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Original Article

Prevalence and Antimicrobial Susceptibility of *Salmonella* Spp. Isolated from Snakes in Peninsular, Malaysia

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Abstract

Salmonellosis is an important zoonotic disease with worldwide distribution. Reptile-associated Salmonellosis in humans is an increasing public health concern. This study was conducted to determine the prevalence and antimicrobial susceptibility of *Salmonella* isolated from snakes. Cloacal swab samples were used for isolation by conventional culture, biochemical and serological test. Confirmations of *Salmonella* were determined by polymerase chain reaction (PCR) using genus specific primers for *invA* genes. A total of 42 snakes were screened for the presence of *Salmonella*, 16 (38%) were positive for *Salmonella*. Among those positive for *Salmonella* serovars, 11 were from the wild snakes while 5 were captive snakes. No significant difference was found in the prevalence of *Salmonella* between wild and captive snakes (p-value = 0.096). The serovars identified were *Salmonella* Typhimurium (n=2), *S.* Corvallis (n=2), *S.* Poona (n=1) and *S.* Mbandaka (n=2), while the rest untypable *S.* enterica (n=9). The resistance to antibiotics observed are as follows; cephalexin (12.5%), cephalothin (12.5%) and amoxicillin-clavulanic acid (6.25%). Interestingly all *Salmonella* isolates were sensitive to chloramphenicol, gentamycin, enrofloxacin, sulphamethazole-trimethoprim and tetracycline. To avoid *Salmonella* transmission, veterinarians and reptile keepers should take hygienic precautions to minimise reptile-associated salmonellosis.

Keywords: Snakes, *salmonella*, antibiotic susceptibility, *invA* genes.

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Introduction

Salmonellosis is an infectious disease affecting both humans and animals. The genus *Salmonella* are facultative anaerobic, non-sporing, rod shaped bacillus and gram negative motile belonging to the family Enterobacteriaceae (Ellermeier and Slauch, 2006). Infections are caused by consumption of contaminated food, person-to-person transmission, waterborne transmission and numerous environmental and animal exposures (Freitas *et al.*, 2010).

The association of reptiles with human Salmonellosis was first reported over a decade ago. Wild and captive snakes are generally known to be asymptomatic carriers of several *Salmonella* serotypes (Corrente, 2003; Jong *et al.*, 2005). Recent increases in the popularity of exotic pet snakes have resulted in an increase in the number of cases of reptile-associated salmonellosis and rapidly becoming emerging public health problems (Buck and Nicholls, 1997).

According to Mermin *et al*., (2004), approximately 1.4 million human cases of *Salmonella* infection occur each year in the USA and it has been estimated that 74,000 cases are as result of exposure to reptiles and amphibians. The prevalence of *Salmonella* infection in snakes varies in different countries of the world; 15% in Trinidad (Gopee *et al*., 2000), 24% in Austria (Pfleger *et al*., 2003), 64.7% in Brazil (Bastos *et al*., 2008), 69.2% in Australia (Scheelings *et al*., 2011) and 69.7% in Taiwan (Chen *et al*., 2010).

Increased antimicrobial resistance in exotic snakes is of growing concern. The emergence and persistence of antibiotic resistance in *Salmonella*. continue to pose serious risks to human health, especially the emergence of multi-drug resistant (MDR) *Salmonella* strains (Joseph *et al*., 2008). In Malaysia, exotic snakes are increasingly popular among reptile enthusiast and many own and raise various breeds and types of snakes. Therefore, this study aimed at determining the prevalence and antimicrobial susceptibility of *Salmonella* spp. in exotic snakes in Klang Valley, Malaysia.

Materials and Methods

Animals

A total of 42 cloacal swab samples from exotics snakes of different breeds were obtained. The samples were collected from 22 wild snakes captured by the wildlife department from different areas around Kuala lumpur and 20 captive snakes at Zoo Negara, also located in Kuala Lumpur, Malaysia. The swabs were transported in transport medium (Cary Blair®) to the laboratory and processed within 24 hours of collection. This study was approved by Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, Universiti Putra Malaysia.

