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

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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 (blaTEM-1, strA, strB, sulII, dfrH1, tetA, and cmlA) 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 eflux pumps (Chen et al., 2004). Usually the genes to be transferred lie on mobile genetic elements.
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 identified 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 Terrestrial 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 antimicrobials 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), 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
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®).

**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

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers sequence (5' to 3')</th>
<th>PCR conditions</th>
<th>Product Size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>tet A</td>
<td>F-GTA ATTCTGAGCAGCTGCTG R-CTGCTC GGA CAACATTCTT</td>
<td>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</td>
<td>957bp</td>
<td>Aarestrup et al., (2003)</td>
</tr>
<tr>
<td>tet B</td>
<td>F-CTCAGTATTTCCAGCCTTGG R-ACCTCCCTGAGCTTTGGAGGG</td>
<td>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</td>
<td>414bp</td>
<td>Aarestrup et al., (2003)</td>
</tr>
<tr>
<td>str A</td>
<td>F-CCAATCGCAGAGTAGAGGCG R-ATCGTCAAGGGATGAAACC</td>
<td>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</td>
<td>548bp</td>
<td>Aarestrup et al., (2003)</td>
</tr>
<tr>
<td>str B</td>
<td>F-ATCGTCAAGGGATGAAACC R-GGATCGTAGAACATATTGC</td>
<td>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</td>
<td>507bp</td>
<td>Gebreyes and Altiers (2002)</td>
</tr>
<tr>
<td>sul II</td>
<td>F-GCGCTCAAGGAGGATGAGGATT R-GCGTTTGATACCGGCACCCGT</td>
<td>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</td>
<td>293bp</td>
<td>Aarestrup et al., (2003)</td>
</tr>
<tr>
<td>dhfr1</td>
<td>F-CGTTCTGAACACGTTCAAGT R-CTGGGGG-ATTCAAGGA AAGTA</td>
<td>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</td>
<td>220bp</td>
<td>Chen et al., (2004)</td>
</tr>
<tr>
<td>blaTEM1</td>
<td>F-ACCAATGCTTAAATCAGTGA R-ACCAATGCTTAAATCAGTGA</td>
<td>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</td>
<td>857bp</td>
<td>Oslen et al., (2004)</td>
</tr>
<tr>
<td>cmdA</td>
<td>F-CGCCAGCGGTGTTGTTTGTAT R-GCGACCTGCGGTAATGTAC</td>
<td>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</td>
<td>393bp</td>
<td>Chen et al., (2004)</td>
</tr>
<tr>
<td>cat1</td>
<td>F-CTTCTGCGCGCTTTCCGTATAAT R-AACGCGCATGGAACCTGAA</td>
<td>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</td>
<td>508bp</td>
<td>Chen et al., (2004)</td>
</tr>
<tr>
<td>ca2</td>
<td>F-AACGCGCATGGAACCTGAA R-ATCCCA-ATG-GCA-TCGTAAAG</td>
<td>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</td>
<td>547bp</td>
<td>Chen et al., (2004)</td>
</tr>
</tbody>
</table>
Table 2. Antimicrobial resistance of *Salmonella* isolates from the dogs, cat and snakes obtained in Malaysia

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

ampicillin (AMP), cephalothin (KP), 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 (*strA, strB, tetA, cmlA, blaTEM1, sulII and dfhrl*) 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 *tetA* and none for *tetB* gene (Figure 1). Of the 5 streptomycin resistance isolates, 4 were positive both *strA* and *strB*, respectively (Figure 2). None of the *Salmonella* isolates were positive for chloramphenicol acetyltransferase genes (*cat1* and *cat2*), instead *cm1A* 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 *sulII* (SD006, SD004 and SD0068) and 3 were positive for *dfhII1* (SD006, SD0068 and D0054), respectively (Figure 4). Only 4 of the 6 ampicillin resistance isolates were positive for *blaTEM1* 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 cm1A (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 sul2 (293bp) and dhfr1 (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.
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 XbaI-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 various 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 SD0064) 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 distinguished 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 5. Representative of PCR amplification of blaTEM1 (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.
Figure 6. Representative PFGE-XbaI profiles of different *Salmonella* serovars from dogs, cats and snakes. Lane H marker strain *Salmonella* Braenderup H9812.

