UNIVERSITI PUTRA MALAYSIA

PREVALENCE, CHARACTERIZATION AND ANTIBIOTIC RESISTANCE OF Salmonella spp. ISOLATED FROM RAW VEGETABLES

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By

NAJWA BINTI MOHD SHAHRIL

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

August 2016

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DEDICATION

Dedicated to my beloved parents, siblings and friends for their eternity love and endless support

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Masters of Science

PREVALENCE, CHARACTERIZATION AND ANTIBIOTIC RESISTANCE OF *Salmonella* spp. ISOLATED FROM RAW VEGETABLES

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August 2016

Chairman : Professor Son Radu, PhD
Faculty : Food Science and Technology

Growing consumption of fresh vegetables has led to an increase in the number of outbreaks of food-borne disease linked to fresh produce including salmonellosis. In Malaysia, some vegetables are eaten raw, known as *ulam* or salad in other countries. Therefore, this research was conducted to study the prevalence and antibiotic resistance of *Salmonella* spp., *Salmonella* Enteritidis (*S.* Enteritidis) and *Salmonella* Typhimurium (*S.* Typhimurium) in *ulam*. This study also aimed to determine the relation among different isolates of same serovar of *Salmonella* spp. by employing random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR) method and the risk acquiring salmonellosis through consumption of *ulam*.

A total of 96 samples of *ulam* were purchased from wet markets and hypermarkets in limited geographical locations of Selangor. Most probable number (MPN) method was combined with multiplex polymerase chain reaction (PCR) and plating method for detection of *Salmonella* spp. Prevalence of *Salmonella* spp., *S.* Enteritidis and *S.* Typhimurium was higher in samples from hypermarkets than wet markets which were 100%, 64.6% and 87.5% respectively at hypermarkets and 95.8%, 43.8% and 75% respectively at wet markets. The density of *Salmonella* spp., *S.* Enteritidis and *S.* Typhimurium ranged from <3 to >2400 MPN/g. MPN-multipex PCR detected more *Salmonella* spp., *S.* Enteritidis and *S.* Typhimurium which were 97.9%, 54.2% and 81.3% respectively, as compared to MPN-plating which were 7.3%, 6.3% and 10.4% respectively.

Six isolates of *S*. Enteritidis and 10 isolates of *S*. Typhimurium were recovered from *ulam*. Both *Salmonella* spp. serovars showed high resistance to amoxicillin/clavunic acid, cephalothin, streptomycin and ciprofloxacin with 100% resistant to ampicillin and erythromycin. All isolates showed resistance to at least three antibiotics tested. *S*. Typhimurium was more resistant compared to *S*. Enteritidis with multiple antibiotic resistances (MAR) index ranging from 0.27 to 0.82 and 0.27 to 0.55 respectively.

RAPD-PCR dendograms showed that most isolates of *S*. Enteritidis and *S*. Typhimurium tend to type together based on same sample type which 3 RAPD types (RAPD type 3 of *S*. Enteritidis and RAPD type 1 and 6 of *S*. Typhimurium) were typed together respectively on same sample type and same sampling locations. The other 3 RAPD types (RAPD type 1 of *S*. Enteritidis, RAPD type 2 and 4 of *S*. Typhimurium) were typed based on the same sample type but different sampling locations. RAPD type 3 of *S*. Typhimurium was typed on different sample types but same sampling location was observed.

The quantitative microbiological risk assessment (QMRA) simulations by single-hit concept showed that overall risk of acquiring salmonellosis due to consumption of *ulam* was higher for *S*. Typhimurium than *S*. Enteritidis. The mean risk of illness per serving per year for *S*. Typhimurium was 1 while for *S*. Enteritidis, the risk was ~1 in the exposed population.

All in all, the prevalence of *Salmonella* spp. in *ulam* is alarming and should be monitored from time to time seems prevention is better than cure.

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

PREVALENS, PENCIRIAN DAN RINTANGAN ANTIBIOTIK *Salmonella* spp. YANG DIPENCILKAN DARI SAYUR-SAYURAN MENTAH

Oleh

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Peningkatan penggunaan sayur-sayuran segar telah membawa peningkatan bilangan wabak penyakit bawaan makanan berkaitan dengan produk segar termasuk salmonellosis. Di Malaysia, beberapa sayur-sayuran dimakan secara mentah, dikenali sebagai ulam atau salad di negara-negara lain. Oleh itu, penyelidikan ini dijalankan untuk mengkaji prevalens dan rintangan antibiotik *Salmonella* spp., *Salmonella* Enteritidis (*S.* Enteritidis) dan *Salmonella* Typhimurium (*S.* Typhimurium) pada ulam. Kajian ini juga bertujuan untuk menentukan kaitan antara pencilan-pencilan yang berbeza pada serovar *Salmonella* spp. yang sama dengan menggunakan kaedah profil amplikasi poliformik DNA rawak- tindak balas berantai polimerasi (RAPD-PCR) dan risiko mendapat salmonellosis melalui penggunaan ulam.

