ANTIBIOTIC RESISTANCE PATTERNS AND GENETIC RELATEDNESS OF *SALMONELLA* SEROTYPES ISOLATED FROM CHICKEN CARCASSES OF RETAIL MARKETS AND SLAUGHTERHOUSES IN KOTA BHARU, KELANTAN

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Antibiotic Resistance Patterns and Genetic Relatedness of Salmonella Serotypes Isolated from Chicken Carcasses of Retail Markets and Slaughterhouses in Kota Bharu, Kelantan

by

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LIST OF ABBREVIATIONS & SYMBOLS

ATCC	American Type Control Culture
BHI	Brain Heart Infusion
BURST	Based Upon Related Sequence Types
DLV	Double Locus Variant
DNA	Deoxyribonucleic Acid
eBG	eBurstGroup
MLST	Multilocus Sequence Typing
MST	Minimum Spanning Tree
PCR	Polymerase Chain Reation
SLV	Single Locus Variant
TBE	Tris-Borate EDTA
UPGMA	Unweighted Pair Group Method With Arithmetic Mean
AMP10	Ampicillin 10µg
AMC30	Amoxycillin/Clavulanic acid 30µg
ATCC	American Type Control Culture
bp	Base Pair
CN10	Gentamycin 10µg
CRO30	Ceftriaxone 30µg
K30	Kanamycin 30µg
S10	Streptomycin 10µg
S3 300	Compound sulphoamides 300µg
SXT 25	Sulphamethoxaole/Trimethoprim 25µg
TE 30	Tetracycline 30µg

Corak Rintangan Antibiotik dan Hubungkait Genetik Serotip *Salmonella* yang Diasingkan Dari Karkas Ayam di Pasar dan Rumah Penyembelihan di Kota Bharu, Kelantan

ABSTRAK

Salmonela bukan-tifoid merupakan antara penyebab utama penyakit bawaan makanan dan produk berasaskan ayam sering dikaitkan sebagai punca jangkitan tersebut. *Salmonela* serotip telah diasingkan dari karkas ayam di pasar runcit dan pusat penyembelihan. Di Kelantan, *Salmonela* telah diasingkan dari 64% ke 65% karkas ayam di pasar runcit dan rumah penyembelihan di Kota Bharu. Namun begitu, hubungkait *Salmonela* serotip daripada karkas ayam di pasar runcit dan rumah penyembelihan tidak pernah dikenalpasti. Objektif penyelidikan ini adalah untuk mengenal pasti corak rintangan antibiotik dan hubungkait genetik *Salmonela* serotip yang diasingkan daripada karkas ayam di pasar dan rumah penyembelihan. *Salmonela* Corvallis, *S.*Enteritidis, *S.*Stanley dan *S.*Typhimurium yang diasingkan dari karkas ayam di pasar dan rumah penyembelihan di Kota Bharu telah digunakan dalam penyelidikan ini. Serotip tersebut telah diuji kerintangan terhadap 13 antibiotik dengan menggunakan kaedah penyebaran disk. Pengelasan molekular terhadap isolat tersebut dilakukan dengan teknik pengelasan pelbagai-lokus jujukan berdasarkan tujuh gen-gen penting (*thr*A, *pur*E, *suc*A, hisD, *aro*C, *hem*D dan *dna*N). Corak rintangan antibiotik bagi *S.*Corvallis dari karkas ayam di pasar dan rumah penyembelihan kebanyakkannya adalah serupa. *Salmonella* Stanley dan *S.*Typhimurium dari rumah penyembelihan adalah lebih rintang terhadap antibiotik berbanding isolat dari pasar. Sebaliknya, *S.*Enteritidis dari pasar adalah lebih rintang terhadap antibiotik berbanding *S.*Enteritidis dari rumah penyembelihan. Daripada 45 *Salmonela* isolat yang diuji, 91%, 82%, dan 69% adalah rintang terhadap *sulfonamides*, *tetracycline* dan *streptomycin*. Secara keseluruhan, 18% daripada isolat yang diuji adalah rintang terhadap lebih daripada tujuh antibiotik yang diuji. *Salmonella* Corvallis isolat 4C, 59, 43 dan 5C dari pasar runcit dan *S.*Corvallis isolat 64, 13C, 69 dan 70 dari rumah penyembelihan berasal dari jenis jujukan yang sama, ST1541. Sebagai kesimpulan, gabungan pengelasan pelbagai lokus jujukan (MLST) dan corak rintangan terhadap antibiotik menunjukkan bahawa *S.*Corvallis daripada karkas ayam dari pasar dan rumah penyembelihan di Kota Bharu mempunyai perhubungan genetik yang menunjukkan bahawa *S.* Corvalis ini berasal dari punca yang sama. Maklumat daripada penyelidikan ini boleh digunakan oleh pihak yang bertanggungjawab untuk meningkatkan aktiviti pencegahan yang sesuai untuk mengurangkan kadar pencemaran *Salmonela* dalam karkas ayam di rumah penyembelihan sebelum karkas ayam tersebut dihantar ke pasar.

