UNIVERSITI PUTRA MALAYSIA

OCCURRENCE OF ANTIBIOTIC RESISTANT Salmonella spp. IN TORTOISES AND TURTLES

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OCCURRENCE OF ANTIBIOTIC RESISTANT *Salmonella* spp.

IN TORTOISES AND TURTLES

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It is hereby certified that I have read this project paper entitled “Occurrence of antibiotic resistant *Salmonella* spp in tortoises and turtles”, by Noorazrena Bt Abdul Aziz and in my opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4901-project.

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Specially dedicated to the ones I love,

My parents

(Mr. Abdul Aziz Marzuki and Mrs. Siti Amaniah Muah)

My siblings

(Badrulhisham, Normahani, Mohd Fuad, Suaibatul Aslamiah, Rosilawati, Fitriah and Azizuddin)

My relatives,

My best friends

(Lokman hakim, Syuhada, Wan Zarina, Norazlina, Melissa, Mariati, Norfaralia, Norma, Siti Aisyah and Iffah)

and My friends.

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ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4901-Project.

OCCURRENCE OF ANTIBIOTIC RESISTANT *Salmonella* spp IN TORTOISES AND TURTLES

By

Noorazrena Bt Abdul Aziz

2008

Supervisor: Prof. Dr. Saleha Bt Abdul Aziz

The popularity of reptiles as pets and exhibit animals in zoological gardens continues to increase in many countries worldwide including Malaysia. Pet reptiles include snakes, iguanas, lizards, tortoises and turtles. The objectives of the study were to determine the occurrence of *Salmonella* spp in tortoises and turtles and to determine the antimicrobial pattern of *Salmonella* isolates against six antibiotics namely ampicillin, enrofloxacin, tetracycline, ciprofloxacin, streptomycin, and erythromycin. A total of 90 cloacal samples from chelonians (turtles and tortoises) were collected from a number of recreational parks, aquarium shops and individual owners for isolation and identification of *Salmonella* spp. A total of 15 water samples were collected from turtle

aquariums, from a number of recreational parks, aquarium shops and individual owners.

Fifteen (33.3%) of the turtles, 10(22.2%) tortoises and 7(46.7%) water samples were positive for the presence of *Salmonella*. Of the 32 isolates, 84.4% showed multiple-resistance while 15.6% demonstrated resistance to one antibiotic. The isolates showed thirteen antibiotic resistance patterns. The highest resistance rate was towards erythromycin (81%), followed by streptomycin (78%), ampicillin (56%), enrofloxacin (25%), ciprofloxacin (22%) and tetracycline (16%). The presence of multiple resistant *Salmonella* poses potential danger to chelonians handlers, owners and public. The emergence of antimicrobial resistant bacteria was possibly because of the overuse or misuse in not only human medicine but also veterinary medicine and from 'contaminated' environment. The *Salmonella* serotypes identified in this study were *S. newport, S. pomona, S. typhimurium, S. tenessee, S. arizona, S. brezany* and *S. corcallis*. The high percentage of *Salmonella* in turtles and tortoises in this study showed a potential risk for transmission of *Salmonella* to humans. Since *Salmonella* infections can be fatal in humans especially those with immature or weakened immune systems, it has been recommended that persons at high risk of *Salmonella* infection should avoid contact with this pet reptile. In addition, recommendations to reduce the risk of transmission of *Salmonella* to humans from such animals should be made. Vets and pet storeowners should provide information to purchasers and owners about the increased risk of acquiring salmonellosis from pet chelonians. Reducing the potential risk of contracting

Salmonella infections from exotic pets is a measure of responsible ownership, public awareness and education.

Keywords: Occurrence, *Salmonella*, Tortoise, Turtle, Antibiotic resistance

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4901-Projek.

KEHADIRAN TAHAN ANTIBIOTIK *Salmonella* DIASINGKAN DARIPADA KURA-KURA DAN PENYU.

Oleh

Noorazrena Bt Abdul Aziz

2008

Penyelia: Prof. Dr. Saleha Bt Abdul Aziz

Populariti reptilia sebagai haiwan peliharaan dan haiwan pameran dalam taman rekreasi terus bertambah di kebanyakan negara termasuk Malaysia. Reptilia-reptilia kesayangan ini termasuklah ular, iguana, cicak, kura-kura dan penyu. Objektif kajian ini adalah untuk menentukan kejadian *Salmonella* spp. dalam kura-kura dan penyu serta menentukan pola antimikrob *Salmonella* yang diasingkan terhadap enam antibiotik iaitu ampicillin, enrofloxacin, tetracycline, ciprofloxacin, streptomycin, and erythromycin. Sebanyak sembilan puluh sampel kloaka daripada chelonian (penyu dan kura-kura) telah diperolehi daripada taman

rekreasi, kedai-kedai akuarium dan pemilik persendirian bagi pengasingan dan pengenalpastian *Salmonella* spp.

Sebanyak 15 sampel air yang menempatkan penyu yang diperolehi daripada taman rekreasi, kedai-kedai akuarium dan pemilik persendirian telah juga disampel. Lima belas (33.3%) penyu, 10(22.2%) kura-kura dan 7(46.7%) sample air didapati positif untuk *Salmonella*. Daripada 32 yang terasing, 84.4% menunjukkan ketahanan terhadap lebih daripada satu antibiotik manakala 15.6% ditunjukkan ketahanan terhadap satu antibiotik sahaja. Asingan menunjukkan tiga belas pola tahan antibiotik. Kadar ketahanan yang tertinggi ialah ke arah erythromycin (81%), diikuti streptomycin (78%), ampicillin (56%), enrofloxacin (25%), ciprofloxacin (22%) dan tetracycline (16%). *Salmonella* yang tahan terhadap lebih daripada satu antibiotik menunjukkan potensi yang berbahaya kepada pemilik kedai akuarium dan orang awam yang memiliki penyu atau kura-kura. Kemunculan bakteria tahan antimikrob adalah mungkin kerana penggunaan berlebihan atau penyalahgunaan antimikrob bukan sahaja pada perubatan manusia malah perubatan veterinari dan juga daripada persekitaran yang tercemar. Serotip *Salmonela* yang dikenal pasti dalam kajian ini adalah *S. newport*, *S. pomona*, *S. typhimurium*, *S. tenessee*, *S. arizona*, *S. brezany* dan *S. corcallis.* Peratusan tinggi *Salmonella* dalam penyu dan kura-kura dalam kajian ini menunjukkan satu potensi risiko kepada manusia terutamanya kepada seseorang yang mempunyai imun sistem yang lemah. Oleh sebab jangkitan *Salmonella* boleh membawa maut pada manusia, maka dicadangkan bahawa orang yang berisiko tinggi terhadap jangkitan *Salmonella* harus mengelak

daripada berhubung dengan reptilia peliharaan ini. Faktor kebersihan perlu dilakukan bagi mengurangkan risiko pemindahan *Salmonella* kepada manusia daripada haiwan. Pegawai veterinar dan pemilik kedai akuarium perlu menyediakan maklumat kepada pembeli-pembeli dan orang awam mengenai risiko yang tinggi untuk mendapat salmonelosis daripada kura-kura dan penyu. Kesedaran, rasa tanggungjawab dan pendidikan kepada pihak awam mengenai jangkitan *Salmonella* daripada haiwan esotik kesayangan adalah satu cara yang boleh dijalankan.