Bacteriological Examination

The isolation and identification of Salmonella were performed after selective enrichment in Rappaport–Vassiliadis-Soy peptone (Oxoid, UK) broth and incubated at 37°C for 24 hrs. A loopful of enriched broth was streaked on Xylose-lysine desoxycholate (XLD) and Brillian green ager (BGA) (Oxoid, UK) agar plates and incubated at 37°C for 24 hrs. All presumptive Salmonella colonies were subcultured onto nutrient agar (Oxoid, UK) at 37°C for 24 hrs, and further confirmed by biochemical tests as recommended by the guidelines of the ISO 6579 (2002). These biochemical tests included the Triple Sugar Iron (TSI), Sulfide Indole Motility (SIM), Simmons citrate, and Urease test reactions. The Slide agglutination test (SAT) as done on presumptive Salmonella isolates using a Salmonella polyvalent O antiserum (Gp A - S) (Remel Europe®). Following this, further serotyping of the Salmonella was performed at the Veterinary Research Institute (VRI), Ipoh, Malaysia according to the Kauffmann-White classification scheme using a battery of somatic and flagellar antisera (OIE Terrestial Manual, 2008).

Polymerase Chain Reaction for Confirmation of Salmonella

The crude DNA was prepared by using a suspension of a loopful of well isolated colonies in 100 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 the supernatant was transferred into clean microfuge

tubes and used as the DNA template for the PCR. The primer was a genus specific primer for Salmonella invA gene (Rahn *et al.*, 1992) having the following nucleotide sequence Forward (5'-3'): GTG AAA TTA TCG CCA CGT TCG GGC AA and Reversed (5'-3'): TCA TCG CAC CGT CAA AGG AAC C). Amplification was performed in 50 µl reaction volumes containing 5 µl DNA template, 25 µl top Taq master mix (Qiagen), 5µl of 1x coral load (Qiagen), 1 µl each of invA forward and reverse primers and 13 µl of RNAse free water (Qiagen). The reaction was performed in thermal cycler (Eppendorf®, USA) under the following cycling conditions: an initial incubation at 94°C for 60 seconds, followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 55 °C for 30 seconds and elongation at 72°C for 45 secs, followed by a 7 minute final extension period. 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®).

Antibiotic Susceptibility Tests

Antimicrobial susceptibility was done using the Kirby Bauer disk diffusion method on Muller-Hinton agar (Oxoid, UK) with commercial antibiotic disks (Oxoid, UK) as recommended by the Clinical and Laboratory Standard Institute (CLSI, 2009). 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 (30µg), cephalothin (30µg), enrofloxacin (5 µg). For each isolate, the zone of inhibition around each disk was measured after incubation at 37◦C for 24 hours. The results were interpreted as sensitive, intermediate or resistant according to CLSI, 2009 standards.

Statistical Analysis

Statistical analysis of results was performed with SPSS version 20 (SPSS Inc. Chicago USA). The linear binary regression test was used for statistical analysis on comparison of Salmonella isolation. All statistical associations were considered significant at $p < 0.05$.

Results

Out of 42 snake samples, 16 (38%) were tested positive for the presence of Salmonella. Among the positive samples, 5 were from captive snakes and 11 from wild snakes. All presumptive Salmonella isolates contained invA gene by producing the 284 bp amplicon (Figure 1) confirming the identity of isolates as Salmonella. The prevalence and distribution of Salmonella serovars are shown in Table 1.

Table 1: Prevalence and distribution of *Salmonella* isolated from cloacal swabs collected from snakes.

