two classes of antimicrobials and 34% (11/32) were multidrug-resistant (MDR) (resistance to 3 or more antimicrobials). All the *Salmonella* isolates were susceptible to gentamycin, amikacin, ceftiofur and ciprofloxacin.

Table 1. Primer sequences used for amplification of antimicrobial resistance genes

Genes	Primers sequence (5‘ to 3‘)	PCR conditions	Product Size	Reference
tet A	F-GTA ATTCTGAGCACTGTCG R-CTGCCT GGA CAACATTGCTT	3 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 62°C and 1 min at 72 °C; 7 min at 72 °C	957bp	Aarestrup *et al.*, (2003)
tet B	F-CTCAGTATTCCAAGCCTTTG R-ACTCCCCTGAGCTTGAGGGG	3 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 62°C and 1 min at 72°C; 7 min at 72°C	414bp	Aarestrup *et al.*, (2003)
str A	F-CCAATCGCAGATAGAAGGC R-ATCGTCAAGGGATTGAAACC	3 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 53°C and 1 min at 72°C; 7 min at 72°C	548bp	Aarestrup *et al.*, (2003)
str B	F-ATCGTCAAGGGATTGAAACC R-GGATCGTAGAACATATTGGC	3 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 53°C and 1 min at 72°C; 7 min at 72°C	507bp	Gebreyes and Altiers (2002)
sul II	F-GCGCTCAAGGCAGATGGCATT R-GCGTTTGATACCGGCACCCGT	3 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 53°C and 1 min at 72°C; 7 min at 72°C	293bp	Aarestrup *et al.*, (2003)
*dhfr*I	F- CGGTCGTAA CACGTTCAAGT R-CTGGGG-ATTTCAGGA AAGTA	3 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 53°C and 1 min at 72°C; 7 min at 72°C	220bp	Chen *et al.*, (2004)
*bla*TEM1	F-ACCAATGCTTAATCAGTGAG R-ACCAATGCTTAATCAGTGAG	3 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C; 10 min at 72°C	857bp	Oslen *et al.*, (2004)
*cml*A	F-CGCCACGGTGTTGTTGTTAT R-GCGACCTGCGTAAATGTCAC	10 min at 95°C; 30 cycles of 30 s at 95°C, 1 min at 55°C and 1 min at 72°C; 7 min 72°C	393bp	Chen *et al.*, (2004)
*cat*1	F-CTTGTCGCCTTGCGTATAAT R-AACGGCATGATGAACCTGAA	10 min at 95°C; 30 cycles of 30 s at 95°C, 1 min at 55°C and 1 min at 72°C; 7 min 72°C	508bp	Chen *et al.*, (2004)
*ca*2	F-AACGGCATGATGAACCTGAA R-ATC-CCA-ATG-GCA-TCGTAAAG	10 min at 95°C; 30 cycles of 30 s at 95°C, 1 min at 55°C and 1 min at 72°C; 7 min 72°C	547 bp	Chen *et al.*, (2004)

Table 2. Antimicrobial resistance of *Salmonella* isolates from the dogs, cat and snakes obtained in Malaysia

Antimicrobials	Dogs (n=15) resistant (%)	Cats (n=1) resistant (%)	Snakes (n=16) resistant (%)	Total (n=32) resistant (%)
Aminoglycoside				
Amikacin	0 (0)	0 (0)	0 (0)	0 (0)
Kanamycin	2 (13.3)	0 (0)	0 (0)	2 (6.3)
Gentamycin	0 (0)	0 (0)	0 (0)	0 (0)
Streptomycin	5 (33.3)	0 (0)	0 (0)	5 (15.6)
Neomycin	2 (13.3)	0 (0)	0 (0)	2 (6.3)
Phenicols				
Chloramphenicol	5 (33.3)	0 (0)	0 (0)	5 (15.6)
β-Lactams				
Ampicillin	6 (40)	0 (0)	0 (0)	6 (18.7)
Amoxicillin–clavulanate	0 (0)	0 (0)	1 (6.3)	1 (3.12)
Cephalothin	2 (13.3)	0 (0)	2 (12.5)	4 (12.5)
Ceftiofur	0 (0)	0 (0)	0 (0)	0 (0)
Cephalexin	0 (0)	0 (0)	2 (12.5)	2 (6.3)
Tetracycline	13 (86.7)	0 (0)	0 (0)	13 (40.6)
Quinolones and fluoroquinolone				
Nalidixic acid	7 (46.7)	0 (0)	0 (0)	6 (21.9)
Ciprofloxacin	0 (0)	0 (0)	0 (0)	0 (0)
Enrofloxacin	4 (26.7)	0 (0)	0 (0)	4 (12.5)
Sulfonamides				
Sulfamethoxazole-Trimethoprim	6 (40)	0 (0)	0 (0)	6 (18.7)