Antibiotic Resistance Patterns and Genetic Relatedness of Salmonella Serotypes Isolated from Chicken Carcasses of Retail Markets and Slaughterhouses in Kota Bharu, Kelantan

ABSTRACT

Non-typhoidal *Salmonella* is one of the most important causes of foodborne illness and chicken products were frequently implicated as the source of the infection. *Salmonella* serotypes had been isolated from chicken carcasses of retail markets and slaughterhouses. In Kelantan, *Salmonella* had been isolated from 64% and 65% of chicken carcasses from retail markets and slaughterhouses in Kota Bharu respectively. However, the relatedness of the*Salmonella* serotypes from the chicken carcasses of the retail markets and slaughterhouses had not been determined. The objectives of the research were to determine the antibiotic-resistance pattern (ARP) and clonal relatedness of *Salmonella* serotypes isolated from chicken carcasses of retail markets and slaughterhouses. *Salmonella* Corvallis, *S.*Enteritidis, *S.*Stanley and *S.*Typhimurium isolated from chicken carcasses of retail markets and slaughterhouses in Kota Bharu were used in this study. The serotypes were tested for their antibiotic susceptibility against 13 antibiotics by using disc diffusion method. Molecular typing of the isolates was conducted by multilocus sequence typing (MLST) of the seven housekeeping genes (*thr*A, *pur*E, *suc*A, hisD, *aro*C, *hem*D and *dna*N). ARP of *S.*Corvallis from chicken carcasses of retail markets and slaughterhouses were mostly similar. *Salmonella* Stanley and *S.*Typhimurium from slaughterhouses were more resistant to antibiotics compared to those from retail markets. In contrast, *S.*Enteritidis from retail markets were more resistant compared to those from slaughterhouses. Of 45 *Salmonella* isolates tested, 91%, 82%, and 69% were resistant to sulphonamides, tetracycline and streptomycin respectively. Overall, 18% of the isolates were resistant to more than seven antibiotics tested. *Salmonella* Corvallis 4C, 59, 43 and 5C from retail markets, and *S.* Corvallis isolates 64, 13C, 69 and 70 from slaughterhouses belonged to the same sequence type (ST), ST1541. In conclusion, combination of MLST and ARP revealed that *S.*Corvallis from chicken carcasses of retail markets and slaughterhouses in Kota Bharu were clonally related indicating that the serotypes originated from the same sources. The information from this study can be used by relevant authorities to enhance appropriate intervention to reduce *Salmonella* contamination in chicken carcasses at slaughterhouses before the chicken carcasses are distributed to retail markets.

CHAPTER 1

INTRODUCTION

1.1 Overview

Non-typhoidal *Salmonella* causes global health burdens and morbidity. A study by Majowic et al., (2010) resported that every year approximately 93.8 millions cases of *Salmonella* gastroenteritis occurred globally with 155,000 deaths. Of the 93.8 millions cases, 80.3 millions were foodborne. In the United States, non-typhoidal *Salmonella* caused approximately 1.2 million illnesses every year resulted in 23,000 hospitalizations and 450 deaths. The medical cost resulted from the infection was approximately $365 millions annually (CDC, 2013). In Malaysia the prevalence of *Salmonella* food poisoning decreased from 36.61 per 100,000 in 2000 to 14.72 per 100,000 in 2003 (Thong, 2006). Infections or outbreaks of foodborne salmonellosis were frequently reported to be associated with consumption of chicken meat or foods containing chicken meat. In Thailand in 2009, army reserve force students were hospitalized due to abdominal pain and diarrhea. Epidemiological investigation revealed that consumption of green chicken curry was associated with the illness (odd ratio, 4.5; 95% confidence interval, 0.5 – 42.1) (Sitthi *et al.*, 2012).

