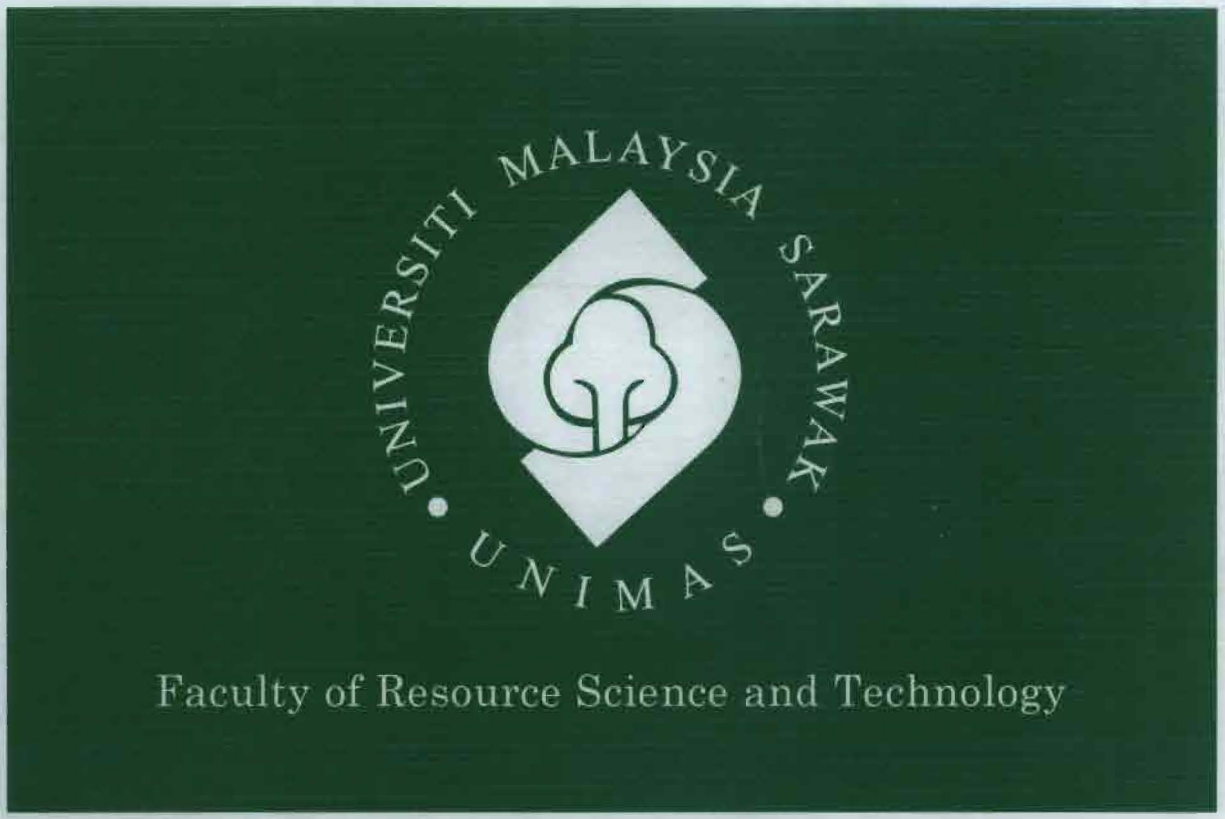

PREVALENCE OF *Salmonella spp.*, *Salmonella* TYPHIMURIUM, *Escherichia coli* AND ESCHERICHIA COLI O157: H7 FROM BATS, BIRDS, SOIL AND WATER IN THE SELECTED STUDY SITES AT SARAWAK

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Bachelor of Science with Honours
(Resource Biotechnology)
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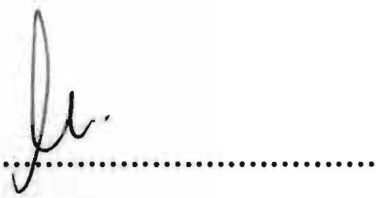
DECLARATION

I hereby declare that this thesis entitled "Prevalence of *Salmonella*, *Salmonella* Typhimurium, *Escherichia coli* and *Escherichia coli* O157: H7 from bats, birds, soil and water in the selected study sites at Sarawak" submitted to the Faculty of Resource Science and Technology is a presented of my original work except for the citations and references which have been duly acknowledged and never been before or concurrently submitted for any other degree of qualification or other institutions. This work was done under supervise of Dr. Lesley Maurice Bilung and submitted to partially fulfill the requirement for the degree of Bachelor of Science with Honours in Resource Biotechnology.