Kuncikata: Kejadian, *Salmonella*, Kura-kura, Penyu, Tahan Antibiotik.

1.0 INTRODUCTION

The popularity of reptiles as pets and exhibit animals in recreational parks continues to increase in many countries worldwide including Malaysia. Pet reptiles include snakes, iguanas, lizards, tortoises and turtles. As long as these animals remain popular, veterinarians will be asked to field questions regarding *Salmonella* in reptiles.

An estimated 90% of all reptiles, which include chelonians, carry and shed various pathogenic bacteria in their feces. For this reason, exotic pets, cold-blooded vertebrates in particular turtles and tortoises, represent important reservoirs of *Salmonella* infection as well as other pathogenic bacteria (Woodword et al., 1997). Reptiles are often asymptomatic carriers of *Salmonella* spp. (Kaufmann et al., 1966).

The first case of turtle-associated salmonellosis in humans was reported in 1943 and the frequency of cases increased over the next 20 years (William and Heldson, 1965). It was not until 1963 that the first case of turtle-associated salmonellosis in a child was reported (Hersey and Manson,1963). *Salmonella hartford* was recovered from a 5 month-old infant with diarrhea, vomiting, and fever. An investigation of the environment of the infant resulted in the isolation of the same serotype from the family's pet turtle.

In 1996, the Center for Disease Control (CDC) estimated that reptiles accounted for 3% to 5% of the 2 million to 6 million cases of human salmonellosis in the U.S. (Cambre and McGuill, 2000). In most documented reptile-associated cases of salmonellosis, the strain of *Salmonella* isolated from the patient was common to the pet reptile, suggesting the source of infection. The increased incidence of reptile- associated salmonellosis has been associated with the increased popularity of these animals as pets during the past decade. Not only does this create the well-documented

threat of salmonellosis in children who receive them as pets (William, 1965), but it could negatively impact indigenous wild turtles when released pets spread disease into the environment (Stuart, 2000). The unsanitary conditions and lack of knowledge on turtle and tortoise care by the pet storeowners and their purchasers cause few of them to survive for long in captivity.

Many studies on occurrence of *Salmonella* in turtles and tortoises of various species have been done in other countries, but published studies were lacking in Malaysia. Thus, the objectives of this present study were:

1) to isolate and identify *Salmonella* spp. in tortoises and turtles.
2) to determine the antibiotic resistance pattern of the *Salmonella* isolates.

2.0 LITERATURE REVIEW

2.1 Chelonian

Chelonian are living fossils representing a very ancient reptile order. Chelonian is under phylum Chordata and class Sauropsida. This animal is classified as Testudines order. In this present study, the term chelonian was used in reference to both turtles and tortoises.

Table 1: Scientific classification of chelonians

Kingdom:	Animalia
Phylum:	Chordata
Class:	Sauropsida
Order:	Testudines

Although the word "turtle" is widely used to describe all members of the order Testudines, it is also common to see certain members described as terrapins, tortoises or sea turtles as well. Precisely how these alternative names are used, if at all, depends on the type of English being used. In this present study, the turtles refer to the freshwater chelonians species whereas tortoises refer to the chelonians for land-dwelling species.

In this present study, seven species of turtles and four species of tortoises were randomly sampled from the recreational parks, aquarium shops and individual owners.

Orange-Headed Temple Turtle *(Heosemys grandis)*

Orange Headed Temple Turtle is called because it has orange spots on the face. The carapace has a single, well-shaped keel and spiny marginals. The forelimbs are covered with scales and the toes are webbed. The plastron is yellow with radiating dark lines on every plate. As the turtle grows older this pattern fades away. The anterior and posterior plastron are serrated. In adult females the plastron is slightly movable. In contrast to most turtle species (sub-) adult female grandises are on average smaller than males, and have a shorter tail. Males usually have a concave plastron, and a thicker tail than females. The habitats for orange-headed temple turtle normally at swampy wetlands, deciduous forest and mountain streams. It spends much time on land partially hidden under shrubbery. The distribution for this turtle is around Myanmar, Vietnam, Cambodia, Laos, Thailand, Malaysia and Singapore.

Black pond turtle *(Geoclemys* *hamiltonii)*

Black Pond turtle or Spotted Pond turtle is a species of turtle found in South Asia. The carapace is much elevated, with three interrupted keels or series of nodose prominences corresponding to the vertebral and costal shields; posterior border strongly serrated in the young, feebly in the adult. The tail is extremely short where as the shell is dark brown or blackish, elegantly marked with yellow spots and radiating streaks. It is distributed in Southern Pakistan (Indus and Ganges river drainages), northeastern India (Assam) and Bangladesh.

Malaysian Giant Turtle or Bornean River Turtle (*Orlitia borneensis)*

The Malaysian giant turtle is the largest of the freshwater turtles and can weigh anywhere between 36 kg to 50 kg or more with a shell length of about 80 cm. The majority of these animals are dark colored, the shells being black, brown, or dark gray and not patterned. The neck, limbs, and tail are the same, black, brown, or gray. The head is dark with a single light colored line starting from the mouth and extending to the back of the head. The bottom of the shell is yellowish or a light brown. The shell is narrow, flat and oval shaped. The top is rounded and smooth. The head is large and broad with a slight protruding snout. This turtle has webbed toes, fingers, and outer forelimbs with band-like scales on them and a carapace and plastron that are strongly joined together. The females' tail is shorter and thinner than the males tail. There are several differences between the shell of a young *Orlitia borneensis* and the grown adult. The Malaysian giant turtle is distributed in Malaysia, Borneo, and also Indonesia. They specifically reside in the deep waters of lakes and rivers. This turtle is an omnivore, eating small fish, plants and sometimes snakes.