Figure 7. Dendrogram showing the results of cluster analysis of the PFGE patterns of XbaI-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

<table>
<thead>
<tr>
<th>Strains code</th>
<th>Salmonella serovars</th>
<th>Sources</th>
<th>Resistotypes (Profiles)</th>
<th>Resistance genes present</th>
<th>Integrons (Kb)</th>
<th>PFGE profiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD003</td>
<td>Corvallis</td>
<td>Dog</td>
<td>S, TE, NA</td>
<td>tetA</td>
<td>N</td>
<td>Xba10</td>
</tr>
<tr>
<td>SD004</td>
<td>Corvallis</td>
<td>Dog</td>
<td>TE</td>
<td>tetA</td>
<td>0.6</td>
<td>Xba9</td>
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<tr>
<td>SD006</td>
<td>Corvallis</td>
<td>Dog</td>
<td>C, KF, AMP, SXT, TE, NA</td>
<td>cmlA, dhfr1</td>
<td>N</td>
<td>Xba19</td>
</tr>
<tr>
<td>SD0037</td>
<td>Corvallis</td>
<td>Dog</td>
<td>ENR, S, TE</td>
<td>tetA, StraA, StraB</td>
<td>1.5</td>
<td>Xba12</td>
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<tr>
<td>SD0047</td>
<td>Corvallis</td>
<td>Dog</td>
<td>C, KF, AMP, SXT, TE</td>
<td>tetA, dhfr1, sul2</td>
<td>0.3, 1.2</td>
<td>Xba18</td>
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<tr>
<td>SD0048</td>
<td>Corvallis</td>
<td>Dog</td>
<td>TE</td>
<td>tetA</td>
<td>N</td>
<td>Xba11</td>
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<tr>
<td>SD0057</td>
<td>Typhimurium</td>
<td>Dog</td>
<td>TE, NA</td>
<td>tetA</td>
<td>0.6</td>
<td>Xba1</td>
</tr>
<tr>
<td>SD0028</td>
<td>Typhimurium</td>
<td>Dog</td>
<td>C, KF, AMP, SXT, TE</td>
<td>tetA, blaTEM1, sul2</td>
<td>N</td>
<td>Xba21</td>
</tr>
<tr>
<td>SD0062</td>
<td>Mbandaka</td>
<td>Dog</td>
<td>AMP, SXT, TE</td>
<td>tetA, blaTEM1</td>
<td>N</td>
<td>Xba22</td>
</tr>
<tr>
<td>SD0064</td>
<td>Corvallis</td>
<td>Dog</td>
<td>TE</td>
<td>tetA</td>
<td>0.5</td>
<td>Xba9</td>
</tr>
<tr>
<td>SD0068</td>
<td>*S. enterica</td>
<td>Dog</td>
<td>C, K, N, AMP, SXT, TE, NA</td>
<td>cmlA, tetA, blaTEM1, sul2</td>
<td>0.3, 1.5</td>
<td>–</td>
</tr>
<tr>
<td>SD0072</td>
<td>Corvallis</td>
<td>Dog</td>
<td>ENR, S, TE, NA</td>
<td>tetA, StraA, StraB</td>
<td>1.5</td>
<td>Xba13</td>
</tr>
<tr>
<td>D0038</td>
<td>Mbandaka</td>
<td>Dog</td>
<td>S, TE, NA</td>
<td>tetA, StraA, StraB</td>
<td>N</td>
<td>Xba7</td>
</tr>
<tr>
<td>D0051</td>
<td>Mbandaka</td>
<td>Dog</td>
<td>Sensitive</td>
<td>N</td>
<td>N</td>
<td>Xba20</td>
</tr>
<tr>
<td>D0054</td>
<td>Agona</td>
<td>Dog</td>
<td>C, K, N, ENR, S, AMP, SXT and NA</td>
<td>cmlA, StraA, StraB blaTEM1, sul2</td>
<td>0.3, 1.2 N</td>
<td>Xba8</td>
</tr>
<tr>
<td>C0065</td>
<td>Ruiru</td>
<td>Cat</td>
<td>Sensitive</td>
<td>N</td>
<td>N</td>
<td>Xba15</td>
</tr>
<tr>
<td>SKC001</td>
<td>Typhimurium</td>
<td>Snake</td>
<td>Sensitive</td>
<td>N</td>
<td>N</td>
<td>Xba2</td>
</tr>
<tr>
<td>SKC003</td>
<td>Poona</td>
<td>Snake</td>
<td>CL and KF</td>
<td>N</td>
<td>0.3</td>
<td>–</td>
</tr>
<tr>
<td>SKC009</td>
<td>*S. enterica</td>
<td>Snake</td>
<td>Sensitive</td>
<td>N</td>
<td>N</td>
<td>Xba16</td>
</tr>
<tr>
<td>SKC010</td>
<td>Corvallis</td>
<td>Snake</td>
<td>Sensitive</td>
<td>N</td>
<td>N</td>
<td>Xba3</td>
</tr>
<tr>
<td>SKC011</td>
<td>Corvallis</td>
<td>Snake</td>
<td>Sensitive</td>
<td>N</td>
<td>N</td>
<td>Xba4</td>
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<tr>
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<td>Sensitive</td>
<td>N</td>
<td>N</td>
<td>Xba5</td>
</tr>
<tr>
<td>SKW002</td>
<td>Corvallis</td>
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<td>Sensitive</td>
<td>N</td>
<td>N</td>
<td>Xba9</td>
</tr>
<tr>
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<td>Mbandaka</td>
<td>Snake</td>
<td>Sensitive</td>
<td>N</td>
<td>N</td>
<td>Xba7</td>
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<tr>
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<td>Sensitive</td>
<td>N</td>
<td>N</td>
<td>Xba17</td>
</tr>
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<td>Typhimurium</td>
<td>Snake</td>
<td>Sensitive</td>
<td>N</td>
<td>N</td>
<td>Xba17</td>
</tr>
<tr>
<td>SKW006</td>
<td>Mbandaka</td>
<td>Snake</td>
<td>Sensitive</td>
<td>N</td>
<td>N</td>
<td>Xba14</td>
</tr>
<tr>
<td>SKW007</td>
<td>Mbandaka</td>
<td>Snake</td>
<td>Sensitive</td>
<td>N</td>
<td>N</td>
<td>Xba14</td>
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<tr>
<td>SKW008</td>
<td>Corvallis</td>
<td>Snake</td>
<td>Sensitive</td>
<td>N</td>
<td>N</td>
<td>Xba6</td>
</tr>
<tr>
<td>SKW009</td>
<td>*S. enterica</td>
<td>Snake</td>
<td>Sensitive</td>
<td>N</td>
<td>N</td>
<td>Xba23</td>
</tr>
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<td>Corvallis</td>
<td>Snake</td>
<td>Sensitive</td>
<td>N</td>
<td>N</td>
<td>Xba5</td>
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<tr>
<td>SKW011</td>
<td>Typhimurium</td>
<td>Snake</td>
<td>CL, KF and AMC</td>
<td>N</td>
<td>0.2, 1.5</td>
<td>Xba1</td>
</tr>
</tbody>
</table>

