UNIVERSITY PUTRA MALAYSIA

PREVALENCE AND GENOTYPIC CHARACTERIZATION OF ANTIBIOTIC-RESISTANT SALMONELLA ISOLATED FROM DOGS, CATS AND SNAKES IN KLANG VALLEY, MALAYSIA

MUSTAPHA GONI ABATCHA

FPV 2014 19
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By

MUSTAPHA GONI ABATCHA

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Master of Science

May 2014
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DEDICATION

This thesis is dedicated to my parents and families.
ABSTRACT

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

PREVALENCE AND MOLECULAR CHARACTERIZATION OF ANTIBIOTIC-RESISTANT SALMONELLA ISOLATED FROM DOGS, CATS AND SNAKES IN KLANG VALLEY, MALAYSIA

By

MUSTAPHA GONI ABATCHA

May 2014

Supervisor : Assoc. Prof. Zunita Zakaria, PhD
Faculty : Veterinary Medicine

Salmonellosis is a major zoonotic disease with worldwide occurrence and distribution. Salmonella species are ubiquitous in nature found in environment, humans and in intestines of wide range of animals. Most Salmonella infections in humans result from the ingestion of contaminated animal derived foods and contact with carrier animals. In veterinary medicine, antibiotics are used in livestock production, disease prevention and as supplement in feed additives. Multidrug resistant (MDR) in Salmonella is a cause of great concern in both clinical and veterinary medicine, because it limits the therapeutic options available for their treatment.

The overall goals of the study were to determine the prevalence and antibiotic susceptibility of Salmonella in dogs, cats and snakes and to investigate the presence of resistant genes and class 1 integrons by Polymerase Chain Reaction (PCR). In addition, the Salmonella isolates were also characterised using Pulsed Field Gel Electrophoresis (PFGE) to determine genetic diversity.

A total of 330 samples collected from 162 dogs, 126 cats and 42 snakes were examined for presence of Salmonella. The types of samples collected were rectal swabs from diarrheal and non-diarrheal dogs and cats at the University Veterinary Hospital at Universiti Putra Malaysia, Society for the Prevention of Cruelty to Animals (SPCA), Selangor and Dewan Bandaraya, Kuala Lumpur (DBKL), and cloacal swabs from captive and wild snakes from the Wildlife Department and Zoo Negara. Thirty-two non-repeat isolates of Salmonella enterica were identified via conventional culture, biochemical, serological and PCR methods. Of the 32 (9.7%) Salmonella isolated from the samples, the prevalence of Salmonella shedding is 9.2% (15/162) of dogs, 0.8% (1/126) of cats and 38% (16/42) of the snakes. All the Salmonella isolates were found to carry invA gene after PCR amplification. Salmonella serovars identified were S.
Typhimurium (n=5), S. Corvallis (n=10), S. Mbandaka (n=5), S. Agona (n=1), S. Poona (n=1) and S. Ruiri (n=1), and the remaining (n=9) of the isolates were untypable using the available antisera and regarded as *Salmonella enterica*.

The *Salmonella* strains were evaluated for susceptibilities towards 12 commonly used antimicrobial from seven classes including aminoglycosides, beta-lactam, phenicols, sulfonamides, cephalosporin, fluoroquinolone and tetracyclines. Fifty percent of the *Salmonella* strains were found to be resistant to the antimicrobial tested and 28% were multidrug resistant (MDR). Resistance to the following antibiotics was common among the isolates: tetracycline (40.6%), sulphamethazole-trimethoprim (18.7%), ampicillin (18.7%), chloramphenicol (15.6%), streptomycin (6.25%), enrofloxacin (12.5%), cephalexin (6.25%), cephalothin (6.25%) and amoxicillin-clavulanic acid (3.12%) was commonly seen in the *Salmonella* isolates.

Based on the resistance phenotypes, antibiotic resistant *Salmonella* strains were selected for further characterisation for their antimicrobial resistance genes. Among 10 different resistance genes investigated in 16 antibiotic resistant isolates, seven genes were detected (*blaTEM-1, strA, strB, sulII, dfrhl, tetA, and cmlA*). The DNA sequence analysis of the resistance genes amplicons showed 90-100% homology with the respective genes in GenBank. Eleven of the tested *Salmonella* strains had class 1 integrons ranging from 0.2 to 1.5 kb. The results showed that resistance genes of streptomycin (*strA, strB*), ampicillin (*blaTEM1*), sulfonamides (*Sul2*), chloramphenicol (*cm/A*), trimethoprim (*dhfrl*) and tetracycline (*tetA*) were carried on chromosomal DNA.