In Hong Kong, non-typhoidal *Salmonella* was second pathogen that commonly caused food poisoning outbreak and chicken meats are among the

foods that were associated with the outbreak (HP, 2011). *Salmonella* Typhimurium and *Salmonella* Enteritidis had shown to cause food poisoning associated with consumption of contaminated chicken meat in Egypt (Rabie, *et al.*, 2012). In Malaysia, *Salmonella* food poisoning has been reported by the local news. For example, 158 pupils from boarding school in Kuala Nerang, Kedah had food poisoning after they ate „Ayam masak merah" contaminated with *Salmonella* as a result of improper preserved chicken meats. In Sungai Petani, Kedah, food poisoning due to chicken meats caused few deaths with hundreds fell ill. In Terengganu, 5 year-old boy died and sixty people had food poisoning as a result of eating fried chicken contaminated with *Salmonella*. Many initiatives have been recommended to prevent *Salmonella* food poisoning such as improving hygiene during processing, prevention of cross-contamination (Buncic and Sofos, 2012) and adequate cooking (Byelashov and Sofos, 2009). Information on genetic relatedness of *Salmonella* serotypes from different sources and its antimicrobial resistance patterns is also one of the efforts to prevent public health against *Salmonella* infection. This is because, getting to know the genetically related isolates distribution trends is prerequisite for us to give a brief idea on tracing back on where the isolates were originated from and the informations are important to study trend of the *Salmonella* spread occurrance.

In Malaysia, *Salmonella* has been isolated from chickens in the farms. For example, Ong et al (2014) reported that of 12,664 samples from poultry farms tested, 11.9% were positive for *Salmonella*. The most common serotypes

were *S.* Enteritidis (3.1%) and *S.*Typhimurium (1.3%). Contamination of chicken meat with *Salmonella spp.* at slaughterhouses has also been reported in Malaysia (Arshad and Che Ibrahim., 2014; Rusul *et al*., 1996). In Vietnam, a study by Bao et al. (2006) found that of 319 chicken carcasses from 15 abattoirs, 32.8% were *S.*Emek, *S.*Hadar (19.0 %), *S.*Derby (8.6 %), *S.*Typhimurium (7.8 %) and *S.*London (6.9 %). In a study also in Malaysia, Arshad and Che Ibrahim (2014) demonstrated that of 20 carcasses from slaughterhouses tested, 13 (65%) were contaminated with *Salmonella spp.* The *Salmonella* serotypes isolated were *S.*Corvallis (61.5%), *S.*Enteritidis (23.1%) and *S.*Stanley (15.4%).

In slaughterhouses, spread of *Salmonella* may occurs at various processing stages (Gómez-aldapa *et al.,* 2012; Trampel and Hoffman, 2000; Wotton, 2006). Mixing of the chicken carcasses during scalding with temperature around 50°C and 52°C, which is low enough to kill the pathogens make the cross contamination among the chicken carcasses are unavoided (Wotton, 2006). De-feathering steps after scalding process produce lots of aerosols during feather removal by rubber fingers on the plucking machine or rotating scrappers also contribute in scattering and dissemination of the bacteria (Gómez-aldapa *et al*., 2012; Musgrove *et al*., 1996). Moreover, accumulation of *Salmonella spp.* on the rubber finger of the machine will combine with organic materials that will lead to formation of biofilms. The biofilm formation on the rubber finger also make those carcasses being highly contaminated (Wotton, 2006).

Prevalence of *Salmonella spp.* in chicken meat from retail premises had been reported previously (Arumugaswamy *et al.*,1995; Freitas *et al.*, 2010). Contamination of chicken meats at retail stores is a public health burden in developed and developing countries such as Russia (Alali *et al.*, 2012), Belgium (Dione *et al.*,2009), India (Suresh, *et al*, 2011), Egypt (El-Aziz, 2013) and Vietnam (Ta *et al.*, 2014). Previously, in Malaysia, *S.* Kentuckey *S.* Blockley, *S.*Enteritidis, *S.*Chinicol, *S.* Muenchen and *S.* Agona were the dominant serotypes isolated from chicken meats bought from retail premises (Arumugaswamy *et al.*, 1995; Rusul *et al.*, 1996). The prevalence of *S.*Typhimurium, *S.*Corvallis, *S.*Weltevreden and *S.*Enteritidis from chicken carcasses from retail markets were then reported (Arshad *et al.*, 2012; Modarressi and Thong, 2010).