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LIST OF ABBREVIATIONS

D	Domain
DNA	Deoxyribonuclei acid
dNTP	Deoxynucleotide triphosphate
ddH_2O	Double distilled water
fliC	Flagellar antigen gene
LB	Luria broth
LB Agar	Luria Bertani Agar
LPS	Lipoposaccharides
$MgCl_2$	Magnesium Chloride
mL	Milliliter
mm	Milimeter
mM	Milimolar
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
Rpm	Revolution per minute
Spp.	Species (plural)
U	Unit
UV	Ultra violet
µL	Microliter
µm	Micrometer
°C	Degree Celsius
%	Percent
-	To

$\leq$	Less than or equal to
$\geq$	More than or equal to
X	Times

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Prevalence of *Salmonella* spp., *Salmonella* Typhimurium, *Escherichia coli* and *Escherichia coli* O157: H7 from Bats, birds, soil and water in the selected study sites at Sarawak

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ASTRACT

A total of 235 samples collected from bats, birds, water and soil of Sebangkoi Recreation Park, Kubah National Park and Bako National Park in Sarawak were studied for the presence of *Salmonella* spp., *Salmonella* Typhimurium, *Escherichia coli* and *Escherichia coli* O157: H7. Among the samples, 12% (28/235) and 14% (33/235) were identified with the presence of *Salmonella* spp. and *E. coli* respectively. Further confirmation using Polymerase Chain Reaction (PCR) indicated that 11% (3/28) of *Salmonella* Typhimurium, 3% (1/33) of *E. coli* O157: H7 and 18% (6/33) *E. coli* O157 were present in the samples. Besides, antibiotic resistance analysis for *Salmonella* spp. showed that there were 56% (14/25), 92% (23/25), 0% (0/25), 4% (1/25), 8% (2/25), 56% (14/25) and 12% (3/25) of the *Salmonella* isolates were resistant to ampicillin, cephalothin, chloramphenicol, doxycycline, gentamicin, nitrofurantoin and streptomycin respectively. On the other hand, the *E. coli* isolates had 18% (6/33), 42% (14/33), 3% (1/33), 0% (0/33), 9% (3/33), 18% (6/33) and 30% (10/33) resistant to ampicillin, cephalothin, chloramphenicol, doxycycline, gentamicin, nitrofurantoin and streptomycin respectively. Then, the lowest and the highest multiple antibiotic resistance (MAR) index for *Salmonella* spp. and *E. coli* isolates were in the ranged from 0.1 to 0.7 which were resistant to at least one and the most five of the antimicrobial agents used. Furthermore, the MAR analysis revealed that 87% (20/23) of *Salmonella* isolates and 67% (12/18) of *E. coli* isolates had MAR index more than 0.2 whereas 13% (3/23) of *Salmonella* isolates and 33% (6/18) of *E. coli* isolates had MAR index less than 0.2.

Key words: *Salmonella* Typhimurium; *Escherichia coli* O157:H7; Polymerase Chain Reaction (PCR); antibiotic susceptibility test; multiple antibiotic resistant (MAR)