Red-eared Slider (*Trachemys scripta elegans*)

The red-eared Slider is a semi-aquatic turtle (terrapin) belonging to the family *Emydidae*. It is a native of the southern United States, but has become common in various areas of the world due to the pet trade. They are very popular pets in the United States, the Netherlands, Canada, and England. Red-eared Sliders are omnivores and eat a variety of animal and plant materials in the wild including, but not limited to fish, crawfish, carrion, tadpoles, snails, aquatic insects and numerous aquatic plant species. Female grow to be 10-12 inches in length and males 8-10 inches. They are almost entirely aquatic, but do leave the water to bask in the sun and lay eggs. These reptiles are deceptively fast and are also excellent swimmers. They hunt for prey and will attempt to capture it when the opportunity presents itself. They are very aware of predators and people and generally shy away from them.

Alligator snapping turtle *(Macroclemys temmincki)*

The alligator snapping turtle is one of the largest freshwater turtle in the world. These creatures can weigh up to 90kg and the shell can reach three quarters of a metre in length. The large head, which may be 25cm across, has powerful sharp-edged jaws capable of crushing and severing a finger. They are usually sluggish, placid animals but will bite if handled roughly. The shell is olive brown with a series of raised ridges running front to back similar to the scales of an alligator, hence its common name. The species' range has dwindled somewhat in recent times due to wetland clearance and hunting for food. It is now almost totally confined to the Mississippi River system in the southern USA. Most of an alligator snapping turtle's life is spent in the water. The alligator snapper's main food is fish.

Asian Leaf Turtle (*Cyclemys dentate)*

The Asian Leaf Turtle is a species of turtle found in South Asia. They are also common in the pet trade. The turtle can be found in North India, Bangladesh, Myanmar (Burma), Thailand, Cambodia, Vietnam, West Malaysia, Indonesia (Sumatra, Java, Borneo, Bali), Philippines (Palawan: Calamian Islands etc.), and China. It appears in the pet trade fairly often and is being captive bred. It is a hardy pet. A medium sized turtle, it grows to 9". It is rather flat and shaped like a broad oval, nearly round. There is a single strong keel on the carapace. It is usually brown although other color variations exist. Posterior marginals of the carapace are strongly serrated in juveniles. Adults form a single plastral hinge. *C. dentata* is webbed-footed and highly aquatic, especially as juveniles. Adults may spend more time on land and prefer an area with plants. These turtles require an aqua-vivarium, at least 50% water. This species is environmentally sensitive. Water must be filtered and kept scrupulously clean. Elevated levels of ammonia (from standing, dirty water) can cause a variety of serious illnesses. In the wilds, *C. dentata* inhabit streams in mountain forests, up to 3500' above sea level. They prefer cool water (68 - 74 degrees F.) and low light intensities, although they do occasionally bask. Youngsters usually prefer to eat in the water, while adults will readily eat on land. They are omnivorous.

Long neck turtle *(Chelodina longicollis)*

Also referred to as a snake-necked turtle, the eastern longneck's shell will eventually grow to around 25cm in length, with its neck almost the same length. The upper shell or carapace can vary in colour from light reddish-brown to almost black, while the lower shell or plastron is usually creamy-yellow, sometimes with other dark brown markings. The feet have strong claws and are webbed for swimming. The jaws are made of hard, horn-like material and, if provoked, can deliver a painful bite. This is an extremely common turtle in eastern Australia, while other long-necked species occur in northern and western Australia. They inhabit almost any type of relatively slow moving water body from farm dams to major rivers and lakes. These turtles prey mostly on fish, tadpoles, frogs and crayfish. The long neck is used like a snake to rapidly strike at passing prey. Large food items are torn apart by the strong front claws.

Galapagos tortoises *(Geochelone nigra)*

Galapagos tortoises may reach a maximum shell length of over a metre and weigh up to 180 kg in weight. There are some 14 distinct subspecies inhabiting different islands in the Galapagos Island archipelago. The shape of the shell varies quite markedly between the subspecies. Some are domed, some are flat, and others have a raised area above the neck. The latter feature is thought to be an adaptation to allow the tortoises to browse on the lower branches of trees and shrubs. They have large, strong legs and are quite capable of climbing over quite large rocks. Galapagos tortoises are restricted to the Galapagos Islands, some 900km west of Ecuador off the South American coast. Galapagos tortoises spend much of their time resting in mud wallows and waterholes. This keeps the reptile cool while also assisting to support its great bulk. They migrate to the moist, cooler highlands during the hot, dry season and move back to the lowlands during the rainy weather. Galapagos tortoises eat almost any green vegetation they can find. In captivity the tortoises will eat carrots, bamboo stalks, hibiscus leaves and flowers and other foods that are high in fibre.

Star tortoise *(Geochelone eleyans* or *Testudo eleyans)*

It is the smallest Geochelone species with the colour of the head varying yellow to tan and bulging in the nasal region. It has black spots on the crown whereas the carapace elongated with stongly elevated or smooth carapace plates. The size of the female can measure up to 10 inches and are larger than males. It is distributed in Malay Peninsula, Sumatra, Java, Borneo, Ceylon, India, Sri Lanl,,a, Pakistan. They are also found in sand dunes, brush wood, scrub forests, park jungle, also in human-altered habitats including waste areas, deserts, scrub lands, plantations.

Elongated Tortoise (*Indotestudo elongata*)

The Elongated Tortoise is native to Asia. They are sometimes known as Lemon Headed or Yellow Headed Tortoises. Although the Elongated Tortoise is diurnal, meaning they are active during the day, they are most active in the very early morning and late evening. They are native to tropical regions of Asia and are most active during the rainy season, when the weather is wet and warm. They do not bask often, nor do they soak in water often, although they enjoy both of these activities from time to time. The Elongated Tortoise is omnivorous, feeding on snails, worms, and slugs as well as on plants. In captivity, mature Elongated Tortoises are usually kept outside. The Elongated Tortoise is found primarily in tropical regions of Southeast Asia. They are most commonly seen in India, Burma, Bangladesh, Cambodia, Vietnam, Laos, Malaysia, and Thailand.

Asian brown tortoise (*Manouria emys)*

This tortoise is the largest inhabiting Asia, and the fourth largest in the world. Two subspecies are currently recognised: *M. e. emys* is commonly known as the Asian brown tortoise, and is characterised by a light to dark brown upper shell (carapace); while *M. e. phayrei* is commonly known as the Burmese brown tortoise, and is larger and darker in colour, having a charcoal to black upper shell (carapace). The forelimbs have five large claws and are covered on the front with large, heavy, overlapping scales. By contrast, the hindlimbs end in four pointed claws and bear a conspicuous cluster of very large tubercular scales on the thigh either side of the tail. These are very large that the species is sometimes referred to as the six-footed tortoise.