Salmonella Typhimurium and *S.*Enteritidis are well known to cause Salmonellosis in human and the most prevalence serotypes found in chicken meats from retails in Turkey (Yildirim, *et al*, 2011), Australia (Fearnley *et al.*, 2011), Egypt (El-Aziz, 2013) and Malaysia (Arshad *et al.*, 2012; Thong and Modarressi, 2010). Retail premises such as wet market, supermarket and roadside stalls are among the popular premises chosen by consumer to buy the source of proteins, such as chicken meats, beef, fishes and others. This is due to the flexibility and convenient circumstances provided by variety of these retail premises. By taking into account such retail premises is a direct medium toward consumers, supplying contaminated chicken meats is a direct hazard to the consumers.

*S.*Enteritidis, *S.*Typhimurium, *S.*Corvallis and *S.*Stanley have also been reported to be actively associates with food-borne outbreak related to consumption of contaminated chicken meats and this become a public health concern (Archambault *et al*., 2006; Hendriksen *et al.*, 2011). Isolation of *S.* Corvallis from chicken meats and food containing chicken products sold at retail premises are common lately and newly emergence serotypes also have frequently isolated from human clinical samples (Cavaco *et al.*, 2007; Modarressi and Thong, 2010; Yoshida *et al*., 2014). In Thailand, *S.*Corvallis and *S.*Stanley were among prevalence serovar isolated from raw chicken meats and human having diarrheal diseases (Bodhidatta *et al*., 2013). Due to the ubiquitous nature of *Salmonella*, a typing scheme capable of more detail strain identification is essential for epidemiological studies, because the ability to distinguish these *Salmonella* isolates is very important to trace the source of infections and outbreaks.

Several methods have been used for deciphering the relatedness among the *Salmonella* isolates but some have low discriminating power, demanding a considerable amount of expertise, time and quipment. Multilocus sequence typing (MLST) very useful for genetic profiling and also easy to interpret as well for result comparison between laboratories and providing the best phylogenetic relationship inferences. Research by Thong and Modarressi in 2010 from Malaysia has identified presence of multi-drug resistant *Salmonella spp.* from animal food origin such as raw beef and chicken meats sold at retail premises. They have discovered eleven serovars recovered from 88 *Salmonella*

isolates (Thong and Modarressi, 2010). Sixty-six of these *Salmonella* isolates shows resistance to tetracycline (73.8%), followed by sulfonamide (63.6%), streptomycin (57.9%), nalidixic acid (44.3%), trimethoprim–sulfamethoxazole (19.3%), ampicillin (17.0%), chloramphenicol (10.2%), cephalotin (8.0%), kanamycin (6.8%), ciprofloxacin (2.2%) gentamycin (2.2%), cefoxitin (2.2%), amoxicillin–clavulanate (1.0%) and amikacin (1.0%). Fifty nine out of 88 isolates (67%) were multi-drug resistant (exhibit resistance toward more than 3 antibiotics). Twenty six of 34 *S.*Corvallis isolates shown highest percentage of resistancy. Meanwhile five isolates of fifteen multidrug resistant *S.*Typhimurium were resistance to more than eight antibiotics (Thong and Modarressi, 2010).

Study conducted by Donado-Godoy and partners in 2014 regarding prevalence of *Salmonella* serovar and their antimicrobial resistant phenotypes on chicken meats sold from variety retail stores and premises in Colombia also revealed the contamination of the chicken meats with *Salmonella* serovar having multiple drug resistant profiles. A total of 354 of 378 (94%) *Salmonella* isolates were resistant to at least one antibiotics, 133 (35.2%) resistant to five antibiotic, 95 (24.6%) resistant to six to 10 antibiotics and 128 (33.9%) were resistant to 11 to 15 antibiotics (Donado-Godoy *et al*., 2014)

1.2 Problem Statement

Salmonella infection in humans had been frequently reported to be associated with consumption of chicken meat or food containing chicken meat (Sitthi *et al*., 2012;Ogata *et al.*, 2009). *Salmonella* also frequently reported being isolated from chickens in farms, slaughterhouses and retail markets in Malaysia (Ong *et al.*, 2014; Arshad *et al.*, 2014; Arshad *et al.*, 2012*;* Thong *et al.*, 2010). Thus, contamination of *Salmonella* in chicken carcasses which were sold at retails such as roadside stall, wet market and supermarket is a direct food poisoning hazard to the consumers. Addition to that, there is no research conducted yet to investigate prevalence of multiple antibiotic resistance as well the genetic relatedness of *Salmonella* from chicken carcasses of retail premises and slaughterhouses in Kota Bharu, Kelantan.