Molecular typing of the strains exhibited different plasmid profiles and PFGE patterns. Thirty *Salmonella* isolates were typable by PFGE generating 21 distinct pulsed-field profiles. The pulsotypes consisted of 12 to 19 *XbaI*-restricted fragments with sizes ranging from 22.5kb to 1135 kb. A wide diversity was found among the strains as evidenced by F-values, which ranged from 0.46 to 0.96. The dendrogram at >80% genetic similarity generated 9 clusters. This study confirmed that dogs might act as reservoir for antimicrobial resistance *Salmonella*. With this information, there is need for public campaign by the authority on the importance of zoonotic *Salmonella* to stakeholders in Malaysia.

*Key words: Salmonella, serotyping, invA gene, resistance genes, DNA sequencing and PFGE*
ABSTRAK
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

PREVALEN DAN PENCIRIAN MOLEKUL SALMONELLA RINTANG ANTIBIOTIK YANG DIASINGKAN DARI ANJING, KUCING DAN ULAR DI LEMBAH KLANG, MALAYSIA.

Oleh

MUSTAPHA GONI ABATCHE

Mei 2014

Penyelia : Assc. Prof. Madya Zunita Zakaria, PhD
Fakulti : Perubatan Veterinar


Matlamat kajian ini adalah untuk menentukan prevalens serta corak kerintangan antibiotik dalam *Salmonella* yang dipencilkan dari anjing, kucing dan ular; menyiasat kehadiran gen kerintangan dan integron kelas 1 dengan kaedah Polymerase Chain Reaction (PCR). Di samping itu, *Salmonella* yang diasingkan juga dicirikan menggunakan Pulsed Field Gel elektroforesis (PFGE) untuk menentukan kepelbagaian genetik.

Kehadiran *Salmonella* diperiksa dari sejumlah 330 sampel yang diambil dari 162 anjing, 126 kucing dan 42 ular. Swab rektum daripada anjing dan kucing yang mengalami cirit-birit dan juga yang tiada simptom cirit-birit di Hospital Universiti Veterinar, Universiti Putra Malaysia, Persatuan Pencegahan Penganiayaan Haiwan (SPCA), Selangor dan Dewan Bandaraya Kuala Lumpur (DBKL), dan swab kloaka dari ular peliharaan dan liar dari Jabatan Hidupan Liar dan Zoo Negara. Tiga puluh dua pencilan *Salmonella enterica* telah diasingkan dan dikenalpasti melalui kaedah konvensional,
ujian biokimia, serologi dan PCR. Secara keseluruhannya, 32 (9.7%) *Salmonella* dikesan daripada sampel. Kelaziman Salmonella adalah 9.2% (15/162) daripada anjing, 0.8% (1/126) daripada kucing dan 38% (16/42) daripada ular. Semua pencilan didapati membawa gen *InvA*. Serovars *Salmonella* yang dikenalpasti adalah *S. typhimurium* (n=5), *S. Corvallis* (n=10), *S. Mbandaka* (n=5), *S. Agona* (n=1), *S. Poona* (n=1) dan *S. Ruiru* (n=1). Selebhihnya iaitu (n=9) adalah tidak dapat ditipkan menggunakan antisera sediada dan dianggap sebagai *Salmonella enterica*.

Corak kerintangan *Salmonella* telah diuji menggunakan 12 agen antimikrob termasuk aminoglycosides, beta-lactam, phenicols, sulfonamides, cephalosporin, flouroquinolone dan tetracyclines. Lima puluh peratus daripada *Salmonella* adalah rintang kepada agen antimikrobial yang diuji dan 28% didapati adalah MDR. Corak kerintangan terhadap antibiotik berikut adalah biasa di kalangan pencilan: tetrasiklin (40.6%), sulhamethazol-trimetoprim (18.7%), ampirisin (18.7%), kloramphenicol (15.6%), streptomisin (6.25%), enrofloxacin (12.5%), sephalexin (6.25%), sephalotin (6.25%) dan amoxicillin-klavulanik asi (3.12%) telah biasa dilihat dalam diasingkan *Salmonella*.