2.2 *Salmonella*

Salmonella is a rod-shaped, non-sporeforming and gram-negative, facultative anaerobe from the family Enterobacteriaceae. Most of the described species are pathogenic. *Salmonella* are typically nonlactose fermenters, although *S. arizonae* routinely ferment this sugar (LeMinor et al., 1984). The current classification of the genus *Salmonella* includes two species, *S. enterica* and *S. bongori*, and six subspecies of *S. enterica* including *enterica* (subspecies I), *salamae* (subspecies H), *arizonae* (subspecies III), *diarizonae* (subspecies IIIb), *houtenae* (subspecies IV), and *indica* (subspecies V) (Poppof et al.; 1997). Subspecies I is routinely isolated from humans, whereas the other subspecies are frequently isolated from poikitotherms and the environment (Mark and Simon, 2001). *Salmonella* survives well in the environment (Roszak et al., 1984, Polo et al., 1999 and Baudart et al., 2000). Infection in animals and humans with *Salmonella* may result in serious disease or give rise to a reservoir for other species and contacts within that environment. The interaction of *Salmonella* with a host gives rise to a number of clinical presentations including inapparent infection, recovered carrier state, enteritis, septicemia, and combinations of disease syndromes (Mark and Simon, 2001).

2.3 *Salmonella* spp. and chelonians (turtles and tortoises).

The presence of *Salmonella* spp. was detected in 39.1% (38/97) of the reptiles, 62.5% (15/24) in lizards, 53.3% (8/15) in snakes and 25.8% (15/58) in chelonians (Isabel and Claude, 2001). *Salmonella* are routinely isolated from apparently healthy reptiles (Mark and Simon, 2001) commonly lives in the intestine of vertebrates, and has been frequently reported in reptiles (Chiodini and Sundberg, 1981and Mermin et al., 2004). It can be disseminated via faeces to soil, water, foods and feeds and to other animals including human (Adams and Moss, 2000). The first report of a *Salmonella* from a chelonian involved *Salmonella newport*, which was isolated from the liver, spleen, lungs, and intestine of a Galapagos tortoise (*Geochelone elephantopus)* at necropsy (McNeil et al., 1946). In a survey of disease of chelonians curried out at the Zoological Society of London, few turtle died of *Salmonella* infection (McNeil et al., 1946). *Arizona*, which is now considered a species of *Salmonella*, found to be excreted by 10.5% of 691 tortoises from southeast and southwest Bulgaria (Dimov, 1965). In Germany, the feces of 125 clinically healthy tortoises were cultured and 28 different *Salmonella* spp. were cultured from 55 tortoises (44%) (Elliott R. Jacobson, 2007). Of 127 turtles tested in a large zoological collection, 37 were positive for *Salmonella* spp. (Otis and Behler, 1973).

2.4 Public health significance.

In the United States, pet turtles are an important source of salmonellosis until commercial distribution of pet turtles less than 4 inches long was banned in 1975 (Cohen, 1976). This ban led to a 77% reduction in the frequency of turtle-associated *Salmonella* serotypes isolated from humans during 1970-1976 (Cohen, 1976). The popularity of other reptiles as pets is growing and has raised concerns about their impact on public health. This demonstrates that reptile-related salmonellosis continues to pose a substantial threat to human health. Approximately 93,000 (7%) cases per year of *Salmonella* spp. infections are attributable to pet reptile or amphibian contact (Mermin et al., 1997). An estimated 3% of households in the United States have a reptile (CDC, 1999). Many reptiles are colonized with *Salmonella* spp. and intermittently shed the organism in their feces (Burnham, Atchley and DeFusco, 1998). Ingesting *Salmonella* after handling a reptile or objects contaminated by a reptile and then failing to wash their hands properly infect persons. Either direct or indirect contact with infected reptiles and their environment can cause human illness (Freidman, Torigian and Shillam, 1998).

Most persons who contract reptile-associated salmonellosis are infants and young children. In 1994, 413 (81%) of 513 *S. marina* cases occurred in children aged less than 1 year, whereas 4301 (14%) of 30,723 reported salmonellosis cases occurred in children aged less than 1 year (Mermin et al., 1997). During 1989-1998, 516 (24%) of 2150 *Salmonella* isolates with reptile-associated serotypes were from children aged less than 4 years, whereas 50,755 (19%) of 267,131 other serotypes were from this age group (CDC, 1999). Infants and immunocompromised persons are more susceptible to illness and many reptile-associated *Salmonella* infections involve serious complications, including septicemia and meningitis (Angulo and Swerdlow, 1995).

Salmonella can be transmitted to humans by direct or indirect contact with a turtle or its feces. No reliable methods are available to guarantee that a turtle is free of *Salmonella*. Most turtles are colonized with *Salmonella* and shed the bacteria intermittently in their feces. In addition, turtles not shedding *Salmonella* species under normal circumstances have been shown to actively shed the bacteria when stressed (DuPonte et al., 1978). Moreover, water in turtle bowls or aquariums can amplify any *Salmonella* shed by turtles. Adams and Moss, (2000) found that *Salmonella* organisms were recovered from samples of water collected from the containers in which pet turtles were kept for sale to the public in New York City. For these reasons, precaution should be made not only in handling chelonians also handling the water and environment.

2.5 Antibiotic resistance pattern in *Salmonella*

The emergence of multidrug resistance in *Salmonella* serotypes is causing growing concern because of the high potential of human involvement through food and animal contact (Nastasi, 2001). World Health Organization was released a report in Infectious Disease Report 2000 to show that the multi-drug-resistant phenothypes have been increasingly described among *Salmonella* species worldwide (Nastasi, 2001). This facts might be due to the use of antimicrobials in human medicine, veterinary medicine, animal husbandary, as well as agricultural and aquaculture practices.

Turtle farming has been an important economic activity in the state of Louisiana since the 1930s (Siebeling et al., 1975) The sale of small carapace turtles, *Pseudemys* (*Trachemys*) *scripta elegans*, was banned in 1975 in the United States' domestic pet market by the Food and Drug Administration (FDA) due to concerns about their excretion of the human pathogen *Salmonella* spp. (Cohen et al., 1980). In spite of the domestic ban on the sale of baby turtles, the export market has continued to thrive. Around 12 million hatchlings enter the export market each year for Western Europe, South America, and Asia (María et al, 2006). Prior to export, hatchings must be certified *Salmonella* free by independent FDA-approved laboratories (Siebeling et al., 1975). In order to eradicate *Salmonella* spp. from turtle eggs, turtle farmers were attempts to treat turtles, turtle eggs, and turtle breeding ponds with antibiotics to eliminate *Salmonella* but have not been successful and have resulted in a high prevalence of antibiotic resistance (Mitchell et al., 2007)

One research was done by Diaz (2006) which showed certain techniques to eliminate *Salmonella* from turtles have been unsuccessful and have resulted in *Salmonella* isolates with increased antibiotic resistance.