ABSTRAK

Sebanyak 235 sampel daripada kelawar, burung, air dan tanah dari Taman Rekreaksi Sebangkoi, Taman Negara Kubah dan Taman Negara Bako di Sarawak telah dikumpul untuk kajian kewujudan *Salmonella* spp., *Salmonella* Typhimurium, *Escherichia coli* and *Escherichia coli* O157: H7. Sebanyak 12% (28/235) dan 14% (33/235) sampel telah dikenalpasti mengandungi *Salmonella* Typhimurium dan *E. coli* O157: H7 masing-masing. Kenalpasti lanjutan dengan menggunakan Reaksi Rantaian Polimerasi (PCR) telah menunjukkan 11% (3/28) *Salmonella* Typhimurium, 3% (1/33) *E. coli* O157: H7 dan 18% (6/33) *E. coli* O157 wujud dalam sampel-sampel tersebut. Analisis tahan antibiotik untuk *Salmonella* spp. strain telah menunjukkan 56% (14/25), 92% (23/25), 0% (0/25), 4% (1/25), 8% (2/25), 56% (14/25) and 12% (3/25 tahan dengan ampicillin, cephalothin, chloramphenicol, doxycycline, gentamicin, nitrofurantoin dan streptomycin masing-masing. Sebaliknya, terdapat 18% (6/33), 42% (14/33), 3% (1/33), 0% (0/33), 9% (3/33), 18% (6/33) and 30% (10/33) *E. coli* strain tahan dengan ampicillin, cephalothin, chloramphenicol, doxycycline, gentamicin, nitrofurantoin and streptomycin masing-masing. Index tahan kepelbagaian antibiotik (MAR) paling tinggi and rendah untuk *Salmonella* spp. dan *E. coli* strain adalah antara 0.1 dan 0.7 di mana mereka tahan antara satu hingga lima antibiotik tersebut. Selain itu, analisis MAR menunjukkan 87% (20/23) *Salmonella* spp. dan 67% (12/18) *E. coli* mempunyai MAR index tinggi daripada 0.2. Manakala, 13% (3/23) *Salmonella* spp. dan 33% (6/18) *E. coli* mempunyai MAR index rendah daripada 0.2.

Kata kunci: *Salmonella* Typhimurium; *Escherichia coli* O157:H7; reaksi rantai polimerasi (PCR); ujian tahan antibiotik; tahan kepelbagaian antibiotik (MAR)

CHAPTER 1

INTRODUCTION

Human, animals, soil and water are known as reservoir for pathogenic microorganisms including bacteria (Decker, 2003). An infection that transmitted from animals to human is called zoonosis or zoonotic disease. Soil borne disease and waterborne disease are infection that caused by etiological agents in human after contacting with contaminated soil and water (Gerba, 1988; Jeffery and van der Putten, 2011). Based on Garrity *et al.* (2005), the members under the family of Enterobacteriaceae are found in animals, soil and contaminated water. According to CDC (2012), the two most frequently foodborne causing bacterial pathogen are *Salmonella and* Shiga toxin-producing *Escherichia coli* (STEC). Foodborne outbreak reported from 2006 to 2012 in the United States of America due to *Salmonella* Typhimurium and *E. coli* O157: H7 were 8 and 13 cases, respectively (CDC, 2012).

Salmonella Typhimurium and *E. coli* O157: H7 are bacteria in the family of Enterobacteriaceae. They are Gram negative bacteria with straight rod shape and have peritrichous flagella that cover their cell surface for motility. Besides that, the bacteria under the genus of *Salmonella* (0.7-1.5 X 2.0-5.0 μm) and *Escherichia* (1.1-1.5 X 2.0-6.0 μm) have almost identical range of sizes. In addition, their survival temperature and pH range are 25°C to 35°C and 6.5 to 8.0, respectively (Sharma *et al.*, 2009). Moreover, these bacteria infect the gastrointestinal tract of human and animals (Bopp *et al.*, 1999; Garrity *et al.*, 2005). Furthermore, they are transmitted through fecal-oral pathway and can cause gastroenteritis as well as foodborne outbreak globally. Infection and disease by these

bacteria can be prevented by practicing good sanitation practices in terms of food preparation and personal hygiene.

This study was carried out to detect *Salmonella* Typhimurium and *E. coli* O157: H7 from bats, birds, water and soil at various selected sites in Sarawak, Malaysia by using Polymerase Chain Reaction (PCR). The selected study sites included Sebangkoi Recreational Park, Sarikei; Kubah National Park, Matang; and Bako National Park, Bako. A total of 197 swab samples were collected from the anus or cloaca, oral cavity or faeces of bats and birds. In addition, 18 water samples were collected from the water source found at each of the study sites while 20 soil samples were collected randomly. All the samples were streaked on both XLD and EMB agar. Previous studies showed that *Salmonella* Typhimurium were detected in bird and chicken (Sato, 1996; Vigo *et al.*, 2009). In addition, *Salmonella* bacteria were isolated from 84 faeces of pigs and 45 farms (Asai *et al.*, 2002). A study by Perales and Audicana (1989) reported that 83 of 256 samples from coastal waters were detected with *Salmonella*. Furthermore, *Salmonella* Typhimurium was detected in soil samples after absence for nine weeks (Davies and Wray, 1996). For *E. coli* O157: H7, it was isolated and detected in birds and mammals (Jay-Russell *et al.*, 2010) as well as soil and water samples (Campbell *et al.*, 2001).