Sejumlah 96 sampel ulam dibeli dari pasar dan pasar raya di tempat-tempat secara geografi yang terhad di Selangor. Kaedah jumlah paling mungkin (MPN) telah digabungkan dengan multipleks tindak balas berantai polimerasi (PCR) dan kaedah piring untuk mengesan *Salmonella* spp. Prevalens *Salmonella* spp., *S.* Enteritidis dan *S.* Typhimurium adalah lebih tinggi di pasar raya dari pasar di mana masing-masing 100%, 64.6% dan 87.5% di pasar raya dan masing-masing 95.8%, 43.8% dan 75% di pasar. Ketumpatan *Salmonella* spp., *S.* Enteritidis dan *S.* Typhimurium berada antara <3 sehingga >2400 MPN/g. MPN-multipleks PCR boleh mengesan lebih *Salmonella* spp., *S.* Enteritidis dan *S.* Typhimurium berbanding MPN-piring. MPN-multipleks PCR mengesan masing-masing 97.9%, 54.2% dan 81.3% *Salmonella* spp., *S.* Enteritidis dan *S.* Typhimurium sementara MPN-pring mengesan masing-masing hanya 7.3%, 6.3% dan 10.4% *Salmonella* spp., *S.* Enteritidis dan *S.* Typhimurium.

Enam belas pencilan *S*. Enteritidis dan *S*. Typhimurium telah didapati daripada ulam. Kedua-dua *Salmonella* spp. serovar menunjukkan rintangan tinggi terhadap amoxicillin/asid clavunic, cephalothin, streptomycin dan ciprofloxacin dengan 100% rintangan terhadap ampicillin dan erythromycin. Semua pencilan menunjukkan rintangan pada sekurang-kurangnya tiga antibiotik yang diuji. *S*. Typhimurium adalah lebih rintangan berbanding *S*. Enteritidis dengan kerintangan antibiotik pelbagai (MAR) indeks masing-masing antara 0.27 ke 0.82 dan 0.27 ke 0.55.

Dendogram RAPD-PCR menunjukkan kebanyakan pencilan *S*. Enteritidis dan *S*. Typhimurium cenderung untuk taip bersama berdasarkan jenis sampel yang sama dimana 3 RAPD taip (RAPD taip 3 *S*. Enteritidis dan RAPD taip 1 dan 6 *S*. Typhimurium) ditaipkan bersama masing-masing pada jenis sampel dan lokasi persampelan yang sama. 3 RAPD taip yang lain (RAPD taip 1 *S*. Enteritidis, RAPD taip 2 dan 4 *S*. Typhimurium) ditaipkan berdasarkan jenis sampel yang sama tetapi berbeza lokasi persampelan. RAPD taip 3 *S*. Typhimurium telah ditaip pada jenis sampel yang berbeza tetapi lokasi persampelan yang sama diperhatikan.

Penilaian risiko kuantitatif mikrobiologi (QMRA) simulasi oleh konsep satu-hit menunjukkan keseluruhan risiko mendapat salmonellosis disebabkan pada penggunaan ulam lebih tinggi untuk *S*. Typhimurium dari *S*. Enteritidis. Purata risiko penyakit per hidangan per tahun untuk *S*. Typhimurium adalah 1 manakala untuk *S*. Enteritidis, risikonya adalah ~1 pada populasi yang terdedah.

Kesimpulannya, prevalens *Salmonella* spp. pada ulam adalah membimbangkan dan perlu dipantau dari masa ke semase memandangkan pencegahan lebih baik daripada mengubati.

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I certify that a Thesis Examination Committee has met on 22 August 2016 to conduct the final examination of Najwa binti Mohd Shahril on her thesis entitled "Prevalence, Characterization and Antibiotic Resistance of *Salmonella* spp. Isolated from Raw Vegetables" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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Supervisory Committee: Assoc. Prof. Dr. Yaya Rukayadi