This is when antibiotics are used, most bacteria will be eradicated, but any bacteria that happen to be resistant to the antibiotic survive and multiply, resulting in antibiotic-resistant bacteria. In the past several years, Gentamicin was the antibiotic commonly used in the procedure to produce *Salmonella*-free turtle eggs (María et al, 2006). Ultimately, turtles were found that harboured *Salmonella* and other strains of bacteria that were resistant to Gentamicin as well as some related antibiotics (Plasmid-Mediated High-Level Gentamicin Resistance among Enteric Bacteria Isolated from Pet Turtles in Louisiana) (D'Aoust et al., 1990). This only makes the situation worse, since bacterial infections resulting from turtles carrying resistant strains could be harder to treat.

In other studies, 24% and 32% *Salmonella* isolates were found to be multidrug resistance in 1984 and 1989, respectively (Anon, 2000). Many authors think that the problem is because of overuse or misuse of antibiotics in not only human medicine but also veterinary medicine and agriculture. If the organisms no longer respond to antibiotic therapy, this can lead to life threatening infections in human.

3.0 MATERIALS AND METHODS

3.1 Cloacal samples

In this study, 90 adult tortoises and turtles were obtained from recreational parks, aquarium shops in Klang Valley and the individual owners that kept turtle or tortoise as their pets. Forty-five healthy tortoises and forty-five healthy turtles were randomly selected from these recreational parks, aquarium shops and individuals.

Table 2: Samples collected from different sources.

Animal	Turtles			Tortoises		
Source of samples	Recreational parks	Aquarium shops	Individual	Recreational parks	Aquarium shops	Individual
No. of samples collected	15	15	15	15	15	15
Total	45			45		

The cloacal region, a shared gastrointestinal, urinary and reproductive orifice of each animal was carefully examined. The cloacal samples were collected using sterile custom-made cotton swabs. One cloacal swab for each animal; the swab was placed into separate sterile Bijou bottles containing 2 ml of pre-enriched broth of buffered peptone water (BPW). Each bottle was carefully labelled.

3.2 Water sample

Fifteen water samples were collected from the recreational parks, aquarium shops and individual owners. The samples were put into a sterile Universal bottle aseptically.

3.3 Isolation and identification procedure

The samples were brought to the Vet Public Health laboratory in Faculty of Veterinary Medicine, Universiti Putra Malaysia for isolation and identification of *Salmonella*. The swab samples from the buffered peptone water were incubated at 37°C for up to 24 hour. After pre-enrichment stage, 1 ml of the solution was transferred to the Rappaport Vassilidis enrichment broth and further incubated up to 24 hour at 37°C.

One loopful of the culture from Rappaport Vassilidis (RV) enrichment broth was cultured onto xylose-lysine-deoxycholate (XLT4) agar and Brilliant-Green Phenol Red Lactose Sucrose (BPLS) agar and incubated at 37ºC for 24-48 hours. Gram staining and Oxidase test will be done on presumptive *Salmonella* colonies. Subcultures on XLT-4 and BPLS agar were done to obtain pure culture. Pure cultures were cultured onto blood agar for biochemical tests and gram staining.

3.4 *Salmonella* identification test

Pure colonies suspected for *Salmonella* were subjected to standard biochemical tests for identification.

Biochemical tests include:

i. Urease test – 1-2 colonies from each pure culture were picked up with sterilized wire loop and stabbed into agar slant and the surface will be streaked. The test tubes were incubated at 37^{o}C up to 48 hours.

ii. Triple Sugar Iron (TSI) – The same procedure as in urease test, but using TSI agar slant and incubated for 18-48 hours.

iii. Lysine Iron (LSI) - The same procedure as in Urease test, but the test tube contains LSI agar slant were incubated at 37^{o}C for 16-18 hours.

iv. Sulphide Indole Motility (SIM) - The same procedure was repeated as in Urease test, but without streaking the surface. The stabbed SIM agars in the test tubes were incubated at 37^{o}C for 24 hours. For Indole test, Kovac's Indole Reagent was dropped into the test tube.

Colonies showing typical characteristics of *Salmonella* spp. from the biochemical tests (alkaline slant, acid butt with hydrogen sulphide production) were then subjected to a slide agglutination test (SAT) for genus determination. Slide Agglutination Test was performed using *Salmonella* polyvalent 'O' and 'H'

Antiserum A-S. A drop of normal saline with a loop of presumptive *Salmonella* colony were mixed thoroughly on a sterilized microscope slide to produce a dense suspension. Then, a drop of *Salmonella* polyvalent antisera was applied onto the bacterial suspension. The slide was held between fingers and gently rocked back and forth for at least 1 minute. If distinct agglutination occurs, the isolate was regarded as positive (Plate 3). The positive *Salmonella* colonies were cultured on Nutrient agar for antibiotic sensitivity test.

3.5 Antibiotic sensitivity test

A loopful of pure culture from Nutrient agar which was mixed with 5 ml of 0.9% normal saline (NaCl) in a universal bottle until they reached turbidity of 0.5 McFarland standard suspension. A swab of the mixture was evenly streaked onto a Mueller Hinton Agar (MHA) plate and left to semi-dry.

Susceptibility of the organisms to antimicrobial agents was tested by a standard disc diffusion method. The discs contained the following antibiotics; ampicillin (10µg), enrofloxacin (5µg), tetracycline (30µg), ciprofloxacin (5µg), streptomycin (10µg), and erythromycin (15µm). The plates were incubated for 24 hours at 37°C. The sensitivity or resistance of each tested strain to these antibiotics were determined by measuring the diameter of the inhibition zone.

Salmonella serotyping

The colonies confirmed for *Salmonella* spp. were sent to the Veterinary Research Institute (VRI) in Ipoh on nutrient agar slant for serotyping

4.0 RESULTS

4.1 Isolation of *Salmonella* from cloacal and water samples

A total of 90 cloacal samples comprising 45 cloacal samples of turtles and 45 cloacal samples of tortoises were collected from recreational parks, aquarium shops and individuals for isolation and identification of *Salmonella*.

Table 3: Occurrence of *Salmonella* in turtles and tortoises

Animal	Source of samples	No. Of samples collected	Positive number for *Salmonella*	Percentage of samples positive (%)
Turtles	Recreational parks	15	6	40%
	Aquarium shops	15	7	46.7%
	Individual owners	15	2	13.3%
		45	15	33.3%
Tortoises	Recreational parks	15	4	26.7%
	Aquarium shops	15	5	33%
	Individual owners	15	1	6.7%
		45	10	22.2%
Total		90	25	27.8%

Out of 90 samples for isolation and identification of *Salmonella*, 15 (33.3%) of turtles and 10 (22.2%) of tortoises were positive for *Salmonella*.