1.3 Research questions

1. Are the selected *Salmonella* serotypes isolated from chicken carcasses of retail markets and slaughterhouses in Kota bharu, Kelantan resistance to multiple antibiotics?
2. Are those selected *Salmonella* serotypes isolated from chicken carcasses of retail markets and slaughterhouses in Kota Bharu, Kelantan genetically related to each other?

1.4 Hypothesis

1. The selected *Salmonella* serotypes isolated from chicken carcasses of retail markets and slaughterhouses in Kota Bharu, Kelantan resistant to multiple antibiotics.
2. The selected *Salmonella* serotypes isolated from chicken carcasses of retail markets and slaughterhouses in Kota Bharu Kelantan also genetically related to each other.

1.5 Objectives

1. To determine the antibiotic resistance patterns (Antibiograms) of *Salmonella* serotypes isolated from processed chickens from retail markets and slaughterhouses in Kota Bharu.
2. To determine genetic relatedness of the *Salmonella* serotype isolated from the processed chickens of retail markets and slaughterhouses in Kota Bharu, Kelantan using MLST Sequence type (STs) profiles.

CHAPTER 2

LITERATURE REVIEW

2.1 History of *Salmonella*

Karl Joseph Eberth, decribed the finding of bacillus which was suspected as the main caused of typhoid fever in 1880. Four years later, a pathologist name Georg Theodor August Gaffky confirmed the bacillus and named it Gaffky-Eberth bacillus in 1884 (Rabsch *et al.*, 2013). In 1885, Daniel Elmer Salmon and Theobald Smith (Smith assistant) reported the isolation of bacteria linked to „hog cholera‟ or „swine fever‟ and named it *Salmonella* Choleraesuis. The bacteria was similar to *Salmonella serovar* Typhi, where at that time the bacillus were thought could cause enteric disease in humans and farm animals (Grimont, *et al*., 2000). Name of *Salmonella* are given as an honour to an American veterinary surgeon, Daniel Elmer Salmon (Fàbrega and Vila, 2013; Rabsch *et al*., 2013).

There were three precious findings happened in 1896. Firstly, serum from animals that were immunized exhibit agglutination against typhoid bacillus which isolated from two separate researches by Pfeiffer and Kolle, and also during research by Gruber and Durham. Second, a serum from a typhoid patient that exhibit agglutination against typhoid bacillus during Widal and Grunbaum research also known as Widal serodiagnostic test. Third finding was when two

isolates recovered from patients with clinical symptoms of typhoid but negative upon widal serodiagnostic named „bacille paratyphique" (Grimont *et al.*,2000).

2.2 Salmonella

Salmonella is in the family *Enterobacteriaceae* with phenotypic characteristic of straight rod, generally motile with peritrichous flagella, aerobes and anaerobes. Most of *Salmonella* that belong to subspecies 1 (enterica) can be confirmed with biochemical tests such as the fermentation of glucose, mannitol and dulcitol, inability to ferment sucrose, salicin and lactose, inability to hydrolyse urea, O-nitrophenyl-β-D-galactopyranoside (ONPG)-positive and production of H_2S. However, composite media such as triple sugar iron agar (TSI) often be used for *Salmonella* confirmation. The medium contains glucose, lactose and sucrose, an H_2S detection system and an indicator also included in the medium. Single colony of isolate can be inoculated to the TSI medium by stabbing into the centre of the butt and continuing down to the base and then streaking the inoculum on to the slope followed with incubation at 37°C within 18-24 hours. Organisms that able to ferment glucose, but not lactose or sucrose, will show an initial acid (yellow) slant in a short period indicates glucose is utilized. Under aerobic condition, TSI slant becomes alkaline (red) because of protein breakdown in the medium where as under anaerobic conditions the butt of the tube, the medium remains acid (yellow) , production of hydrogen sulfide, H_2S is characterized by a blackening of the medium. (Jones *et al.*, 2000).