The objectives of this study are:

i. To study the occurrence of *Salmonella* and *E. coli* from bats, birds, soil and water samples

ii. To detect the presence of *Salmonella* Typhimurium from wild animals, soil and water samples by using specific PCR targeting *fliC* gene

iii. To detect the presence of *E. coli* O157: H7 from wild animals, soil and water samples by using multiplex PCR targeting *stx1, stx2, rfbE and* $fliC_{h7}$ genes

iv. To determine the antibiotic resistance profile of *Salmonella* spp. and *E. coli* from the selected study sites

CHAPTER 2

LITERATURE REVIEW

2.1 Enterobacteriaceae

The family Enterobacteriaceae denotes a group of enterobacteria that infect the gastrointestinal tract. They are Gram negative straight rod shape bacteria with no endospore. Most of them have peritrichous flagella that cover their cell surface and a size of 0.3-1.0 μm X 1.0-6.0 μm. In addition, they are able to survive with or without the presence of oxygen. Examples of enterobacteria are *Salmonella* sp. and *Escherichia coli (E. coli)* (Garrity *et al.*, 2005).

2.2 Taxonomy of *Salmonella* spp. and *Salmonella* Typhimurium

According to *Bergey's Manual of Systematic Bacteriology* (2005), *Salmonella* spp. are prokaryotes under genus of *Salmonella*, family of Enterobacteriaceae, order of Enterobacteriales, class of Gammaproteobacteria, phylum of Proteobacteria, and domain of Bacteria. The genus *Salmonella* consists of two main species, *Salmonella enterica* and *Salmonella bongori* (Grimont and Weill, 2007). The *S. enterica* is divided into six different subspecies, named *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) *and indica* (VI) as shown in Table 2.1.

Table 2.1 Number of serotypes in each species and subspecies (Grimont and Weil, 2007).

S. enterica	2557
S. enterica subsp. enterica	1531
S. enterica subsp. salamae	505
S. enterica subsp. arizonae	99
S. enterica subsp. diarizonae	336
S. enterica subsp. houtena	73
S. enterica subsp. indica	13
S. bongori	22
Total (genus *Salmonella*)	**2579**

Each subspecies is further distinguished into different serovars or serotypes based on somatic (O), capsular (Vi) (if present) and flagellar (H) antigens (Garrity *et al.*, 2005). An example of serovar of *S. enterica* is *S. enterica* subspecies *enterica* serovar Typhimurium which normally abbreviated as *Salmonella* Typhimurium (Ellermeier and Slauch, 2006). *Salmonella* Typhimurium has the antigenic formulae of 1,4,[5],12:i:1,2 which represent the O antigen(s): Phase 1 H antigen(s): Phase 2 H antigen(s) (Garrity *et al.*, 2005; Ellermeier and Slauch, 2006). The somatic (O) antigen is a heat stable antigen found in the lipopolysaccharide (LPS) of the outer membrane of gram negative bacteria (Ellermeier and Slauch, 2006). On the other hands, the flagellar (H) antigen is a heat labile antigen that situated at the bacterial flagella. The capsular (Vi) antigen is a capsular polysaccharide that present only on the surface of *Salmonella* Typhi, *Salmonella* Paratyphi and *Salmonella* Dublin (Garrity *et al.*, 2005; Ellermeier and Slauch, 2006).

2.3 Characteristic of *Salmonella* spp. and *Salmonella* Typhimurium

Salmonella spp. are straight rod shape, non spore forming bacteria with diameter range from 0.7 to 1.5 μm and length range from of 2.0 to 5.0 μm (Garrity *et al.*, 2005). Most of the serotypes have peritrichous flagella on their cell surface for mobility, with only two exceptions, which are immobile *S.* gallinarum and *S.* pullorum. They are also known as

facultative anaerobe bacteria, gram negative bacteria and form colonies with the diameter about 2 to 4 mm (Garrity *et al.*, 2005). Furthermore, they are catalase positive, nitrate reducer, glucose fermentor and oxidase negative (Gillespie, 1994; Tortora *et al.*, 2007).