Table 4: Prevalence of *Salmonella* in chelonians (turtles and tortoises) according to different sources.

Source of samples	Recreational parks	Aquarium shops	Individual Owners	Total
No. of samples collected	30	30	30	90
Positive number for *Salmonella*	10	12	3	25
Percentage (%)	33.3%	40%	10%	27.8%

A high number of chelonians were positive for *Salmonella*, collected from aquarium shops (40%), followed by recreational parks (33.3%) and 10% from individual owners.

Table 5: Occurrence of *Salmonella* spp. from water samples.

Source of water samples	Recreational parks	Aquarium shops	Individual owners	Total
No. of collected sample	5	5	5	15
No. of positive for *Salmonella*	2	4	1	7
Percentage (%)	40%	80%	20%	46.7%

From a total of 15 aquarium water samples from three different sources, a high number of samples positive for *Salmonella*, were those collected from aquarium shops (80%), followed by recreational parks (40%) and 20% from individual owners.

4.2 Identification of *Salmonella*

There was 32 samples confirmed positive for *Salmonella* upon biochemical testing (TSI, LIA, SIM and Urease). Upon gram staining, isolates showed Gram negative and single short rod organisms. The isolates were further confirmed by using the *Salmonella* O Antisera and H Antisera and agglutination indicated positive for *Salmonella*. Most isolates were positive for *Salmonella* O Antisera.

4.3 Antibiotic sensitivity test

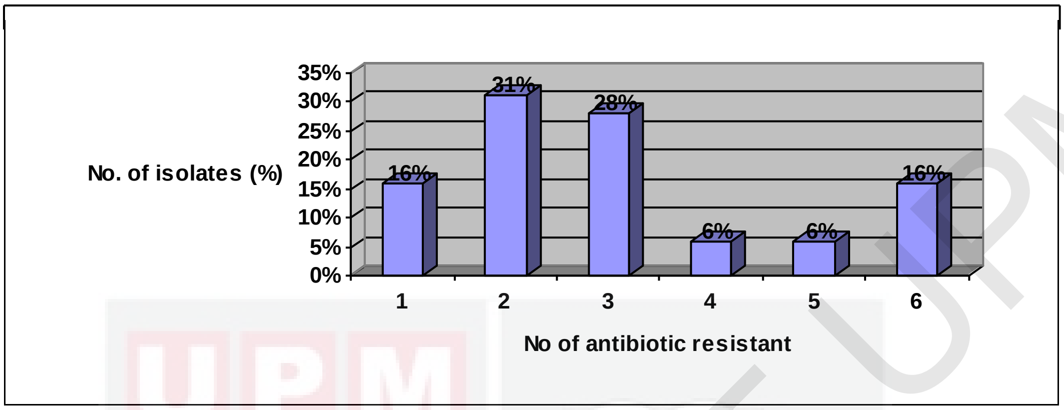
Antibiotic sensitivity test was carried out for *Salmonella* and results are shown in Table 6, Table 7 and Figure 1.

Table 6: Antibiotic Sensitivity Test for *Salmonella* against Six Antibiotics

Antibiotics	Number and Percentage Susceptible (%)	Number and Percentage intermediate (%)	Number and Percentage Resistant (%)
AMP (10µg)	7 (22%)	7 (22%)	18 (56%)
ENR (5µg)	19 (59%)	5 (16%)	8 (25%)
TE(30µg)	20 (62%)	7 (22%)	5 (16%)
CIP (5µg)	25(78%)	0 (0%)	7 (22%)
S (10µg)	5 (16%)	2 (6%)	25(78)%
E (15µg)	4 (13%)	2 (6%)	26 (81%)

Note: (1) Ampicillin (AMP); Enrofloxacin (ENR); Tetracycline (TE); Ciprofloxacin (CIP); Streptomycin (S); Erythromycin (E).

Figure 1: Percentage of *Salmonella* isolates resistant to antibiotics



In this study, 84.4% of the isolates showed resistant to more than one antibiotic tested and 15.6% of the isolates demonstrated resistant to at least one antibiotic. Twenty-seven isolates were resistant to two or more antibiotics, 34% were resistant to two antibiotics, 28% were resistant to three antibiotics, 6% were resistant to four antibiotics, and 6% were resistant to five antibiotics whereas 16% were resistant to all antibiotics tested. The highest resistance rate was towards erythromycin (81%), followed by streptomycin (78%), ampicillin (56%), enrofloxacin (25%), ciprofloxacin (22%) and tetracycline (16%). The isolates showed thirteen antibiotic resistance patterns. The most common multiple-resistance pattern was erythromycin- streptomycin- ampicillin (7 isolates). Refer to table 8.

Table 7: Percentage of *Salmonella* isolates resistance to number of antibiotics

No. Of Antibiotics	No of isolates (%) resistance to antibiotics
1 antibiotic	5 (16%)
2 antibiotics	10 (31%)
3 antibiotics	9 (28%)
4 antibiotics	2 (6%)
5 antibiotics	2 (6%)
6 antibiotics	4 (16%)
Total	32

Table 8: Antibiotic resistance patterns of *Salmonella* isolates

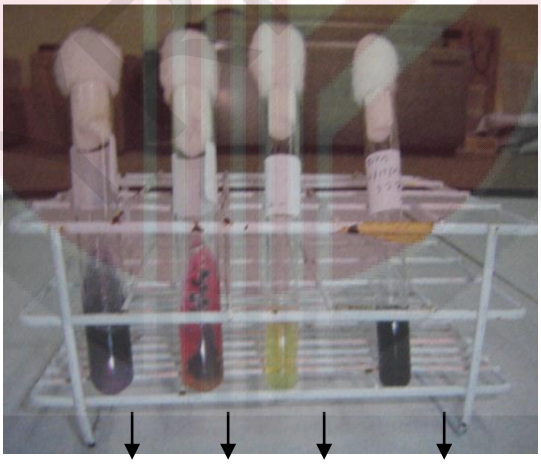
Antibiotics	No. of isolates
E	3
S	2
E-S	5
E-AMP	2
S-AMP	3
E-S-AMP	7
E-S-ENR	1
E-S-CIP	1
E-S-AMP-ENR	1
E-S-AMP-CIP	1
E-S-AMP-ENR-CIP	1
E-S-AMP-ENR-TE	1
E-S-AMP-ENR-CIP-TE	4
Total	32

Note: (1) Ampicillin (AMP); Enrofloxacin (ENR); Tetracycline (TE); Ciprofloxacin (CIP); Streptomycin (S); Erythromycin (E).