ampicillin (AMP), cephalothin (KF), chloramphenicol (C), gentamicin (CN), streptomycin (S), tetracycline (TE), trimethoprim–sulfamethoxazole (SXT), nalidixic acid (NA), ciprofloxacin (CP), kanamycin (K), amikacin (AK), amoxicillin–clavulanic acid (AMC) and ceftiofur (XNL), enrofloxacin (ENF), neomycin (N), Cephalexin (CL)

Antimicrobial resistance genes and class 1 integrons

In this study, 7 of the 10 resistance genes (*str*A, *str*B, *tet*A, *cml*A, *bla*TEM1, *sul*II and *dfhr*1) were detected and identified, conferring resistance to streptomycin, tetracycline, chloramphenicol, ampicillin, sulfonamides, and trimethoprim. The PCR results were consistent with the resistotypes. Among 13 tetracycline-resistant isolates, 12 were positive for *tet*A and none for *tet*B gene (Figure 1). Of the 5 streptomycin resistance isolates, 4 were positive both *str*A and *str*B, respectively (Figure 2). None of the *Salmonella* isolates were positive for chloramphenicol acetyltransferase genes (*cat*1 and *cat*2), instead *cm*1A genes where detected in 3 of the chloramphenicol resistance isolates (*S.* Corvallis, *S.* Agona and *S. enterica*) (Figure 3). Among the 6 sulfamethoxazole-trimethoprim resistant isolates, 3 were positive for *sul*II (SD006, SD004 and SD0068) and 3 were positive for *dhfl*1 (SD006, SD0068 and D0054), respectively (Figure 4). Only 4 of the 6 ampicillin resistance isolates were positive for *bla*TEM1 gene *(S.* Mbandaka, *S.* Typhimurium, *S.* Agona and *S. enterica*) (Figure 5).

Class 1 integrons were present in 68.8% (11/16) of the resistance isolates while 31.3% (5/16) did not show any evidence of detectable integrons. The integrons comprised variable regions with sizes

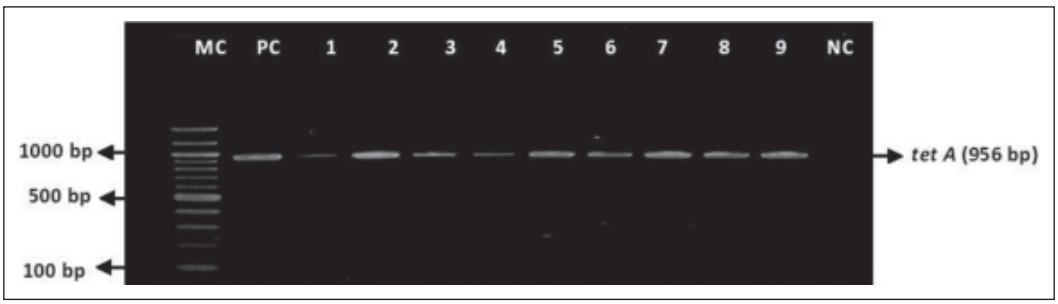


Figure 1. Representative of PCR amplification of *tet*A (958bp) genes in *Salmonella* strains. Lane MC: Molecular ladder 100 bp, Lane PC: Positive control, Lane 1: SD0037, Lane 2: SD0047, Lane 3: SD0056, Lane 4: SD0057, Lane 5: SD0061, Lane 6: SD0063, Lane 7: SD0064, Lane 8: SD0067, Lane 9: SD0072 and NC: Negative control (distilled water).

