PREVALENCE, ANTIBIOTIC RESISTANCE AND BIOFILM FORMATION OF Salmonella IN RAW CHICKEN MEATS AT SELECTED SLAUGHTERHOUSES IN PENINSULAR MALAYSIA

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By

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Salmonella is one of the most common causes of foodborne diseases. The objectives of this research were to isolate and identify *Salmonella* from raw chicken meat, determine its antibiotic resistance and its ability to form biofilm on the surfaces using microtitre plate surface in a growth media at different incubation period. Raw chicken meat was obtained from selected slaughterhouses located in four different zones in Peninsular Malaysia; Northern Zone (States of Perlis, Kedah, Penang and Perak); Central Zone (States of Selangor, Negeri Sembilan and Melaka; Southern Zone (States of Johor) and Eastern Zone (States of Terengganu, Kelantan and Pahang). The samples were collected and isolated at the Veterinary Public Health Laboratory (VPHL), Department of Veterinary Services (DVS), Sepang, Selangor. Isolation and identification of samples were performed using an in-house conventional (VPHL) culture method adopted from the American Association of Analytical Chemists (AOAC, 1995), the Food Safety and Inspection Services of USDA (FSIS, 1998) and the Australian Standard (AS 1766.2.5, 1991). Positive isolates were serotyped at the Veterinary Research Institute, Ipoh Perak using the White-Kauffmann-Le Minor scheme comprising of commercial somatic and flagellar antisera. The antibiotic resistance of 135 *Salmonella* isolates was investigated via the Kirby-Bauer disk-diffusion method, using 12 antibiotics. The biofilm-forming ability of the isolates was assessed using two media; a tryptic soy broth (TSB) and a 1/20 TSB with incubation periods of 24 and 48 hours at 37 °C. Crystal violet staining was used for the quantification of *Salmonella* isolates based on the difference between optical density measurements of the test and negative control samples (ΔOD_{590nm}). It was found that 17.31% (135/780) of the raw chicken meat tested positive for *Salmonella*. Serotyping of the total 135 isolates demonstrated that 87 (64.44%) belonged to 12 different serovars; *S.* Corvallis, *S.* Brancester, *S.* Enteritidis, *S.* Albany, *S.* Typhimurium, *S.* Braenderup, *S.* Hindmarsh, *S.* Hiddudify, *S.* Bellevue, *S.* Duesseldorf, *S.* Cyprus and *S.* Indiana. The results showed that the *Salmonella* isolates had the highest percentage of resistance to erythromycin (87.41%) and tetracycline (85.19%). *Salmonella* isolates were also resistant to sulphamethoxazole/trimethoprim (55.55%), ampicillin (26.63%),

streptomycin (29.63%), enrofloxacin (22.96%) and nalidixic acid (17.04%). In contrast, lower percentage of resistance was observed against gentamicin (7.41%), cephalothin (5.96%), ceftriaxone (3.70%) and amoxicillin-clavulanic acid (2.22%). All *Salmonella* isolates were susceptible to ciprofloxacin (CIP). The multiple antibiotic resistance (MAR) index varied from 0.08 to 0.75. A total of 93 isolates (68.88%, 93/135) were multi-drug resistant. Meanwhile, more than 85% of 135 *Salmonella* isolates were able to form biofilm in TSB and 1/20-TSB media. A greater quantity of *Salmonella* were able to produce biofilm 1/20-TSB (90.37%) compared to TSB (88.15%), respectively. The maximum biofilm (94.81%) formed by *Salmonella* isolates was at 24 hours incubation in 1/20-TSB whereas 88.89% biofilm formed in TSB. The occurrence and antibiotic resistance profiles of *Salmonella* isolates in slaughterhouses could promote awareness on controlling *Salmonella* at all production stages of raw chicken meat in Peninsular Malaysia. The ability of *Salmonella* to form biofilm could indicate common factors promote biofilm formation; thus further work could develop interventions to reduce this incidence.