4.4 *Salmonella* serotypes

Twenty-five (27.8%) sample from chelonians and seven (46.7%) water samples were positive for *Salmonella* spp. *Salmonella* colonies on XLT4 agar and biochemical tests reactions observed is shown in Plates 1 and 2 respectively. Seven *Salmonella* serotypes were identified namely *S.newport*, *S. pomona*, *S. typhimurium, S. tenessee, S. arizona, S. brezany* and *S. corcallis*.

Plate 1: *Salmonella* colonies on XLT4 Agar



LIA TSI Urease SIM

Plate 2: Positive results for biochemical tests

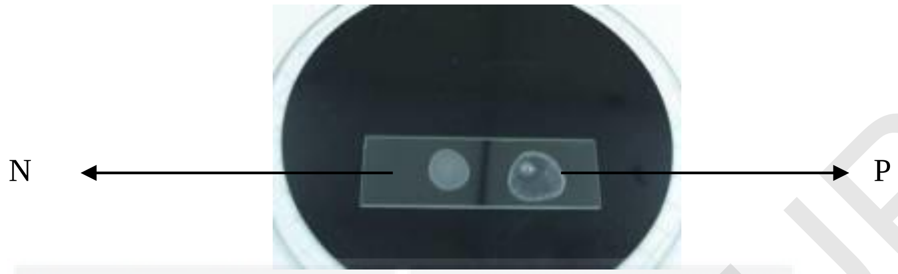


Plate 3: Positive results for *Salmonella* Polyvalent O

Note: Positive (P); Negative (N)

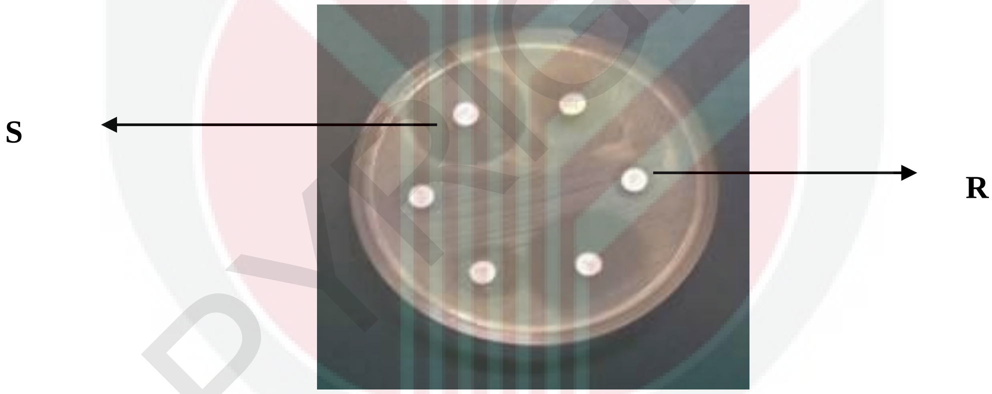


Plate 4: Antibiotic Sensitivity Test

Note: Resistant (R); Sensitive (S)

5.0 DISCUSSION

A high prevalence of *Salmonella* in the faeces of reptiles was expected. An estimated 90% of all reptiles carry and shed *Salmonella* in their faeces (Woodward et al., 1997). For this reason, exotic pets, in particular turtles and iguanas represent an important reservoir for *Salmonella* infections. A variety of *Salmonella* serotypes have been identified from these pets, and their occurrence in humans represents a marker for exposure to reptiles. In humans, *Salmonella* infections can be fatal, and it has been recommended that persons at high risk of *Salmonella* infection, that is, those with immature or weakened immune systems, including babies, children younger than 5 years of age, pregnant women, elderly people, and people with AIDS, should avoid contact with reptiles. Twenty-five (27.8%) and seven (46.7%) samples revealed positive for *Salmonella* spp., which were, isolated from cloacal swab and aquarium water samples respectively and they can be a potential source of bacterial infection to human.

Mismanagement accounts for many disease problems in captive reptiles, and clinical problems may be directly related to captive environment conditions. Reptiles in captivity are seldom maintained under optimum environment conditions. Below optimum environmental conditions may lead to a depressed immune system, (Evans, 1963) with possible invasion of opportunistic pathogens. Direct contact between a turtle or tortoise to another susceptible turtle or tortoise or human is required for transmission of these bacteria.

Salmonellosis is certainly the most frequent and major zoonosis transmitted by reptiles especially turtles (Lamm et al.; 1972). In the present study, a substantial proportion of *Salmonella* positive samples (33.3%) were found and a significantly lower percentage of *Salmonella* positive samples in turtles in comparison with tortoises (22.2%). Turtles may be considered as a very important reservoir for *Salmonella* in the study area.

In the present study, there were seven *Salmonella* serotypes identified and the most common serotype was *S. pomona*. Greenberg *et al*., (1976), reported serotype *S. pomona* widely distributed, and frequently reported in reptiles. Woodword et al., (1997), reported that human salmonellosis by serotype *S. pomona* was isolated from 30 human patients. *Salmonella* isolated from turtle aquarium water suggested that it could be a health hazard to human, who have daily direct contact with the chelonians and its water. Public health officers consider all *Salmonella* isolates to have zoonotic potential.

From the seven serotypes discovered, only *S. pomona, S. newport* and *S. typhimurium* had been reported in previous studies from turtles and tortoises. *Salmonella tenessee, S. arizona, S. brezany* and *S. corcallis* are those, new serotypes discovered in chelonians, hence *Salmonella* serotyping found in this study could be used as an epidemiological marker in this region as well as Malaysia, to study the incidence of *Salmonella* infection in chelonians and the associated transmission to human as recommended by Woodword et al.(1997).

A significantly higher percentage of *Salmonella* positive chelonians were detected in the group of aquarium shops. A very high percentage of nearly 40% of *Salmonella* isolates were found in samples of chelonians from aquarium shops and nearly 33.3% in samples from recreational parks. *Salmonella* survives well in the environment (Roszak et al., 1984, Polo et al., 1999 and Baudart et al., 2000). In the wild or natural habitats, the ingestion of faeces or contaminated water is considered a probable way of colonization of *Salmonella* (Polo et al., 1999, Baudart et al., 2000 and Mermin et al., 2004). Thus, the high incidence of *Salmonella* that found in turtles might originate via oral ingestion. Other than that, cloacal transmission might be a mechanism of transmission among individuals, especially as different males frequently mount individual females for copulation during the breeding season (Roques et al., 2004). In general, the aquatic medium is considered a favourable environment for transmission of *Salmonella* (Polo et al., 1999 and Baudart et al., 2000). In this study area, the results demonstrate that, turtles kept in wild or natural habitats (recreational paks) less prone to *Salmonella* infection than the one kept in aquarium shops. It may be explained by the lower persistence of the bacteria in those living in aquatic medium where they are washed from skin and cloaca, while in aquarium habitats they persist longer due to stagnant water and are directly transmitted among individuals.