Salmonella Typhimurium is a rod shaped bacterium with the diameter about 1.3 μm and length about 4.0 μm (Abbineni and Mao, 2010). This bacterium has peritrichous flagella on its cell membrane surface which plays an important role in the cell motility. The flagella are made up of three components that are basal body, filament and hook. Each flagellar filament is made up of about 20,000 flagellin subunits with the molecular weight of 50,000 (Turner *et al.*, 2000; Cruz Ramos *et al.*, 2004). The flagellin is a globular protein encoded by *flic* gene and composed of four linearly linked domains (Niu *et al.*, 2007; Abbineni and Mao, 2010). The domains are D0, D1, D2 and D3, which arranged from internal to external of the filament, forming the shape of upper case of Greek gamma (Γ) (Niu *et al.*, 2007). The D2 and D3 are highly variable domains, which mean that they are genetically modifiable by inserting foreign gene into the variable region of *flic* gene (Abbineni and Mao, 2010). The diameter and length of the filament are approximately 12 to 25 nm and 2 nm respectively (Kamiya and Asakura, 1976; Berry and Armitage, 1999).

Besides that, *Salmonella* Typhimurium is a non endorespore forming bacterium which can be killed by extreme stress conditions such as overheat, water stress, toxic chemical or radiation exposure (Jana, 2005; Tortora *et al.*, 2007). Moreover, it is known as gram negative bacterium with the cell wall made up of thinner peptidoglycan and an outer membrane (Tortora *et al.*, 2007; Abbineni and Mao, 2010). Furthermore, *Salmonella* Typhimurium can be facultative anaerobic or aerobic bacteria whereby they can survive in the presence or absence of oxygen (Jana, 2005; Tortora *et al.*, 2007).

2.4 Habitat of *Salmonella* spp. and *Salmonella* Typhimurium

Most *Salmonella* spp. inhabit in the intestinal tracts of organisms such as human and animals (Bopp *et al.*, 1999). *Salmonella* grow in the intestine and gall bladder of the digestive system (Stecher *et al.*, 2007; Gonzalez-Escobedo *et al.*, 2011). Besides, they are found in the lymph nodes of the lymphatic system (Arthur *et al.*, 2008). A study conducted by Arthur *et al.* (2008) stated that lymph nodes from the flanks of cow and bull carcasses had the highest prevalence of Salmonella bacteria. As stated by UC Food Safety (2012), *Salmonella* spp. can survive in the temperature range of 5°C to 45°C and pH range of 4 to 9 with the optimum growth temperature and pH ranges of 35°C to 43°C and 7.0 to 7.5 respectively. Other than the body systems, *Salmonella* spp. are detected in water, food and natural environment due to fecal contamination (Grimont *et al.*, 2000). According to New Mexico Department of Health (2009), *Salmonella* spp. are excreted in faeces from the infected organisms or salmonellosis recovered organisms to the surrounding environment. About 1 million of *Salmonella* bacteria per gram can be detected in the faeces excreted by an infected person (Tortora *et al.*, 2007). Although *Salmonella* spp. are not free living organisms, they can survive up to several years in the environment (Bopp *et al.*, 1999).

Salmonella Typhimurium colonizes mainly the digestive system of human and warm blooded animals (Ellermeier and Slauch, 2006). Based on studies by Brown *et al.* (1979) and Menghistu *et al.* (2011), *Salmonella* Typhimurium were detected in the gastrointestinal tracts of warm blooded animals such as mice and poultry. Besides, it lives in circulatory system and lymphatic system too. The bacteria were detected in blood of typhoid patients and lymph nodes of bovine (Song *et al.*, 1993; Brichta-Harhay *et al.*, 2012).

The optimum growth temperature and pH of *Salmonella* Typhimurium is 37°C and 6.5 to 7.5 respectively (Jana, 2005; Dunkley *et al.*, 2007). According to a study conducted by Al Tayib *et al.* (2007), *Salmonella* Typhimurium can survive in the pH range within 5.0 to 9.0. However, the bacteria die when exposed to acidic condition of pH 3.0 or lower and alkaline condition of pH 10.0 or higher (Lin *et al.*, 1995; Álvarez-Ordóñez *et al.*, 2011).

Other than in the living organism, this bacterium is also found in natural environment such as water and soil (Guillot and Loret, 2010). Previous studies conducted by Knight *et al.* (1990) and Platz (1980) showed that *Salmonella* Typhimurium were detected from water and soil. The bacteria are excreted in the stool of infected persons or carriers to the natural environment (Arvanitakis, 2008).