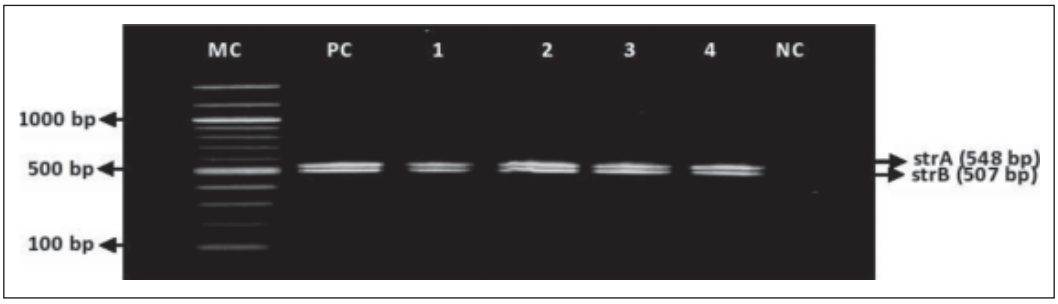


Figure 2. Representative of PCR amplification of *str*A (548bp) and *str*B (507bp) genes. Lane MC: Molecular ladder 100 bp, Lane PC: Positive control, Lane 1: SD0037, Lane 2: SD0073, Lane 3: D0038, Lane 4: D0054 and lastly lane NC: Negative control.

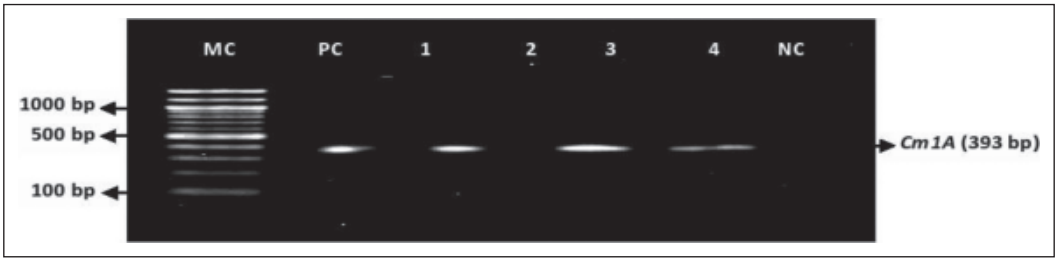


Figure 3. Representative of PCR amplification of *cm1*A (393bp) genes. Lane MC: Molecular ladder 100 bp, Lane PC: Positive control, Lane 1: SD006, Lane 2: SD0068, Lane 3: D0054, lastly lane NC: Negative control.

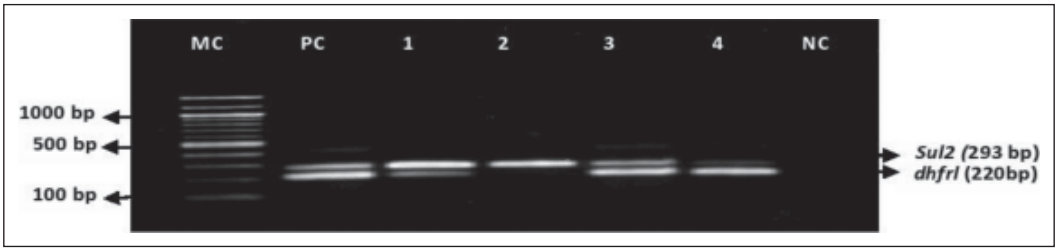


Figure 4. Representative of PCR amplification of *sul*2 (293bp) and *dhfr*I (220bp) genes. Lane MC: Molecular ladder 100 bp, Lane PC: Positive control, Lane 1: SD006, Lane 2: SD0047, Lane 3: SD0068, Lane 4: D0054 and lastly lane NC: Negative control.

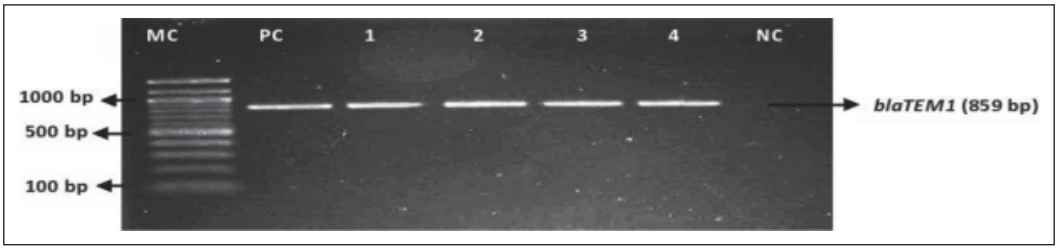


Figure 5. Representative of PCR amplification of *bla*TEM1 (859bp) genes. Lane MC: Molecular ladder 100 bp, Lane PC: Positive control *Salmonella*, Lane 1: SD0061, Lane 2: SD0063, Lane 3: D0068, Lane 4: D0054 and lastly lane NC: Negative control.