Salmonella are probably saprophytic organisms in reptiles (Chiodini and Sundberg, 1981), the animals may shed these bacteria over long periods without any sign of disease. Also the high number of isolates with so-called ‘exotic’ serovars may suggest that the reptiles could carry the *Salmonella* from the aquarium shops to their new owners (individual owners). By contrast, *Salmonella* were found less frequently in pet chelonians. In this group, saw a significantly lower prevalence of *Salmonella* in animals kept by individual as their pet. *Salmonella* were isolated in three out of 30 turtles and tortoises. Moreover, two out of three *Salmonella* positive tortoises were kept together with *Salmonella* positive aquarium water. These facts may suggest that the risk of *Salmonella* infection in chelonians of individual owners is relatively low, particularly when no *Salmonella* positive animals are kept. In addition, this might be due to the regular cleaning and good hygiene management of the chelonians kept as pets by the owners. Moreover, the chelonians kept in optimum condition and less stress shed less *Salmonella* in the environment.

The serotypes of *Salmonella* between the positive chelonians and the water within the same enclosure were similar. These facts indicate an increased risk of horizontal transmission of *Salmonella* between all animals held by one owner and under similar enclosure, once a single animal is infected. Hence even one chelonian shedding *Salmonella* spp. is able to contaminate its aquarium water and infect other healthy animal via ingestion of the aquarium water. However in this present study although it shows the lower occurrence of *Salmonella* in individual owners, public must always be aware of the risks. Exotic pets often have free access to areas in the house where families and children live. Kitchen or bathroom sinks are used to clean reptile accessories or caging material (Woodward et al., 1997). For this reason, a horizontal transmission of *Salmonella* between animals and human is possible. Thus, reptile pets clearly represent an important reservoir for human *Salmonella* infection under these conditions. The danger of transmission to humans may be increased because *Salmonella* infections are usually asymptomatic in reptiles. In our study, there was no significant difference regarding the health status of *Salmonella* positive reptiles and those without *Salmonella* detection. Reptile-associated salmonellosis is a serious public health problem.

The emergence of antibiotics-resistant bacteria has become an increasing problem all over the world (Usera et al., 2002). Drug resistance of pathogenic bacteria diminishes the effectiveness of antimicrobial treatment and can lead to the use of less safe, ineffective or expensive alternatives. Strains of *Salmonella* that are resistant to antimicrobial agents are a worldwide problem (Usera et al., 2002). The World Health Organization reported an increase in the incidence of antimicrobial resistant *Salmonella* strains and the emergence of multidrug-resistant strains isolated from animal and human. More than 80% of the strains isolated from animal and human sources showed resistance to tetracycline, sulfonamides, streptomycin and chloramphenicol. Resistance to ampicillin was associated with resistance to at least four antimicrobial agents in 78% of the human and 83% of the animal isolates. The most common combination of multiple resistance included ampicillin, sulfonamides, streptomycin, chloramphenicol and tetracycline (Usera et al., 2002). Work of Mitchell et al.; (2007) shows that the antimicrobial resistance of *Salmonella* is acquired by recombination and transfer of DNA between strains and that some strains have all the genetic material needed for transfer of resistance genes.

In this study, 84.4% of the isolates showed resistant to more than one antibiotic tested and 15.6% of the isolates demonstrated resistant to at least one antibiotic. Twenty-seven isolates were resistant to two or more antibiotics, 34% were resistant to two antibiotics, 28% were resistant to three antibiotics, 6% were resistant to four antibiotics, and 6% were resistant to five antibiotics whereas 16% were resistant to all antibiotics tested. The isolates showed thirteen antibiotic resistance patterns. The most common multiple-resistance pattern was erythromycin- streptomycin- ampicillin (7 isolates).

Most isolates showed resistance to erythromycin, streptomycin, ampicillin, enrofloxacin and followed by tetracycline. The emergence of the antimicrobial resistance may be due to the overuse or misuse of antibiotics in human and veterinary medicine and also from ‘contaminated’ environment.

6.0 CONCLUSION

Twenty-eight percent of cloacal swab of chelonians and 47% of water samples were positive for *Salmonella* in this study; this suggest that *Salmonella* in chelonians certainly pose a public health concern, which could be a potential source of human salmonellosis. About 84.4% of the isolates showed resistant to more than one antibiotic tested and 15.6% of the isolates demonstrated resistant to at least one antibiotic. The emergence of multi-drug resistance in *Salmonella* spp. is also a public health concern. It is because the increase of morbidity and mortality resulting from failing antimicrobial treatment and increase in cost of health care, the development of new and efficacious drugs and also due to the prolonged treatment of diseases.

The zoonotic significance of owning *Salmonella* infected reptiles and reptile-associated salmonellosis at recreational parks cannot be ignored; because it may occur through environmental contamination. Veterinarians working in private practice and in recreational institutions can serve a vital role in the prevention of reptile-associated salmonellosis. Pet reptile owners and visitors to recreational parks should be informed of the potential risks of maintaining and handling these animals. Immunocompromised individuals and young children should avoid contact with reptiles. Hand washing should be recommended following any contact with a reptile or reptile enclosure. Educating pet owners on zoonosis prevention can help them make more informed decisions about risks and benefits of owning a pet. However, the overall benefits of the human–animal bond must be considered and in general immunocompromised patients should be able to continue to enjoy the significant benefits of pet ownership.

7.0 RECOMMENDATION

Additional recommendations for reducing the risk of transmission of *Salmonella* to humans from exotic pets include the following:

1. Handlers (breeders, distributors, owners) of exotic pets should wash their hands thoroughly immediately after direct or indirect contact. Secondary transmission of *Salmonella* to those who have not had direct contact with exotic pets can occur by contact with handlers who have not thoroughly washed their hands.
2. Aquariums and housing cages for exotic pets should not be cleaned in sinks or be located in vicinities where food may be prepared for human consumption.
3. Exotic pets should be confined to aquariums or housing cages and should not be permitted free access to areas occupied by families and children (kitchens, recreation rooms, day-care centers, classrooms).
4. Exotic pets should not be fed raw meats, including raw ground beef. This could create the potential for the spread of other dangerous enteric pathogens such as *Escherichia coli* O157:H7.
5. Veterinarians and pet storeowners should provide information to potential purchasers and owners about the increased risk of acquiring salmonellosis from exotic pets.

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9.0 APPENDIX

Malayan Giant Turtle

Orange Headed Temple Turtle

Black Pond Turtle

Red-eared Slider

Alligator Snapping Turtle

Asian Leaf Turtle

Long-necked Turtle

Galapagos Tortoise

Asian Brown Tortoise

Elongated Tortoise

Star Tortoise