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

MUSTAPHA GONI ABATCHA

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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

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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 *inv*A 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.* Ruiru (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, flouroquinolone 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, dfrhI, 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 *Xba*I-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, *inv*A 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 ABATCHA

Mei 2014

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Salmonellosis adalah penyakit zoonotik utama dan ianya adalah tersebar di seluruh dunia. *Salmonella* hadir di mana-mana sahaja di alam sekitar manusia dan di dalam usus pelbagai jenis haiwan. Jangkitan *Salmonella* yang pada manusia kebiasaannya berpunca akibat daripada pemakanan berasal haiwan yang tercemar dan juga melalui kontak dengan haiwan pembawa. Dalam bidang perubatan veterinar, antibiotik digunakan dalam pengeluaran ternakan, pencegahan penyakit dan sebagai suplemen dalam makanan. Kerintangan multi antibiotik (MDR) dalam *Salmonella* telah menjadi kebimbangan besar dalam bidang perubatan klinikal dan haiwan. Ini adalah kerana ia boleh menghadkan pilihan terapi untuk rawatan.

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 *Inv*A. 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). Selebihnya iaitu (n=9) adalah tidak dapat ditipkan menggunakan antisera sediaada 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 terhdapap antibiotik berikut adalah biasa di kalangan pencilan: tetrasiklin (40.6%), sulphamethazol-trimetoprim (18.7%), ampisilin (18.7%), kloramphenicol (15.6%), streptomisin (6.25%), enrofloxacin (12.5%), sephalexin (6.25%), sephalotin (6.25%) dan amoxicillin-klavulanik asid (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, Str*A, *str*B, *sul*II, *dfrh*I, *tet*A, *dan cml*A diantara 10 gen rintangan. Antibiotic yang dijui. 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 (*Str*A, *str*B), ampicillin (*blaTEM1*), sulfonamides (*Sul2*), kloramphenicol (*cm1A*), trimethoprim (dhfrl) dan tetrasiklin (*tet*A) 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 *Xba*I 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 kempen awam oleh pihak berkuasa yang mengenai kepentingan zoonotik *Salmonella* kepada pihak berkepentingan di Malaysia.

Kata kunci: *Salmonella,* serotip, *inv*A gen, gen rintangan, DNA sequencing dan PFGE

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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.

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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:

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Declaration by graduate student

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Thong Kwai Lin, PhD
(Member)

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>	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	Enyme-linked immunosorbent assay
ENR	Enrofloxacin
EtBr	Ethidium Bromide
EtOH	Ethanol
FDA	Food and Drug Administration
Fig.	Figure
g	Gram
HCL	Hydrocloric 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

No.	Number
PCR	Polymerase Chain Reaction
PFGE	Pulsed field gel electrophoresis
Pmol	picomole
Psi	Pound per square inch
R	Resistant
Ref.	Reference
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
S	Streptomycin
SIM	Sulpha Indole Motility
SDS	Sodium dodecyl sulphate
Spp.	Species
Sxt	Sulfonamides-trimethoprim
TE	Tetracycline
TBE	Tris-borate-EDTA
TE	Tris-EDTA
Tn	Transposon
Tris	Tris (Hydroxymethyl) methylamine
UPGMA	Unweighted pair group arithmetic means methods
µl	Microlitre
UV	Ultraviolet
V	Volt
WHO	World Health Organization
w/v	Weight per unit volume
XLD	Xylose Lysine Desoxycholate

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 Heildelberg, 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, Bovismorbis 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 reptiles 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 Electroporesis (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 (*blaTEM1*, *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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