from 0.2kb, 0.3kb, 0.5kb, 0.6, 1.2kb and 1.5kb. Seven different integron profiles (IPs) were identified out of the 11 isolates (Table 4). Among all profiles, IP-1 was found only in *S.* Agona (D0054) and *S. corvallis* serovars (SD0047) with variable regions of 0.3kb and 1.2kb. Other profiles appeared in several isolates; IP-2 with variable regions of about 0.3kb and 1.5kb, found in *S. enterica* (SD0068) and *S.* Mbandaka (SD0063), IP-3 (variable regions of about 0.6kb) in *S.* Corvallis (SD004) and *S.* Typhimurium (SD0057), IP-4 (variable regions of about 1.5kb) in *S.* Corvallis (SD006 and SD0072). IP-5 contained one integron, with size of 0.5kb in *S.* Corvallis, IP-6 with one integron 0.3 kb in *S.* Poona (SKC003) and finally IP-7 with variable regions of about 0.2kb and 1.5 kb found in *S.* Typhimurium (SKW011).

Genotyping of the *Salmonella* isolates

Thirty *Salmonella* isolates were typable using PFGE generating 23 distinct pulsotypes. The pulsotypes consisted of 12 to 19 *Xba*I-restricted fragments with sizes ranging from 22.5kb to 1135 kb (Figure 6). A wide diversity was found among the strains as evidenced by F-values, which ranged from 0.54 to 1.00. The dendrogram at 70% similarity generated 5 major clusters containing 22 *Salmonella* isolates and 16 pulsotypes. The clusters, A, B, C, D and E, comprised mainly of isolates *Salmonella* serovars Corvallis (n=12), Typhimurium (n=3), Mbandaka (n=4), Agona (n=1), Ruiru (n=1) and non typeable *S. enterica* (n=1) (Figure 7). Clusters B and D were predominantly serovars Corvallis from snakes and dogs. Among these, Cluster (B) comprised of six *S.* Corvallis from variouse wild and captive snakes and consisting of 4 pulsotypes (F=0.75-1.0), and were sensitive to antimicrobials. Except for three isolates (SKW010, SKW001 and SKW002) from wild snakes with indistinguishable profile, and the other *S.* Corvallis isolates from wild and captive snakes were genetically different. Clusters (D), comprising six serovars Corvallis and of these two isolates (SD004 and SD064) were indistinguishable, and all were from stray dogs and consisting of 4 pulsotypes (F=0.80-1.0). The cluster is well correlated with antimicrobial resistance phenotypes and almost exclusively comprised isolates exhibiting core resistance to tetracycline. The *Salmonella* Typhimurium (n=3) were distingushed into clusters A with 2 different pulsotypes. The PFGE profiles of *S.* Typhimurium isolates recovered from stray dogs (SD0057) and wild snake (SKW011) were identical (Figure 6), while the other *S.* Typhimurium isolates from captive snake (SKC001) were genetically different. Cluster C comprised isolates from 2 different serovars and host; among is *S.* Mbandaka from a pet dog (D0038) and *S.* Mbandaka from wild snakes (SKW003) with indistinguishable profile, the isolates from wild snakes (drug sensitive) while that of stray dog *S.* Mbandaka (drug resistant).

A summary of the phenotypic and genotypic characteristics of *Salmonella* from dog, cat and snake given in Table 3.