2.5 Salmonellosis by *Salmonella* spp. and *Salmonella* Typhimurium

Salmonellosis is digestive system diseases or infections caused by *Salmonella* spp.. About 10 thousands to 100 millions of *Salmonella* bacteria are needed to cause *Salmonella* infection (David, 2012). *Salmonella* invade the epithelial cells lining on the intestinal tract and enter the host cells by membrane ruffling (Tortora *et al.*, 2007). After that, they grow inside the vesicles formed in the cells causing inflammatory response that result diarrhea. Sometimes, *Salmonella* move across the epithelial cells to enter the blood circulatory system and the lymphatic system (Tortora *et al.*, 2007).

Normally, the symptoms of salmonellosis begin within 12 to 36 hours after exposure to *Salmonella* bacteria (NIAID, 2011; Tortora *et al.*, 2007). The symptom(s) include headache, loss of appetite, nausea, vomiting, abdominal pain or cramps, diarrhea

and fever. There are two types of fever related to *Salmonella* spp., typhoid fever and paratyphoid fever. World Health Organization (2012) stated that typhoid fever is caused by *Salmonella* typhi while paratyphoid fever is caused by *Salmonella* paratyphi.

Non-typhoidal salmonellosis is a type of food poisoning caused by *Salmonella* bacteria including *Salmonella* Typhimurium (Arvanitakis, 2008). It is transmitted to a healthy organism through oral-fecal pathway (Oliveira *et al.*, 2006). Normally, the symptoms of non-typhoidal salmonellosis appear within 6 to 48 hours after exposure to the bacteria (Arvanitakis, 2008). An example of non-typhoidal salmonellosis is gastroenteridis. The symptom(s) of gastroenteridis include headache, loss of appetite, nausea, vomiting, abdominal pain or cramps, diarrhea and moderate fever which can resolve within few days without special treatment. Another example of disease is bacteremia (Arvanitakis, 2008). Bacteremia is an uncommon type of non-typhoidal salmonellosis caused by serotype *Salmonella* Choleraesuis, *Salmonella* Typhimurium and *Salmonella* Heidelberg. The symptom(s) of bacteremia are fever, nausea, vomiting, diarrhea, abdominal discomfort, cardiovascular infection, metastatic infection or immune complex deposition (Kim *et al.*, 2004).

2.6 Salmonellosis outbreak

According to Public Health Agency of Canada (2011) and CDC (2012), salmonellosis due to *S. enterica* occurs globally. In the United States, 42 outbreak of salmonellosis were reported from 2006 to 2012 with the largest outbreak in 2010; 1,939 people infected with *Salmonella* Enteritidis due to contacting with contaminated egg shells (CDC, 2012).

Besides that, nine salmonellosis outbreaks due to *Salmonella* Typhimurium had occurred in United States from 2006 to 2012.

In KwaZulu-Natal, South Africa, *Salmonella* Enteritidis were isolated from 18 patients and food samples (Niehaus *et al.*, 2011). Various *Salmonella* strains were also isolated from poultry droppings, fresh beef, meat retailers' aprons and tables as well as in the blood, stool and urine of the typhoid patients from five hospitals in Akwa, Nigeria (Itah and Uweh, 2005; Orji *et al.*, 2005). In Jakarta, Indonesia, *Salmonella* Typhimurium were the most frequently detected serovar in children with non-typhoidal *Salmonella* infection from January 2003 to August 2005 (Bukitwetan *et al.*, 2007).

In Selangor, Malaysia, a typhoid outbreak occurred due to sewage disposal in Sungai Congkak (Anita *et al.*, 2011). Four cases of food poisoning in Meradong, Sarikei, Sarawak due to *Salmonella* bacteria was reported by Sarawak Health Department (2012). *Salmonella* Typhimurium was the fourth common serovars isolated in Malaysia from 2002 to 2005 as stated by Haruo *et al.* (2006).

2.7 Prevention and treatment of salmonellosis

Salmonellosis can be prevented by practicing a good sanitation in terms of personal and public health dimensions during food preparation (Underman, 2011). Raw meats, eggs and unpasteurized milk should be cooked thoroughly before consumption (Bullock *et al.*, 2006). Hands, kitchen work surfaces and utensils should be washed with soap and water immediately after each food preparation and meal. Hands must also be washed immediately after contacting with reptiles, pets, or feaces of pets. In addition, infected