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LIST OF ABBREVIATIONS

%eat	% of Malaysia population eating *ulam*
× g	Times gravity
16S rRNA	16 subunit ribosomal ribose nucleic acid
A2	*S.* Typhimurium ATCC 14028
A3	*S.* Enteritidis ATCC 13076
ACSSuT	Ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracycline
AFLP	Amplified fragment length polymorphism
AMC	Amoxycillin/clavunic acid
Amp	Ampicillin
ARDRA	Amplified ribosomal DNA restriction analysis
ATCC	American Type Culture Collection
a_w	Water activity
BGA	Brilliant green agar
BOX-PCR	BOX-A1R-based repetitive extragenic palindromic-PCR
bp	Base pair
BPW	Buffered peptone water
C	Concentration
C	Cholaramphenicol
C. jejuni	*Campylobacter jejuni*
CDC	Centers for Disease Control and Prevention
Cip	Ciprofloxacin
CLSI	Clinical Laboratory Standards Institute
Cn	Gentamicin
D	Dose per serving
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTs	Definitive phage types
E	Exposure (no. of serving/year)
E	Erythromycin
E. coli	*Escherichia coli*
eaeA	Intimin
Ec	Expected no. of cases per year
EPS	Exopolymer substance
ERIC-PCR	Enterobacterial repetitive intergenic consensus-polymerase chain reaction
Fim	Type I fimbriae
fliC $_{h7}$	Flagellar antigen
H_2S	Hydrogen sulfide
HE	Hektoen enteric
HIV	Human Immunodeficiency Virus
hly $_{933}$	Hemolysin
ICMSF	International Commission on Microbiological Specifications for Foods

inv	invasion
K. pneumonia	*Klebsiella pneumonia*
Kf	Cephalothin
L	Long bean
L. monocytogenes	*Listeria monocytogenes*
LIA	Lysine iron agar
Lpf	Long polar fimbriae
LPS	Lipopolysaccharides
LT	Heat labile
MAR	Multiple antibiotic resistances
Max	Maximum
MDR	Multidrug resistance
Med	Median
$MgCl_2$	Magnesium chloride
MH	Mueller Hinton
MIC	Minimum inhibitory concentration
Min	Minimum
MLCB	Mannitol lysine crystal violet brilliant green
MLST	Multilocus sequence typing
MPN	Most probable number
MPN-multiplex PCR	Most probable number-multiplex polymerase chain reaction
MPN-PCR	Most probable number-polymerase chain reaction
MPN-plating	Most probable number-plating
Na	Nalidixic acid
NCRA	National Committee on Risk Analysis
NTS	Non-typhoidal *Salmonella*
P	Prevalence
P	Asian pennywort
PCR	Polymerase chain reaction
P_{eat}	Population of Malaysia eating *ulam*
Pef	Plasmid-encoded fimbriae
PFGE	Pulsed field gel electrophoresis
$P_{ill/pos}$	Probability of illness per positive serving
$P_{ill/serv}$	Probability of illness per serving
$P_{ill/year}$	Probability of illness per year
P_M	Population of Malaysia
QMRA	Quantitative microbial risk assessment
QRDR	Quinolone resistance-determining region
RAPD-PCR	Random amplification of polymorphic DNA-polymerase chain reaction
RFLPs	Restriction fragment length polymorphism
RV	Rappaport-Vassiliadis
S	Serving size
S	Water dropwort
S	Streptomycin
S. Enteritidis	*Salmonella* Enteritidis

S. Paratyphi	*Salmonella* Paratyphi
S. Typhi	*Salmonella* Typhi
S. Typhimurium	*Salmonella* Typhimurium
S. Typhimurium DT104	*Salmonella* Typhimurium definitive phage type 104
SCV	*Salmonella* containing vacuole
sdf	*Salmonella* difference fragment
SE	*S*. Enteritidis
SGI1	*Salmonella* genomic island 1
spp.	Species
SS	Salmonella-Shigella
SSCP	Single strand conformation polymorphism
Sspp	*Salmonella* spp.
ST	*S*. Typhimurium
STM4492	Cytoplasmic protein of *Salmonella* Typhimurium LT2
stx_1, stx_2	Shiga toxin
Sxt	Trimethoprim-Sulphamethoxazole
Taq	*Thermus aquaticus*
TBE	Tris-Borate EDTA electrophoresis buffer
TE	Tris-EDTA buffer
Te	Tetracycline
TSI	Triple sugar iron agar
TT	Tetrathionate
U	Unit
UPGMA	Unweighted pair group linkage analysis method
USA	United States of America
V. *parahaemolyticus*	*Vibrio parahaemolyticus*
VBNC	Viable but non-culturable
VNTR	Variable number of tandem repeat
W	Winged bean
WHO	World Health Organization
XLD	Xylose lysine deoxycholate
α	Alpha
β	Beta

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Consuming vegetables is gaining popularity nowadays as many people are starting to be concerned in leading a healthy lifestyle. Ministry of Health, Malaysia recommends eating plenty of vegetables and fruits every day. According to Malaysian Food Pyramid, it is recommended to take three servings of vegetables and two servings of fruits daily. Malaysian Food Pyramid is a visual tool in designing a healthy diet which consists of four levels of food groups and recommended number of servings per day. Vegetables and fruits provide important sources of vitamins, minerals, fibre and other bioactive compounds such as phytochemicals. Hence, by eating vegetables and fruits daily is able to prevent cardiovascular disease, diabetes, coronary heart disease, stroke, high blood pressure and cancers. A raw eaten vegetables known as *ulam* in Malay language is one of the healthier choices than cooked vegetables. It is because, it can prevent nutrient loss since cooking process will damage certain nutrients and phytochemicals in vegetables (MOH, 2010).