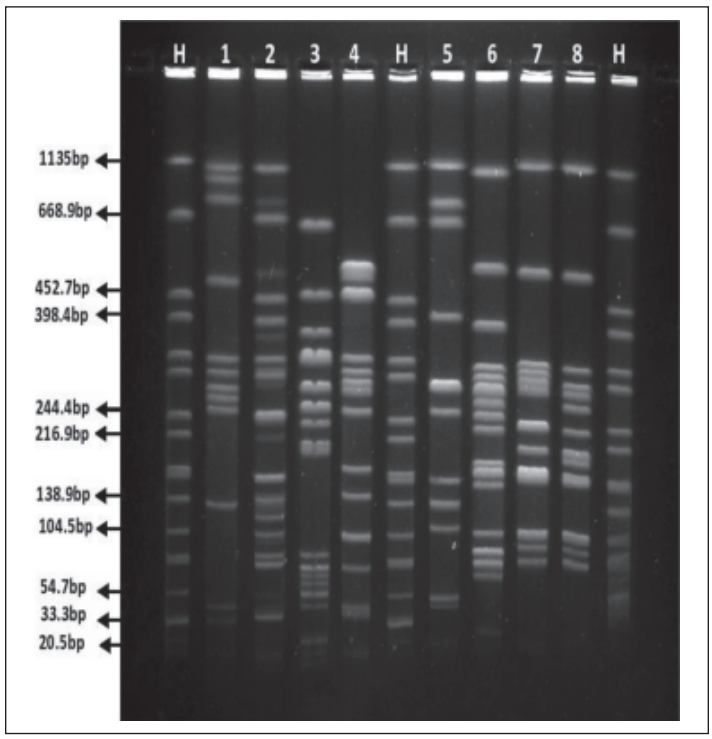
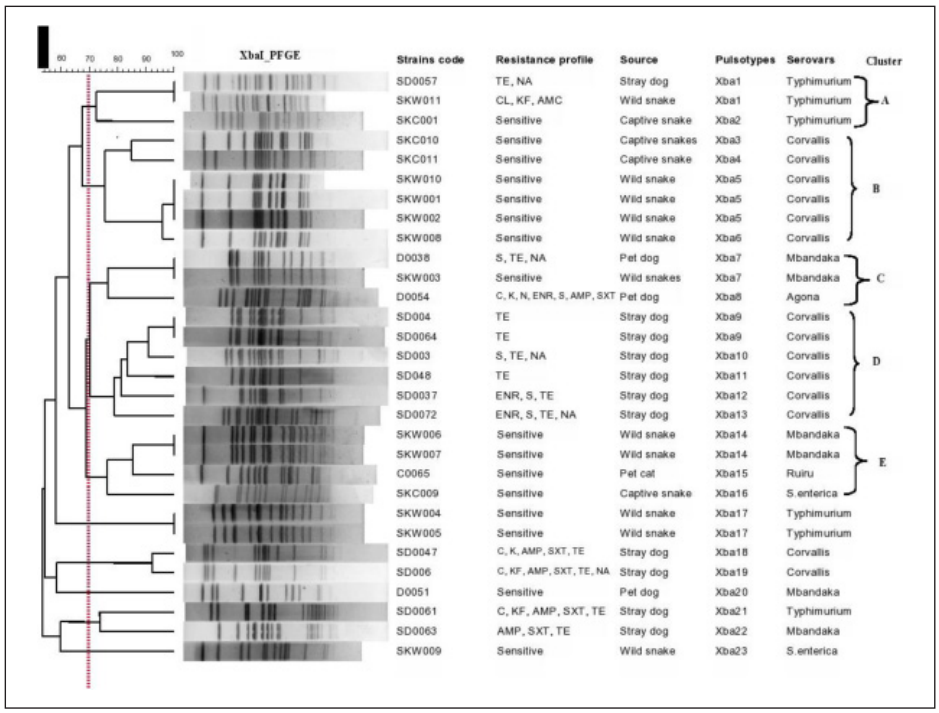


Figure 6. Representative PFGE-*XbaI* profiles of different *Salmonella* serovars from dogs, cats and snakes. Lane H marker strain *Salmonella* Braenderup H9812.



Strains code	Resistance profile	Source	Pulsotypes	Serovars	Cluster
SD0057	TE, NA	Stray dog	Xba1	Typhimurium	A
SKW011	CL, KF, AMC	Wild snake	Xba1	Typhimurium	
SKC001	Sensitive	Captive snake	Xba2	Typhimurium	
SKC010	Sensitive	Captive snakes	Xba3	Corvallis	B
SKC011	Sensitive	Captive snake	Xba4	Corvallis	
SKW010	Sensitive	Wild snake	Xba5	Corvallis	
SKW001	Sensitive	Wild snake	Xba5	Corvallis	
SKW002	Sensitive	Wild snake	Xba5	Corvallis	
SKW008	Sensitive	Wild snake	Xba6	Corvallis	
D0038	S, TE, NA	Pet dog	Xba7	Mbandaka	C
SKW003	Sensitive	Wild snakes	Xba7	Mbandaka	
D0054	C, K, N, ENR, S, AMP, SXT	Pet dog	Xba8	Agona	
SD004	TE	Stray dog	Xba9	Corvallis	D
SD0064	TE	Stray dog	Xba9	Corvallis	
SD003	S, TE, NA	Stray dog	Xba10	Corvallis	
SD048	TE	Stray dog	Xba11	Corvallis	
SD0037	ENR, S, TE	Stray dog	Xba12	Corvallis	
SD0072	ENR, S, TE, NA	Stray dog	Xba13	Corvallis	
SKW006	Sensitive	Wild snake	Xba14	Mbandaka	E
SKW007	Sensitive	Wild snake	Xba14	Mbandaka	
C0065	Sensitive	Pet cat	Xba15	Ruiru	
SKC009	Sensitive	Captive snake	Xba16	S.enterica	
SKW004	Sensitive	Wild snake	Xba17	Typhimurium	
SKW005	Sensitive	Wild snake	Xba17	Typhimurium	
SD0047	C, K, AMP, SXT, TE	Stray dog	Xba18	Corvallis	
SD006	C, KF, AMP, SXT, TE, NA	Stray dog	Xba19	Corvallis	
D0051	Sensitive	Pet dog	Xba20	Mbandaka	
SD0061	C, KF, AMP, SXT, TE	Stray dog	Xba21	Typhimurium	
SD0063	AMP, SXT, TE	Stray dog	Xba22	Mbandaka	
SKW009	Sensitive	Wild snake	Xba23	S.enterica	