Berdasarkan fenotip kerintangan antimikrob, pencirian lanjut bagi pengesanan gen rintangan antimikrobial dikaji. Sejumlah tujuh gen kerintangan yang dikesan iaitu *blaTEM-1*, *StrA*, *strB*, *sulII*, *dfhr1*, *tetA* dan *cm1A* diantara 10 gen rintangan. Antibiotic yang diuji. Analisis urutan DNA gen rintangan menunjukkan 90-100% homologi dengan gen masing-masing dalam GenBank. Sebelas *Salmonella* didapati mempunyai integron kelas 1 bersaiz 0.2-1.5 kb. Hasil kajian menunjukkan bahawa gen rintangan streptomisin (*StrA*, *strB*), ampicillin (*blaTEM1*), sulfonamides (*Sul2*), kloramphenicol (*cm1A*), trimethoprim (*dhfr1*) dan tetrasiklin (*tetA*) adalah dibawa oleh plasmid. Kesemua 30 pencilan *Salmonella* dapat ditipkan oleh PFGE dengan menghasilkan dua puluh satu profil. Pulsotip adalah terdiri daripada 12 hingga 19 band *XbaI* dengan saiz antara 22.5kb untuk 1135 kb. Kepelbagaian yang tinggi ditemui di kalangan strain dan ini dibuktikan oleh nilai - F dari 0.46 kepada 0.96. Dendrogram pada > 80% persamaan genetik menjana 9 kelompok. Kajian ini mengesahkan bahawa anjing mungkin bertindak sebagai takungan untuk antimikrob rintangan *Salmonella*. Dengan maklumat ini, terdapat keperluan untuk kemenapersan awam oleh pihak berkuasa yang mengenai kepentingan zoonotik *Salmonella* kepada pihak berkepentingan di Malaysia.

Kata kunci: *Salmonella*, serotip, *invA* gen, gen rintangan, DNA sequencing dan PFGE
ACKNOWLEDGEMENTS

Thanks to almighty God for everything. He has been taking me all the way through the turns and twists of life according to his plan. My next deep gratitude is to my father Alhaji Goni Abatcha, who installed the courage, inspirations, and endurances into my education. I would like to extend my sincere gratitude to my supervisor, Assoc. Prof. Dr. Zunita Zakaria for her invaluable guidance and support throughout my candidature. Her scholarly criticisms, scrutiny and suggestions kept me going against all odds. In addition, I would like to thank Prof. Thong Kwai Lin and Assoc. Prof. Dr. Gurmeet Kaur Dhaliwal for their role as my co-supervisors for their overall assistances, contribution, swift replies and wonderful comments indeed made me a better research student.

I would like to acknowledge the staff at University Veterinary Hospital-Universiti Putra Malaysia, Society for the Prevention of Cruelty to Animals (SPCA) Selangor, Dewan Bandaraya Kuala Lumpur (DBKL), Wildlife Department and Zoo Negara for their cooperation and assistance during the early stage of my work. I am also thankful to the staff of Bacteriology Laboratory, UPM and Laboratory of Biomedical Science and Molecular Microbiology (LBSMM), University Malaya for their friendly and comforting cooperation and unreserved assistances throughout my laboratory works. I also extend my thankfulness to my lab mates and friends, Adamu Kaikabo, Yusuf Yakubu and Ehsan for their assistance and friendly cooperation during my laboratory works.
APPROVAL

I certify that a Thesis Examination Committee has met on 19th May 2014 to conduct the final examination of Mustapha Goni Abatcha on his thesis entitled “Prevalence and Genotypic Characterisation of Antibiotic Resistance *Salmonella* in Dogs, Cats and Snakes in Klang valley, Malaysia” in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

**SITI KHAIRANI BINTI BEJO, Ph.D.**
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

**SON RADU, Ph.D.**
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
/Internal Examiner

**ABDUL RANI BIN BAHAMAN, Ph.D.**
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

**KASING APUN, Ph.D.**
Professor
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
(External Examiner)

Noritah Omar, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:
This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Zunita Zakaria, PhD  
Associate Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Chairperson)

Gurmeet Kaur Dhaliwal, PhD  
Associate Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Member)

Thong Kwai Lin, PhD  
Professor  
Institutes of Biological Science  
Universiti Malaya  
(Member)

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Signature: ____________________  Signature: ____________________
Zunita Zakaria, PhD                  Gurmeet Kaur Dhaliwal, PhD
(Chairperson)                        (Member)

Signature: ____________________
Thong Kwai Lin, PhD
(Member)
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LIST OF ABBREVIATIONS