Keywords: Prevalence, antibiotic, biofilm, *Salmonella*, chicken meat, slaughterhouse

Abstrak tesis yang dikemukan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KELAZIMAN, RINTANGAN ANTIBIOTIK DAN PEMBENTUKAN BIOFILEM BAGI *Salmonella* DALAM DAGING AYAM MENTAH DI RUMAH SEMBELIH TERPILIH DI SEMENANJUNG MALAYSIA

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Salmonella adalah salah satu penyebab yang paling biasa kepada penyakit bawaan makanan. Objektif kajian ini adalah untuk pemencilan dan pengenalpasti *Salmonella* dari daging ayam mentah, menentukan profil rintangan antibiotik dan keupayaannya untuk membentuk biofilem pada permukaan menggunakan permukaan plat mikrotiter dalam media pertumbuhan pada tempoh pengeraman berbeza. Daging ayam mentah diperolehi dari rumah sembelih ayam yang terpilih terletak di empat zon yang berlainan di Semenanjung Malaysia adalah Zon Utara (negeri Perlis, Kedah, Pulau Pinang dan Perak), Zon Tengah (negeri Selangor, Negeri Sembilan dan Melaka), Zon Selatan (negeri Johor) dan Zon Timur (Terengganu, Kelantan dan Pahang). Sampel ayam dikumpulkan dan pengenalpastian dijalankan di Makmal Kesihatan Awam Veterinar (MKAV), Jabatan Perkhidmatan Veterinar (JPV) Sepang, Selangor. Pencilan dan pengenalpastian sampel dilakukan menggunakan kaedah kultur komersial dalaman (MKAV) diadaptasi daripada AOAC (1995), FSIS/USDA (1998) dan AS 1766.2.5. (1991). Positif *Salmonella* menjalani serotip di Institut Penyelidikan Veterinar Ipoh, Perak menggunakan skim White- Kaufmann-Le Minor terdiri daripada antisera somatic komersial dan flagellar. Rintangan antibiotik daripada 135 pencilan *Salmonella* dilakukan dengan menggunakan kaedah penyebaran cakera Kirby-Bauer, menggunakan 12 antibiotik. Keupayaan membentuk biofilem oleh pencilan *Salmonella* telah dinilai dalam dua media berbeza; Kaldu Triptik Soya (TSB) dan 1/20-TSB yang dieram selama 24 dan 48 jam pada suhu 37°C. Pewarnaan ungu kristal digunakan dan dikuantifikasi pencilan *Salmonella* berdasarkan perbezaan antara pengukuran kepadatan optik setiap sampel dan sampel negatif (ΔOD590nm). Ia didapati bahawa 17.31% (135/780) dari daging ayam mentah menunjukkan positif *Salmonella*. Serotip daripada 135 pencilan *Salmonella* menunjukkan bahawa 87 (64.44%) tergolong dalam 12 serovar yang berbeza; *S*. Corvallis, *S*. Brancester, *S*. Enteritidis, *S*. Albany, *S*. Typhimurium, *S*. Braenderup, *S*. Hindmarsh, *S*. Hiddudify, *S*. Bellevue, *S*. Duesseldorf, *S*. Cyprus dan *S*. Indiana. Keputusan menunjukkan bahawa pencilan *Salmonella* mempunyai peratusan rintangan