Figure 7. Dendrogram showing the results of cluster analysis of the PFGE patterns of *Xba*I-digested DNA from *Salmonella* strains. The strain code, resistance profile, sources, serovars and cluster are indicated.

Table 3. Phenotypic and genotypic characteristics of dog cat and snake *Salmonella* strains

Strains code	*Salmonella* serovars	Sources	Resistotypes (Profiles)	Resistance genes present	Integrons (Kb)	PFGE profiles
SD003	Corvallis	Dog	S, TE, NA	*tet*A	N	Xba10
SD004	Corvallis	Dog	TE	*tet*A	0.6	Xba9
SD006	Corvallis	Dog	C, KF, AMP, SXT, TE , NA	*cml*A, *dhrl*1	N	Xba19
SD0037	Corvallis	Dog	ENR, S, TE	*tet*A, *Stra*A, *Stra*B	1.5	Xba12
SD0047	Corvallis	Dog	C, KF, AMP, SXT, TE	*tet*A, *dhfr*1, *sul*2	0.3, 1.2	Xba18
SD0048	Corvallis	Dog	TE	*tet*A	N	Xba11
SD0057	Typhimurium	Dog	TE, NA	*tet*A	0.6	Xba1
SD0061	Typhimurium	Dog	C, KF, AMP, SXT, TE	*tetA*, *bla*TEM1, *sul*2	N	Xba21
SD0063	Mbandaka	Dog	AMP, SXT, TE	*tetA*, *bla*TEM1	N	Xba22
SD0064	Corvallis	Dog	TE	*tetA*	O.5	Xba9
SD0068	**S. enterica*	Dog	C, K, N, AMP, SXT, TE, NA	*cml*A, *tet*A, *bla*TEM1, *sul*2	0.3, 1.5	–
SD0072	Corvallis	Dog	ENR, S, TE, NA	*tet*A, *Stra*A, *Stra*B	1.5	Xba13
D0038	Mbandaka	Dog	S, TE , NA	*tet*A, *Stra*A, *Stra*B	N	Xba7
D0051	Mbandaka	Dog	Sensitive	N	N	Xba20
D0054	Agona	Dog	C, K, N, ENR, S, AMP, SXT and NA	*cml*A, *Stra*A, *Stra*B *blaTEM*1, *sul*2	0.3, 1.2 N	Xba8
C0065	Ruiru	Cat	Sensitive	N	N	Xba15
SKC001	Typhimurium	Snake	Sensitive	N	N	Xba2
SKC003	Poona	Snake	CL and KF	N	0.3	–
SKC009	**S. enterica*	Snake	Sensitive	N	N	Xba16
SKC010	Corvallis	Snake	Sensitive	N	N	Xba3
SKC011	Corvallis	Snake	Sensitive	N	N	Xba4
SKW001	Corvallis	Snake	Sensitive	N	N	Xba5
SKW002	Corvallis	Snake	Sensitive	N	N	Xba9
SKW003	Mbandaka	Snake	Sensitive	N	N	Xba7
SKW004	Typhimurium	Snake	Sensitive	N	N	Xba17
SKW005	Typhimurium	Snake	Sensitive	N	N	Xba17
SKW006	Mbandaka	Snake	Sensitive	N	N	Xba14
SKW007	Mbandaka	Snake	Sensitive	N	N	Xba14
SKW008	Corvallis	Snake	Sensitive	N	N	Xba6
SKW009	**S. enterica*	Snake	Sensitive	N	N	Xba23
SKW010	Corvallis	Snake	Sensitive	N	N	Xba5
SKW011	Typhimurium	Snake	CL, KF and AMC	N	0.2, 1.5	Xba1