>  Greater than
≥  Same or greater than
~  Approximately
=  Equals to
°C  Degree Celcius
µl  Microliter
µm  Micrometer
µg  Microgram
%  Percent
AMP  Ampicillin
AMC  Amoxicillin-clavuculanic acid
AAC  Aminoglycoside acetyltransferase
ATCC  American Type Culture Collection
BGA  Brillian Green Agar
bp  basepair
BSA  Bovine Serum Albumin
C  Chloramphenicol
CDC  Centers for Disease control and Prevention
Cip  Ciprofloxacin
CL  Cephalexin
CN  Gentamicin
D  Discriminatory Power
dH2O  Distilled water
ddH2O  Double distilled water
DHFR  Dihydrofolate reductase
DNA                                      Deoxyribonucleic acid
EDTA                                    Ethylenediaminetetraacetic
ELISA                                   Enzyme-linked immunosorbent assay
ENR                                      Enrofloxacin
EtBr                                       Ethidium Bromide
EtOH                                     Ethanol
FDA                                      Food and Drug Administration
Fig.                                      Figure
g                                      Gram
HCL                                      Hydrochloric acid
K                                        Kanamycin
KF                                      Cephalothin
Kb                                      Kilobase pair
LB                                      Luria-Bertani
LIA                                      Lysine Iron Agar
LPS                                    Lipopolisacharide
M                                      Molar
MDR                                     Multi Drug Resistant
MH                                    Mueller-Hinton
mM                                      Milimolar
mg                                      Miligram
ml                                      Mililiter
mm                                      Milimeter
N                                        Neomycin
NA                                      Nutrient agar
NA                                      Nalidixic Acid
NCCLS                                  National Committee for Clinical Standard
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>No.</td>
<td>Number</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed field gel electrophoresis</td>
</tr>
<tr>
<td>Pmol</td>
<td>picomole</td>
</tr>
<tr>
<td>Psi</td>
<td>Pound per square inch</td>
</tr>
<tr>
<td>R</td>
<td>Resistant</td>
</tr>
<tr>
<td>Ref.</td>
<td>Reference</td>
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<tr>
<td>RNase</td>
<td>Ribonuclease</td>
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<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>S</td>
<td>Streptomycin</td>
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<tr>
<td>SIM</td>
<td>Sulpha Indole Motility</td>
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<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<td>Sxt</td>
<td>Sulfonamides-trimethoprim</td>
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<tr>
<td>Tn</td>
<td>Transposon</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (Hydroxymethyl) methylamine</td>
</tr>
<tr>
<td>UPGMA</td>
<td>Unweighted pair group arithmetic means methods</td>
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<tr>
<td>µl</td>
<td>Microlitre</td>
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<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per unit volume</td>
</tr>
<tr>
<td>XLD</td>
<td>Xylose Lysine Desoxycholate</td>
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CHAPTER ONE

INTRODUCTION

1.1 General introduction

Salmonellosis constitutes a major public health burden and represents a significant cost to society in many developed and developing countries. Additionally, Salmonella is a common intestinal inhabitant in a broad range of animals, including mammals, reptiles, birds and insects. Most cases of human infection are associated with the consumption of contaminated food products such as beef, pork, poultry meat, eggs, vegetables, juices and other kind of foods. Infections may also be associated with the contact between humans and infected animals (Freitas et al., 2010).

Animals commonly infected with Salmonella which may pose a risk to humans, include amphibians, birds, cats, dogs, fish, guinea pigs, hamsters, horses, mice, rabbits, lizards, snakes, and turtles. According to literature reports, the last three (lizards, snakes, and turtles) are responsible for the majority of human salmonellosis outbreaks (Bruins et al., 2006; Corrente et al., 2006; CDC, 2008a; Bertrand et al., 2008).

Dogs and cats have been commonly reported to be the carriers of Salmonella spp. worldwide and have the potential to serve as sources of exposure or infection for humans (Van Immerseen et al., 2004; Chang et al., 2011). It was reported that the intestinal carriage of Salmonellae is more common than the prevalence of clinical disease in dogs. The frequency of faecal isolation of Salmonella spp. from clinically healthy dogs was reported to be between 0.0% and 43.0% (Sanchez et al., 2002). According to Polpakdee, et al., (2012), the prevalence of Salmonella amongst dogs in Thailand is 12.4% and in cats is 9.0% with the predominant serovars identified in dogs as S. Stanley, S. Risen, S. enterica serovar (4, 5, 12), S. Weltevreden and S. Typhimurium (14.52%, 12.90%, 11.29%, 11.29% and 9.68 %, respectively) and those in cats as S.Weltevreden, S.Eastbourn, S.Typhimurium, S.Virchow and S.Hvittingfoss (44.44%, 22.22%, 11.11%, and 11.11%, respectively).

Reptiles are very common carriers of Salmonella (Corrente, 2003; Jong et al., 2005). A recent increase in the popularity of exotic reptile pets has resulted in an increase in the number of reptile-associated salmonellosis (Center for Disease Control and Prevention, 1995b). According to Mermin et al. (2004), approximately 1.4 million human cases of Salmonella infection occur each year in the USA within estimated 74,000 being the result of exposure to pet reptiles and amphibians.