tertinggi terhadap eritromisin (87.41%) dan tetrasiklin (85.19%). Pencilan *Salmonella* juga rintang terhadap sulphamethoxazole/trimtopin (55.55%), ampisillin (26.63%), streptomisin (29.63%), enrofloxasin (22.96%) dan asid nalidiksik (17.04%). Sebaliknya, peratus rintangan yang rendah diperhatikan pada gentamisin (7.41%), sefalotin (5.96%), ceftriaxone (3.70%) dan amoxisillin-asid clavulanic (2.22%). Semua pecilan *Salmonella* rentan kepada ciprofloxasin (CIP). Indeks rintangan antibiotik berganda (MAR) bervariasi dari 0.08 hingga 0.75. Sejumlah 93 pencilan (68.88%, 93/135) adalah rintang kepada pelbagai antibiotik. Sementara itu, ebih daripada 85% daripada 135 pencilan *Salmonella* mempunyai keupayaan membentuk biofilem dalam media TSB dan 1/20-TSB. *Salmonella* menghasilkan biofilem lebih besar dalam media 1/20-TSB (90.37%) berbanding dengan TSB (88.15%). Biofilem yang mempunyai peratusan maksimum (94.81%) dibentuk oleh pencilan *Salmonella* pada pengeraman selama 24 jam dalam media 1/20-TSB manakala 88.89% terbentuk dalam media TSB. Penemuan dan profil rintangan antibiotik *Salmonella* di rumah sembelih meningkatkan kesedaran serta mengawal *Salmonella* di semua peringkat pengeluaran ayam mentah di Semenanjung Malaysia. Keupayaan *Salmonella* untuk membentuk biofilem boleh menunjukkan faktor-faktor umum dalam menggalakkan pembentukan biofilem. Oleh itu kajian yang lebih mendalam boleh membentuk langkah-langkah pencegahan bagi mengurangkan insiden ini.

Kata kunci: Kelaziman, antibiotik, biofilem, *Salmonella*, daging ayam, rumah sembelih.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

˚C	Degree in Celcius
µL	Microliter
1/20 TSB	One over twenty Tryptic Soy Broth
AMC	ampicillin/clavulanic acid
AMP	Ampacillin
ANOVA	Analysis of Variance
AOAC	Association of Official Agricultural Chemists
BAM	Bacteriological Analytical Manual
CDC	Centers for Disease Control and Prevention
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standard Institute
CN	Gentamicin
CODEX	Codex Alimentarius Commission
CRO	Ceftrixone
DVS	Department of Veterinary Services
E	Erythromycin
E. coli	*Escherichia coli*
ELISA	Enzyme Linked Immunosorbent Assay
ENR	Enrofloxacin
EPS	Extracellular Polymeric Substance
FDA	Food and Drug Administration
FSIS	Food Safety and Inspection Service
g	grams
H_2S	Hydrogen sulphide
HACCP	Hazard Analysis and Critical Control Point
I	Intermediate
ISO	International Organizational for Standardization
JPV	Jabatan Perkhidmatan Veterinar
KF	Cephalothin
LIA	Lysine Iron Agar
MAR	Multiple Antibiotic Resistance
mL	milliliter
mm	millimeter
MDR	Multidrug-Resistance
MH	Mueller-Hinton Agar
MKAV	Makmal Kesihatan Awam Vaterinar
MOH	Ministry of Health
NARMS	National Antimicrobial Resistance Monitoring System
NA	Nalidixic Acid
NPCB	National Pharmaceutical Control Bureau
nm	nanometer
OD	Optical Density
ONPG	O-nitrophenyl-β-D-galactoside
R	Resistance
RVS	Rappaport-Vasiliadis
S	Streptomycin
S	Susceptible

S.	*Salmonella*
SPSS	Statistical Packages for Social Sciences
SXT	Sulphamethoxazole/trimethoprim
TE	Tetracycline
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TSI	Triple Iron Agar
TTB	Tetrathionate Broth
USA	United States of America
USDA	United States Department of Agriculture
VPHL	Veterinary Public Health Laboratory
WHO	World Health Organization
XLD	Xylose Lysine Decarboxycholate
XLT4	Xylose Lactose Tergitol™ 4

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Salmonellosis, an infectious disease caused by the *Salmonella*. It has been reported as a major foodborne illness affecting populations around the world (Cui et al., 2016; Grant et al., 2016; Wang et al., 2015). This infection has contributed to significant cases of morbidity, mortality, and economic losses (Sallam et al., 2014). Non-typhoidal *Salmonella* is responsible for about 93.8 million illnesses globally; of which an estimated 80.3 million are foodborne and cause about 155,000 deaths every year (Majowicz et al., 2010). According to Malaysia's Ministry of Health, in 1999, 811 (9.4%) out of 8640 cases of food poisoning in Malaysia were due to *Salmonella* infection (Thong et al., 2002). The cases of food poisoning in Malaysia continually increased by 7.87% in 2017 (MOH, 2017) compared to 2016 (MOH, 2016), making it as one of the top communicable diseases with highest incidence and mortality rate.