* *Salmonella enterica* – Serovar could not be determined, designated as untypable, N – no integron/gene

DISCUSSION

In the last few years, *Salmonella* strains obtained from foods, animals and humans demonstrate increasing microbial resistance rates. In the present study, 34% (11/32) of the *Salmonella* strains were multi drug resistant with resistance to three and more anti-microbials. The most frequent anti-microbial resistance among all different *Salmonella* serovars studied was against tetracycline (40.6%), nalidixic acid (21.9%), sulphamethazole-trimethoprim (18.7%), ampicillin (18.7%) followed by chloramphenicol (15.6%), streptomycin (6.25%), enrofloxacin (12.5%), cephalexin (6.25%), cephalothin (6.25%) and amoxicillin-clavulanic acid (3.12%). In this study, tetracycline had higher resistances rates among the *Salmonella* strains. The findings were similar to previous report from Thailand and Taiwan of the *Salmonella* strains in dogs being resistance to tetracycline (Ammari *et al*., 2009; Chang *et al*., 2011). This is probably due to antimicrobial being widely use in human and veterinary medicine and in animal feed, as a growth promoters and additive supplement (Cardoso *et al*., 2006). These findings were similar to previous reports that *Salmonella* strains in dogs were resistant to multiple antimicrobials, including tetracycline, sulfonamides and streptomycin (Leonard *et al*., 2012). Resistance of *Salmonella* isolates to nalidixic acid (21.9%) was particularly high in all serovars except in *Salmonella* Mbandaka. Likewise, Benacer *et al*. (2010) reported a 27.6% resistance to nalidixic acid by the *Salmonella* serovars. Numerous studies have reported the increase in resistance of *S*. Typhimurium strains to nalidixic acid and trimethoprim (Heurtin-Le Corre *et al*., 1999; Antunes *et al*., 2006), and this is probably as a result of the use of these agents in the treatment of invasive gastrointestinal infections (Aarestrup *et al*., 2003) and in animal feeds (Threlfall, 2002). In this study, the most commonly observed MDR *Salmonella* serovars were *Salmonella* Agona (D0054), *Salmonella* Corvallis (SD006, SD0047), *Salmonella* Typhimurium (SD0061, SKW011), *Salmonella enterica* (SD0068) and *S*. Mbandaka (SD0063).

A PCR was applied for accurate detection of antibiotics resistance genes in this study. The results showed that the predominant tetracycline-resistant gene *tetA* was present in all the tetracycline-resistant *Salmonella* strains, except *S*. Corvallis (SD006), and none of the strains harboured *tetB* genes. This finding was similar to that of previous studies, in which a higher prevalence of tetracycline-resistant gene *tetA* was found in zoonotic *Salmonella* strains (Benacer *et al*., 2010). These genes are easily transferred and are widely spread among isolates that are multi-drug resistant (Chen *et al*., 2004; Pezzella *et al*., 2004).

The *str*A and *str*B genes are widely distributed in streptomycin-resistance *Salmonella* strains and are often located in the plasmid (Caratoli *et al*., 2002; Sundin, 2002). Both *str*A and *str*B genes were present in all 4 strains, which were streptomycin resistant. Moreover, the s*trA-strB* genes have been identified in bacteria found in humans, animals, and plants (Caratoli *et al*., 2002; Soudin, 2002). Since tetracycline and streptomycin are among the most used antimicrobials in veterinary medicine, the extensive use of such drugs may have contributed to the successful transfer of these genetic determinants in these pathogens.

Among the chloramphenicol-resistant *Salmonella* strains in this study, none of the chloramphenicol acetyltransferase genes (*cat1* and *cat2*) were detected; instead the *cm1*A genes were found in three *Salmonella* serovars namely *S*. Corvallis (SD006), *S*. enterica (SD0068) and *S*. Agona (D0054). Thong & Modarressi (2011) detected *cmlA* in two isolates (serovars Istanbul and Wandsworth) in food and retail meat. The emergence of chloramphenicol resistance *Salmonella* strains is of great public health concern.