Common a name (Scientific name)	No. sample	No. positive (%)	Serovars isolated
Captive snakes			
Reticulated python *(Phyton reticulates)*	16	4(20%)	*S.* Poona, *S.* enterica *S.* Corvallis (2)
Burmese phyton (*Python molurus bivittatus)*	5	1(5%)	*S.* enterica
Albino buremese phyton (*Python molurus bivittatus)*	1	0 (0)	-
Wild snakes			
Reticulated python **(***Phyton reticulates)*	15	6(27%)	*S.* enterica (6*)*
Radiated Ratsnake *(Coelognathus radiates)*	4	4(18%)	*S.*Typhimurium (2) *S.* Mbandaka (2)
Sumatran Cobra (*Naja sumatrana)*	1	1(4.5%)	*S.* enterica

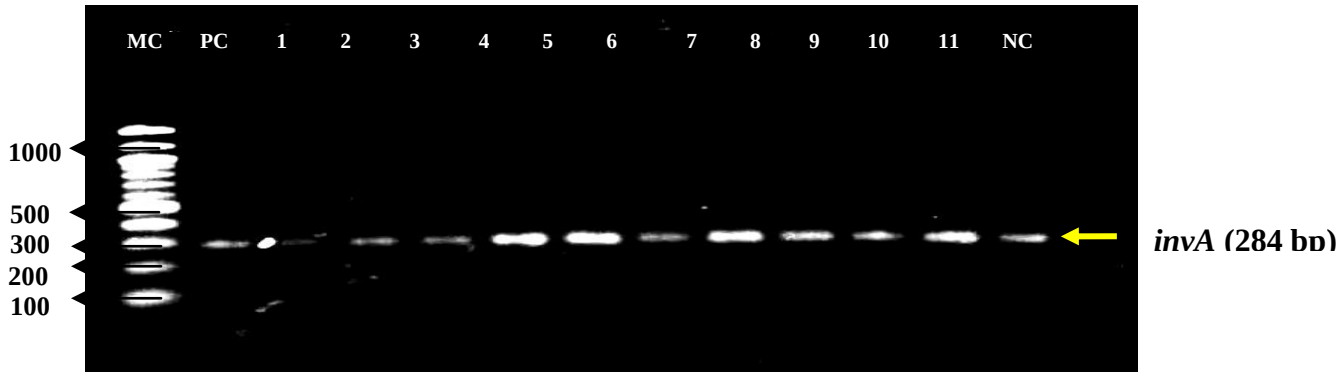

Fig. 1: Representative of PCR amplification of *invA* genes**.** Lane MC: Molecular ladder 100 bp, Lane PC: Positive control *Salmonella* Typhimurium ATCC 14028, Lane 1-11 *Salmonella* strains and lastly lane NC: Negative control.

No significant differences were found in the isolation of Salmonella between wild and captive snakes (p-value = 0.096). Seven of the 16 isolates were serotypes as S. Mbandaka, S. Typhimurium, S. Corvallis and S. Poona, while nine isolates were untypable S. enterica. Seven isolates of the 9 untyped Salmonella are from Phyton reticulates, one isolate from Python molurus bivittatus and one from a Naja sumatrana. The 7 typed isolates were distributed among 4 serovars. The serovars are S. Corvallis (two isolates from Phyton reticulates), S. Typhimurium (two isolates from Coelognathus radiates), S. Mbandaka (two isolates from a Coelognathus radiates and S. Poona (one isolate from Phyton reticulates).

The antimicrobial resistances demonstrated by Salmonella isolates were as follows; cephalexin (12.5%), cephalothin (12.5%) and amoxicillin-clavulanic acid (6.25%), this depicted in Table 2 and Figure 2. In addition, all isolates were 100% sensitives to chloramphenicol, gentamycin, enrofloxacin, sulphamethazole-trimethoprim and tetracycline.

Table 2: Antibiograms sensitivity test of *Salmonella* serovars from snakes (captive and wild).