Salmonella, a Gram-negative bacteria composed of rod-shaped bacilli, belongs to the Enterobacteriaceae family. Over 2,500 *Salmonella* serovars have been identified based on their antigenic formula (Acar et al., 2017; Jaglic et al., 2014; Lampang, Chailangkarn and Padungtod, 2013). The most predominant serovars that have been detected are *Salmonella enterica* ser. Typhimurium and *Salmonella enterica* ser. Enteritidis, both of which were associated with human foodborne illnesses (Modarressi and Thong, 2010). *Salmonella* is a type of commensal pathogen, which frequently colonises the intestinal tract of animals and, subsequently, spread to humans. The bacteria can cause mild to severe systemic infections, especially affecting at-risk populations such as infants, young children, pregnant women, older adults, and people with weakened immune systems. *Salmonella* has been found prevalent in chicken meat, poultry, and in slaughterhouses and farm environments (Chotinun et al., 2015; Cui et al., 2016; Kalambhe et al., 2016; Lamas et al., 2016; Lee et al., 2016). Poultry is considered a potential vehicle for *Salmonella* transmission—once transmitted, the outbreak will be very difficult to control (Ren et al., 2016; Cui et al., 2016; Abbassi-Ghozzi et al., 2012; Adzitey et al., 2012). Besides that, vegetables and seafood could also cause *Salmonella* infection in humans (Najwa et al., 2015).

An increased incidence of the antibiotic resistance of *Salmonella*, particularly multi-drug-resistant (MDR) strains, is a very serious issue occurring around the world (Thung et al., 2016). Antibiotics are used in poultry for growth promotion, therapeutics (treating clinically sick animals), and prophylaxis (preventing or reducing the incidence of infectious disease) purposes (Thai et al., 2012). Antibiotics help animals survive in crowded, stressful and unsanitary condition. However, overuse of antibiotics in feed has given rise to detrimental effects, including antibiotic-resistant bacteria. Antibiotics cause genomic selective pressure by killing susceptible bacteria and allowing antibiotic-resistant bacteria to survive and multiply (Kemal et al., 2016). A few studies have

reported the occurrence of multi-drug resistant *Salmonella* isolated from raw chicken meat (Ren et al., 2016; Alcaine et al., 2016; Mir et al., 2015; Akbar and Anal, 2013; Thai et al., 2012). These bacteria are most frequently resistant to tetracycline, ampicillin, chloramphenicol, streptomycin, nalidixic acid, trimethoprim, and sulphonamides (Ta et al., 2014). Therefore, it is important to monitor the antibiotic resistance of bacteria in both animal and food products (Hur et al., 2011).

The demand for chicken has risen in recent years. From 2000 to 2011, chicken meat intake in Malaysia has expanded from 36 to 39 kg per capita consumption. The reason behind this is that various religions permit the consumption of chicken and because chicken is cheaper than other types of meat in Malaysia (Jayaraman et al., 2013). The chicken slaughtering process includes the process of slaughtering, bleeding, scalding, de-feathering, washing, evisceration, chilling, draining, grading, weighing, and packing (Guran et al., 2017). Unfortunately, chicken meat could become contaminated with *Salmonella* due to improper handling and cross-contamination during the slaughtering process.

Salmonella has the ability to grow on food contact surfaces by forming a biofilm, which can then infect the host. Bacterial biofilm is defined as a large collection of bacterial cells that adhere to each other and to surfaces embedded in a matrix of extracellular polymeric substances (Zhou et al., 2013). The biofilm is essentially made up of bacteria that exist as planktonic cells in bulk solution and sessile cells that form a unit to attach to a surface (Nguyen et al., 2014). One of the characteristics of bacteria is their ability to attach to and subsequently detach from surfaces via a multistep process involving initial attachment, followed by cluster formation, microcolony collection, and ending with maturation (Chmielewski and Frank, 2003). The main component of extracellular polymeric substance (EPS) are polysaccharides, proteins, phospholipids, and teichoic and nucleic acids (Speranza et al., 2011).