In this study, among sulfamethoxazole and trimethoprim resistant *Salmonella* strains, three were positive to *sul*2 (*S*. Agona, *S*. Corvallis and *S*. Typhimurium) and 3 were positive to *dhfl*1 (SD006, SD0068 and D0054) respectively. The entire *sul*2 gene was found

in 2 integron-positive strains. This finding is similar to the findings of Benacer *et al*., (2011) who reported that the *sul*2 gene was found in integron-positive *Salmonella* strains.

Four of the 6 ampicillin resistance isolates carried *bla*TEM1 namely *S*. Typhimurium (SD61), *S*. Mbandaka (SD0063), *S*. enterica (SD6008) and *S*. Agona (SD0054). The *bla*TEM genes have been reported to be the most widely distributed β-lactamase among bacteria in many parts of the world (Rayamajhi *et al*., 2008). Peirano *et al*. (2006) reported that resistance to ampicillin in *Salmonella* is mediated by TEM beta-lactamases. Many organisms are now resistant to ampicillin due to their wide clinical use.

Class 1 integrons were present in 68.8% (11/16) of antimicrobial resistance strains, indicating a wide distribution of this mobile genetic element among Malaysian *Salmonella* isolates. This class 1 integrons is the vehicle for transfer of antibiotic resistant genes. According to Rowe-Magnus (2002), class 1 integrons have been found in several *Salmonella* serotypes, such as *Salmonella* Serotypes Typhimurium, Enteritidis, Ohio, Panama, Virchow, Hadar, and Muenhen. In this study, the integrons were present in 5 different serovars, which were *S*. Agona (n=1), *S*. Corvallis (n=5), *S*. Typhimurium (n=1), *S*. Mbandaka (n=1), *S*. Poona (n=1), and *S*. enterica (n=2). This demostrate that the integrons are not limited to specific *Salmonella* serovars and may occur in any serovars. In addition class 1 integrons are more prevalent in multi-resistance gram negative bacteria, including MDR *Salmonella* strains (Khentong and Chuancheuen, 2008).

In an outbreak investigation, molecular methods have been developed for genetic discrimination of *Salmonella* isolates (Yan *et al*. 2004). PFGE is the most standard typing method for *Salmonella* outbreak investigations and is unique for examining epidemiologically related strains and determining sources (Foley *et al*., 2006). In this study, analysis of the dendrogram at 70% similarity generated 5 major clusters containing 22 *Salmonella* isolates and 16 pulsotypes. There is wide diversity among the strains as evidenced by F-values, which ranged from 0.54 to 1.00. Likewise, cluster (B and D) were predominantly serovars Corvallis from snakes and dogs and in some instances, there did appear to be a relationship between PFGE patterns and antimicrobial resistance profiles. Among these isolates, the most common anti-microbial resistance pattern was Te (tetracycline). The high degree of overall similarity suggests that the strains originated from a single clone. Within this pattern, there are some that exhibited resistance to ENR, S, or NA. *S*. Corvallis strains from snakes (cluster B) are drug sensitive. It is possible that, these differences in antibiotic susceptibility pattern may be due to point mutations or minor genetic changes that were insufficient to alter the PFGE pattern. In these cases, only a large change in the DNA or a mutation occurring within the recognition site for the restriction enzyme used is likely to alter the PFGE pattern (Thong *et al*., 2002).

In conclusion, high rates of resistance were found among the strains indicated by the prevalence of resistance genes among the *Salmonella* strains. The finding of this study suggests that integron-mediated resistance genes contributed to the emergence of MDR phenotypes seen in the *Salmonella* serovars. It should be highlighted that the integrons were not limited to a certain *Salmonella* serovar and can be found in various serotypes. This finding is significant because class 1 integrons, is the means for the transfer of antibiotic resistance genes in bacteria. PFGE analysis showed that the strains were very diverse and certain PFGE pattern clusters correlated well with antimicrobial resistance phenotypes.

Acknowledgments. The authors would like to thank the laboratory technicians and assistants at Bacteriology laboratory, Faculty of Veterinary Medicine, UPM and Laboratory of Biomedical Science and Molecular Microbiology (LBSMM), University Malaya for their friendly and comforting cooperation and unreserved assistances. We also like to

thank RUGS 91848 (UPM) for funding the research project and University of Malaya (grant 57-02-03-1015) for financial support.

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