Antibiotic	Sensitive (%)	Intermediate (%)	Resistance (%)
Ampicillin (AMP) 10µg	14(87.5)	2(12.5)	0(0)
Chloramphenicol (C) 30 µg	16(100)	0 (0)	0(0)
Gentamycin (CN) 120 µg	16(100)	0 (0)	0(0)
Kanamycin (K) 30 µg	15(93.75)	1(6.25)	0(0)
Streptomycin (S) 25 µg	15(93.75)	1(6.25)	0(0)
Neomycin (N) 10 µg	4(25)	12(75)	0(0)
Cephalexin (CL) 30 µg	11(68.75)	3(18.75)	2(12.5)
Cephalothin (KF) 30 µg	11(68.75)	3(18.75)	2(12.5)
Enrofloxacin (ENR) 5 µg	16(100)	0(0)	0(0)
Sulpham-TriM (SxT) 25 µg	16(100)	0(0)	0(0)
Tetracyclin (TE) 30 µg	16(100)	0(0)	0(0)
Amoxicillin-Clav (AMC) 30 µg	13(81.25)	2(12.5)	1(6.25)

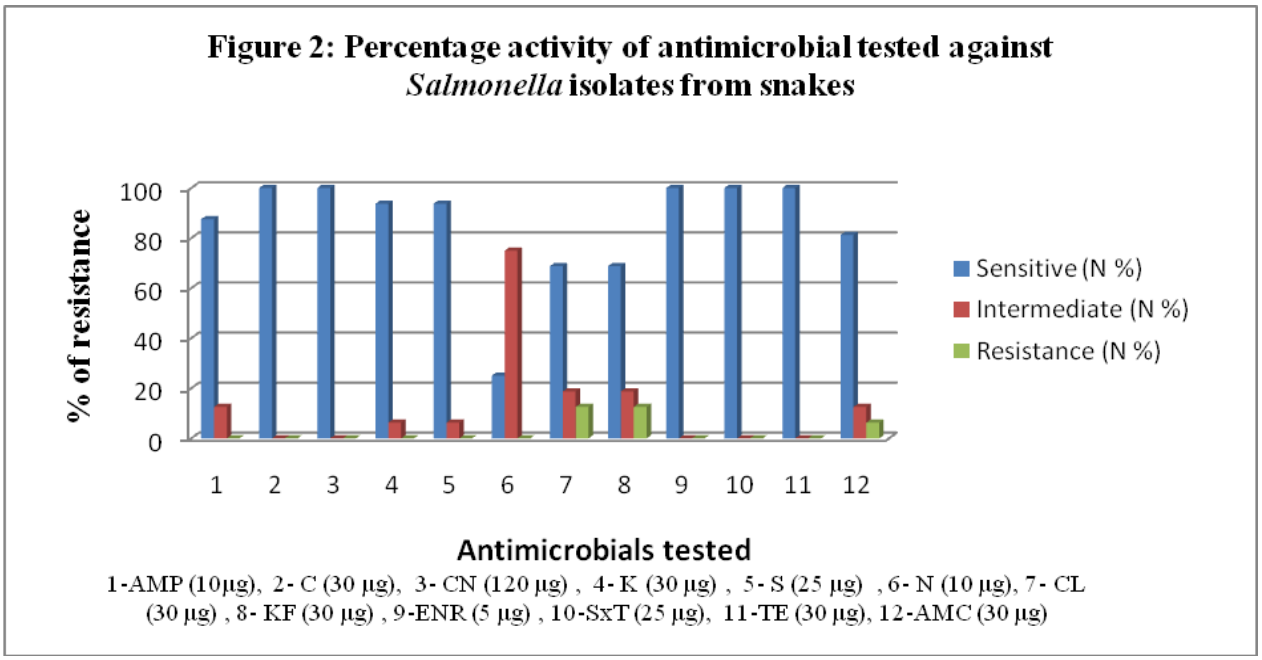

Fig. 2: Prevalence activity of antimicrobial tested against *Salmonella* isolates in snakes
Discussion.