In veterinary medicine antibiotics are used in livestock production, disease prevention and as supplement in feed additives (Soto et al., 1999). The use of antibiotics in animals disrupts the normal flora of the intestine, resulting in the emergence of antibiotic-resistant Salmonella strains and prolonged faeces shedding of these organisms into the environment (Araque et al., 2009). Interest in antimicrobial resistance in companion
animals has increased; the emergence and persistence of antibiotic resistance in *Salmonella* spp. continue to pose serious risks to human health (Joseph *et al*., 2008).

Antibiotic resistant *Salmonella* have been found in dogs and cats. Most of the resistant *Salmonella* strains were serovars Heidelberg, Kentucky and Indiana. *Salmonella* Heidelberg is the most common from dogs in USA and is resistant to amoxicillin/clavulanic, ampicillin, cefoxitin, ceftiofur and ceftriazone (Guardabassi *et al*., 2004; Eric *et al*., 2011). In another study, serotypes Typhimurium, Enteritis, Bovismorbois isolated from cats were resistant to ampicillin, chloramphenicol and tetracycline while 4:i:- strains (resistant to ampicillin, chloramphenicol, sulfonamides, trimethoprim, and sulfamethoxazole/trimethoprim) (Van Immerseel *et al*., 2004). *S*. Typhimurium was the serovar with the widest range of antimicrobial resistance. In exotic reptiles, it was found to be resistant to ampicillin, chloramphenicol, gentamicin, streptomycin, trimethoprim-sulfamethoxazole, and tetracycline (Chen *et al*., 2010). These are traditional antimicrobial agents used clinically in humans.

Recently multidrug resistant (MDR) strains have emerged, presumably due to the extensive use of antimicrobial agents both in humans and animals (Tennant *et al*., 2010). These include the recent identification of MDR *Salmonella* Typhimurium strain from pets and reptile animals (Freitas *et al*., 2010). MDR in *Salmonella* is a cause of great concern in both clinical and veterinary medicine, because it may limit the therapeutic options available for treatment (Glynn *et al*., 1999; Van Duijkeren *et al*., 2003). The fatality rate for people infected with antibiotic-resistant *Salmonella* strains is 21 times greater than for individuals infected with non-antibiotic resistant *Salmonella* strains (Tekeli, 2006).

Since last decade, concerns have arisen on the emergence and spread of multidrug-resistant Typhimurium strains, especially the multidrug-resistant ACSSuT type, in companion and reptile pet animals (Rabsch *et al*., 2001). Recently, *S*. Typhimurium from cats was found to be resistant to a single drug such as ampicillin or chloramphenicol, while most strains from the group-housed cats (same clone) were resistant to ampicillin, chloramphenicol, and tetracycline (Fillip Van *et al*., 2004). Resistance genes were found to be *blaTEM* (ampicillin), *cat* (chloramphenicol), and *tetA* (tetracycline). The resistance genes in the multidrug-resistant strains were found in the integrons which are located in the genomic island of the *Salmonella* (Boyd *et al*., 2001).

To date, very little data has been published on the antimicrobial resistance mechanism among *Salmonella* from dogs, cats and snakes in Malaysia. Based on the review of current literatures, the role of the animals in the dissemination of antimicrobial resistance has not been given accorded attention. Most of the studies on the prevalence and characterization of *Salmonella* serovars by antibiogram, resistance genes, class1 integrons and Pulsed Field Gel Electrophoresis (PFGE) reported in the last few years focused on isolates from food animals. However, studies in companion and exotic reptiles are limited and at present, there is a paucity of data regarding molecular mechanisms, as well the risk factors associated with transmission of antimicrobial resistance to humans.
The aim of this study was to obtain more detailed information and to achieve better discrimination of *Salmonella* strains using a combination of methods. Antimicrobial resistance testing was carried out for detection of antibiotic resistance genes (*bla*TEM1, *straA, straB, cat1, cat2, cmlA, sul2, dfhrl, tetA, tetB*), and class 1 integrons by using Polymerase Chain Reaction. Furthermore, using PFGE to subtype the *Salmonella* strains.

1.2 Study objectives

1. To determine the prevalence and antibiotic resistance pattern of *Salmonella* spp. isolated from dogs, cats, and snakes in Klang Valley, Malaysia.
2. To detect the presence of the antimicrobial resistance genes and class 1 integron in the *Salmonella* isolates.
3. To determine the genetic diversity of the *Salmonella* strains using Pulsed Field Gel Electroporesis.
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