* 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 multidrug resistant with resistance to three and more antimicrobials. The most frequent antimicrobial 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%), cephalaxin (6.25%), cephalothin (6.25%) and amoxicillin-clavulanic acid (3.12%). In this study, tetracycline had higher resistance 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 (Anmari et al., 2009; Chang et al., 2011). This is probably due to antimicrobial being widely used 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 tetrA 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 multidrug resistant (Chen et al., 2004; Pezzella et al., 2004).

The strA and strB genes are widely distributed in streptomycin-resistance Salmonella strains and are often located in the plasmid (Caratoli et al., 2002; Sundin, 2002). Both strA and strB genes were present in all 4 strains, which were streptomycin resistant. Moreover, the strA-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 cm1A genes were found in three Salmonella serovars namely S. Corvallis (SD006), S. enterica (SD0068) and S. Agona (D0054). Thong & Modarressi (2011) detected cm1A 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 sul2 (S. Agona, S. Corvallis and S. Typhimurium) and 3 were positive to dfr1 (SD006, SD0068 and D0054) respectively. The entire sul2 gene was found
in 2 integron-positive strains. This finding is similar to the findings of Benacer et al., (2011) who reported that the sul2 gene was found in integron-positive Salmonella strains.

Four of the 6 ampicillin resistance isolates carried blaTEM1 namely S. Typhimurium (SD61), S. Mbandaka (SD0063), S. enterica (SD6008) and S. Agona (SD0054). The blaTEM 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 integron 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 demonstrate 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 pulsortypes. 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 antimicrobial 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.

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