The presence of Salmonella in exotic snakes constitutes a major public health concern, due to the increasing popularity in snakes as a pet in many Asian societies. In the last few years, a high percentage of human Salmonellosis has been associated with reptiles in many parts of the world (Olsen *et al.*, 2001). To our knowledge, this is the first report on the prevalence of *Salmonella* colonization of snakes in Malaysia. The findings indicate an overall prevalence (38%) of *Salmonella* infection in these snakes. Fifty percent (11/22) of wild snakes and 25% (5/20) captive snakes were infected. The difference in the prevalence of Salmonella in captive and wild snakes may reflect types of feeds and contaminated environment they are exposed to. The wild snakes are free to roam and feed on rodents and other wildlife species, which are known to have a high exposure to Salmonella colonized, thus playing a significant role in the epidemiology of Salmonellosis in human and animals. Previous studies have indicated shedding rates of up to 50% and 62.5%, in Romania (Köbölkuti *et al.*, 2009). In Thailand, the prevalence of Salmonella in wild snakes was 39.2% while that in captive farm snakes was 80% (Chanchaithong *et al.*, 2008). The prevalence of Salmonella in snakes varies across different geographical locations, and these could be due to differences in isolation methods and study sample size in the various countries (Johnson-Delaney, 2006). The serovars found in the present study, some are comparable to those reported in other countries. For example, a Thailand study identified 14 Salmonella serovars. Among were serovar Oslo, Newport and Poona, which were also reported as the common causes of human salmonellosis in Thailand (Bangtrakulnonth, 2004; Chanchaithong *et al.*, 2008). Moreover, in another study in Taiwan, 44 different Salmonella serovars were identified. Of major importance are, S. Heron, S. Bredeney, S. Typhimurium and S. Treforest, which were recovered from human cases of Salmonella infection (Chen *et al.*, 2010). In the present study, 4 different Salmonella serovars were identified; these were S. Typhimurium, S. Corvallis, S. Mbandaka and S. Poona. In Malaysia, Salmonella Typhimurium, Salmonella Corvalis are frequently incriminated in human illness (MOH, 2005); Salmonella Mbandaka has been isolated in humans in Denmark (Torpdahl *et al.*, 2009) and S.Poona in Thailand (Bangtrakulnonth, 2004; Chanchaithong *et al.*, 2008).

Although the overall antimicrobial resistance of the Salmonella strains were not very high (6.25%–12.5%), the resistance was found towards cephalexin, cephalothin and amoxicillin-clavulanic acid which are all traditional antimicrobial agents used clinically for humans. Cephalothin is frequently used for the treatment of bacterial infections with multidrug resistance. In this study 12.5% of the Salmonella isolates were resistant to cephalothin. In this study, most of the Salmonella serovars were susceptible to chloramphenicol, gentamycin, enrofloxacin, sulphamethazole-trimethoprim and tetracycline. This was similarly reported in other findings where Salmonella strains isolated from snakes were generally sensitive to aminoglycosides, quinolones and trimethoprim-sulfamethoxazole (Bastos *et al.,* 2008; Chen *et al.,* 2010; Gopee *et al.,* 2000). According to Bastos, (2012) Salmonella strains carried by free-ranging snakes were also generally sensitive to antibiotics and also multi-resistant strains are uncommon. The virulence invasion (invA) gene was detected by PCR in all Salmonella isolates in our study. The invA gene of Salmonella contains sequences unique to this genus and has been found to be a suitable PCR target with potential diagnostic application and confirmation of genus Salmonella (Malorny *et al.,* 2003; Jamshidi *et al.,* 2008).

In conclusion, with a relatively high prevalence of Salmonella colonization observed in captive and wild snakes, it is necessary to consider control programs to prevent reptiles-associated human Salmonellosis in Malaysia. The results offer valuable information to educate people who are raising or are considering raising snakes as pets and can be applied in future risk assessment of *Salmonella* infection in humans. Good hygiene practices are recommended to personnel employed in zoos and wildlife organizations, in order to minimize the risk of infection.

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