Biofilm in food manufacturing contributes to the transmission of bacteria to food, bio-fouling in pipelines, the rusting or impedance of the heat transfer process or mechanical blockage. Floors, drains, pipelines, walls, conveyors, and racks are the common sites known to harbour *Salmonella.* Normally, bacteria have the capability to attach on surfaces such as plastic, glass, stainless steel, or rubber (Nillian et al., 2016; Agarwal et al., 2011; Sinde and Carballo, 2000). The quantification of biofilms using a microtiter plate method is the most frequent assay used to investigate the formation of biofilms.

1.2 Problem Statement

Salmonella is one of the recognized pathogens as most causative agents of food poisoning which poses a significant public health hazard. Therefore, *Salmonella* can be found in chicken which are known to be a significant reservoir. *Salmonella* exists as a permanent fixture in the alimentary tract and the reproductive system of chickens. Therefore, contaminated chicken meat can easily transmit the bacteria to humans Chicken might also become infected with *Salmonella* due to poor hygiene in the slaughterhouse. In order

to manage this risk to human health, it is essential to tackle this problem at the slaughterhouses to reduce the level of cross-contamination. However, although extensive research on the prevalence of *Salmonella* in chicken has been done, the issues of contamination of *Salmonella* in chicken still occurred. To date, only a limited number of studies have investigated the occurrence of *Salmonella* isolated from raw chicken meat from slaughterhouses in the states of Malaysia. Past research has focused more on certain States in Malaysia with not many studies focused on every State in Malaysia. Therefore, this research extends the body of work in this area including samples from all States in Peninsular Malaysia. In particular, this study investigates the occurrence of *Salmonella* isolated from raw chicken meat at selected slaughterhouses in Peninsular Malaysia. Besides that, the trends of *Salmonella* occurrence was also studied and a database provided. The trend of *Salmonella* contamination in raw chicken meat could help establish prevailing serotypes in this bacterial community. Antibiotics are serve for chicken as a growth promoter and for preventing the chicken from contracting infections. However, the irrational use of antibiotics is one of the factors contributing to the global surge and spread of antibiotic resistance in bacteria. In addition, antibiotic monitoring are in place in the slaughterhouse. However, lacking of schedule monitoring may overlook this control. The widespread overuse and misuse of antibiotics in developing countries have contributed to an increasing trend of drug resistance level in *Salmonella* bacteria. The emergence of multi-drug antibiotic-resistant *Salmonella* is also a cause of global concern because stronger infections could be spread worldwide (WHO, 2018). The use of antibiotics has, therefore, increased to prevent this infection, but this is worrying because the bacteria are able to mutate in order to survive in humans or animals. Therefore, this study provides the current patterns and predictions of multidrug-resistant *Salmonella* isolated from raw chicken meat from selected slaughterhouses in Peninsular Malaysia. The appearance of multi-drug-resistant *Salmonella* isolates is becoming an increasing subject of concern, so extensive research has been carried out in order to create proper antibiotic data to combat *Salmonella* infection. The development and spread of *Salmonella* in the form of biofilm on certain surfaces are considered an urgent public health concern. The bacteria are able to cause outbreaks and once spread, are extremely difficult to contain (Steenackers et al., 2012). Thus, the ability of *Salmonella* to form biofilms at different nutrient availability and incubation period was also investigated in this study. The result of this biofilm research will prove helpful in developing strategies to prevent the development of *Salmonella* biofilm in food production areas.

1.3 Objectives of Study

The objectives of this study were as follows:

1. To isolate and identify *Salmonella* in raw chicken meats from selected chicken slaughterhouses in Peninsular Malaysia.
2. To investigate the antibiotic resistance profiles of *Salmonella* isolates from raw chicken meat and the antibiotic resistance pattern of the multi-resistant isolates.
3. To quantify the ability of *Salmonella* isolates to produce biofilm on surfaces using a microtitre plate surface in a growth media at different incubation periods.

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