Even though the vegetables and fruits are known with the healthy and nutritional values, people also are faced with the increasing of reported foodborne pathogens contamination on fresh produce worldwide such as *Salmonella*, *Escherichia coli* O157:H7, *Vibrio parahaemolyticus*, *Campylobacter* and *Listeria monocytogenes* (Losio *et al*., 2015; Tunung, 2012; Pui *et al*., 2011; Gabriel *et al*., 2007). *Salmonella* can grow on the surface of alfafa sprouts, tomatoes, other fruits and vegetables (Forsythe, 2010). Centers for Disease Control and Prevention (CDC) reported that fresh produce such as cucumbers, bean sprouts, mangoes, cantaloupe, papaya and tomatoes contaminated with *Salmonella* can lead to outbreaks. For instance, in 2012, there was multistate outbreaks regarding *Salmonella* Typhimurium (*S.* Typhimurium) and *Salmonella* Newport (*S.* Newport) linked to cantaloupe throughout 24 states of United States of America (USA). From the outbreaks, about 261 persons were infected with 94 persons were hospitalized and 3 deaths were reported in Kentucky (CDC, 2013).

A foodborne pathogen, *Salmonella* is first discovered in 1885 and it can be divided into two groups: typhoid *Salmonella* and nontyphoid *Salmonella*. Typhoid *Salmonella* causes systemic infections known as a typhoid fever while nontyphoid *Salmonella* causes a self-limiting gastroenteritis. Reservoir of typhoid fever is limited to human whereas animal is for nontyphoid *Salmonella*

(Sánchez-Vargas *et al*., 2011; Bhunia, 2008). *Salmonella* can contaminate fresh produce during harvesting, processing, retailing and preparation at home through cross-contamination and mishandling practice.

Conventional methods and rapid methods specifically polymerase chain reaction (PCR) can be employed to detect the presence of *Salmonella* in food. Conventional methods involve enrichment, plating, biochemical test and serological confirmation provides an acceptable detection limit and specificity but they are time-consuming, laborious and not suitable for large number of samples (Lee *et al*., 2015). Meanwhile, PCR and other PCR-based methods promise rapid detection and high degree of specificity (Saeki *et al*., 2013). In the present study, most probable number (MPN) method was combined with PCR to detect and quantify *Salmonella* in food sample.

Characterization of *Salmonella* spp. is important for epidemiological study in order to track the source of outbreaks and to study the genetic diversity of bacterial pathogens. It involves typing methods such as antibiotic resistance patterns, molecular typing methods including random amplification of polymorphic DNA-PCR (RAPD-PCR) and pulsed field gel electrophoresis (PFGE) (Ranjbar *et al*., 2014).

Malaysian government as well as Malaysians are more concern about the food safety issues nowadays. Hence, Food Safety and Quality Division under Ministry of Health, Malaysia had established National Committee on Risk Analysis (NCRA) in 2002 in order to examine and investigate food safety issues in the country (FAO, 2004). Information on safety of *Salmonella* spp. in *ulam* is still scarce but salmonellosis has been reported in Malaysia. To the best of author's knowledge, there is no published data on prevalence of *Salmonella* spp., *Salmonella* Enteritidis (S. Enteritidis) and *Salmonella* Typhimurium (*S*. Typhimurium) using most probable number-multiplex polymerase chain reaction (MPN-multiplex PCR) technique on these *ulam*. Thus, this study will provide a glance on the Malaysia scenario and alert the related health authorities on safety issues of *ulam* at the retail markets. Furthermore, it also gives us insight into the prevalence of *Salmonella* spp., *S*. Enteritidis and *S*. Typhimurium in *ulam* and risk acquiring *Salmonella* through consumption of *ulam*.

1.2 Objectives

The objectives of this study are:

1. To ascertain the prevalence and contamination level of *Salmonella* spp., *S*. Enteritidis and *S*. Typhimurium in *ulam* at retail level.
2. To examine antibiotic resistance patterns of *S*. Enteritidis and *S*. Typhimurium isolates from *ulam*.
3. To characterize *S*. Enteritidis and *S*. Typhimurium isolates using random amplification of polymorphic DNA-PCR (RAPD-PCR).
4. To estimate the risk of illness (salmonellosis) through consumption of *ulam*.

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