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PREVALENCE OF Salmonella spp. IN CHICKEN MEAT AND ITS PRODUCTS, AND DEVELOPMENT OF PCR-ELISA FOR DETECTION OF Salmonella Enteritidis

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By

MOHD AFENDY BIN ABDUL TALIB

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

November 2017

Dedicated to my beloved mum (Azizah Md. Said), family members, colleagues in MARDI and friends in UPM for their understanding and support

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

PREVALENCE OF *Salmonella* spp. IN CHICKEN MEAT AND ITS PRODUCTS, AND DEVELOPMENT OF PCR-ELISA FOR DETECTION OF *Salmonella* Enteritidis

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

The increase in *Salmonella* infections necessitate the development of methods for the pathogen detection. Conventional culture for *Salmonella* detection is laborious and time-consuming. This has encourage the use of Polymerase Chain Reaction (PCR) and PCR-enzyme-linked immunosorbent assay (ELISA) that offer faster result and better sensitivity. To date, there was no published data on the prevalence and quantities of *Salmonella* spp., *S.* Enteritidis and *S.* Typhimurium in raw, processed and ready-to-eat (RTE) chicken samples by most probable number (MPN)-mPCR & MPN-plating and no published data on the effect of two different matrices of raw chicken meat and chicken sausages towards PCR-ELISA performance. Thus, this study is aimed to discover these gaps.

The multiplex polymerase chain reaction (mPCR) method was established. Three primer sets were successfully designed to simultaneously identify *Salmonella* spp., *S.* Enteritidis and *S.* Typhimurium. The mPCR containing *S.* Typhimurium generated 650 bp and 500 bp amplicons, *S.* Enteritidis generated 500 bp and 103 bp amplicons and *S.* Typhi produced a 500 bp amplicon.

Subsequently, MPN-mPCR was used to determine the prevalence and quantity of *Salmonella* spp., *S.* Typhimurium and *S.* Enteritidis in 130 samples of chicken meat and its products. The prevalence of *Salmonella* spp., *S.* Enteritidis and *S.* Typhimurium obtained is 25.38%, 10% and 6.92%

respectively. The prevalence of *Salmonella* spp. in raw chicken meat was the highest at 45% followed by RTE food at 30% while *S.* Enteritidis is 22.5% and 8% respectively. No contamination was detected in processed chicken meat products. The estimated quantity of *Salmonella* spp., *S.* Enteritidis and *S.* Typhimurium varied from 3 MPN/g to the 1100 MPN/g. Quantity of *Salmonella* spp. was found highest in chicken breast and thigh (1100 MPN/g), *S.* Typhimurium was highest detected in RTE chicken curry (210 MPN/g), while *S.* Enteritidis was highest detected in chicken breast (93 MPN/g).

In subsequent chapter, PCR-ELISA was successfully established and compared with PCR to detect *S.* Enteritidis in raw chicken meat & chicken sausages. The limit of detection (LOD) by PCR-ELISA is 9.4 CFU/mL in raw chicken meat and 2.46 CFU/mL in chicken sausage while PCR detected 9.4 X 10^1 CFU/mL and 2.46 X 10^1 CFU/mL respectively, indicating that the PCR-ELISA is 10-fold more sensitive than the conventional PCR. In fact, the LOD of PCR-ELISA calculated by GraphPad Prism is 15.23 CFU/mL for chicken sausage and 3.28 CFU/mL for raw chicken meat.

The prevalence and quantity of *Salmonella* spp., *S.* Typhimurium and *S.* Enteritidis in the samples discovered in this study indicated that the contaminated chicken samples posed a risk to consumer as it contained high level of contamination which some exceeded the infectious dose (10^3 CFU/mL) of *Salmonella* to human. Meanwhile, PCR-ELISA offer as an alternative and improved assay to the PCR and real-time PCR for detection of *S.* Enteritidis in semi-quantitative and high-throughput manner. The PCR, MPN-mPCR and PCR-ELISA method established in this study, could be used selectively as a screening tools for surveillance and monitoring of foodborne pathogen contamination so that appropriate actions could be taken by the relevant parties.

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

PREVALENS *Salmonella* spp. DALAM DAGING AYAM DAN PRODUKNYA, DAN PEMBANGUNAN PCR-ELISA UNTUK PENGESANAN *Salmonella* Enteritidis

Oleh

MOHD AFENDY BIN ABDUL TALIB

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Pengerusi: Son Radu, PhD
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Peningkatan jangkitan *Salmonella* memerlukan perkembangan kaedah untuk mengesan patogen tersebut. Kultur konvensional untuk mengesan *Salmonella* memakan masa yang lama. Ini telah menggalakkan penggunaan tindakbalas berantai polimerase (PCR) dan PCR-asai imunoserapan terangkai enzim (ELISA) yang menawarkan keputusan yang lebih cepat dengan dan lebih sensitif. Sehingga kini, tiada data yang diterbitkan berkenaan dengan prevalens dan kuantiti *Salmonella* spp., *S*. Enteritidis and *S*. Typhimurium dalam sampel ayam mentah, diproses dan sedia-untuk-dimakan (RTE) menggunakan angka kebarangkalian kebanyakan (MPN)-mPCR & MPN-pemplatan dan tiada data yang diterbitkan mengenai kesan dua matriks yang berbeza, daging ayam mentah dan sosej ayam terhadap prestasi ELISA. Oleh itu, kajian ini bertujuan untuk mengetahui jurang masalah ini.

Kaedah tindakbalas berantai polimerase multipleks (mPCR) telah dibangunkan. Tiga set primer telah berjaya direka untuk mengenal pasti *Salmonella* spp., *S*. Enteritidis dan *S*. Typhimurium secara serentak. mPCR yang mengandungi *S*. Typhimurium menghasilkan 650 bp dan 500 bp amplikon, *S*. Enteritidis menghasilkan 500 bp dan 103 bp amplikon dan *S*. Typhi menghasilkan 500 bp amplikon.

Seterusnya, MPN-mPCR digunakan untuk menentukan prevalens dan kuantiti *Salmonella* spp., *S*. Enteritidis and *S*. Typhimurium dalam 130 sampel,

daripada daging ayam mentah dan produk berasaskan ayam. Prevalens *Salmonella* spp., *S.* Enteritidis and *S.* Typhimurium masing-masing adalah 25.38%, 10% dan 6.92%. Prevalens *Salmonella* spp. dalam daging ayam mentah adalah yang paling tinggi pada 45% diikuti oleh makanan RTE pada 30% manakala prevalens *S.* Enteritidis adalah 22.5% dan 8% masing-masing. Tiada pencemaran yang dikesan dalam produk daging ayam yang diproses. Kuantiti *Salmonella* spp., *S.* Enteritidis dan *S.* Typhimurium berbeza daripada 3 MPN/g hingga 1100 MPN/g. Kuantiti *Salmonella* spp. didapati paling tinggi dalam dada dan paha ayam (1100 MPN/g), *S.* Typhimurium dikesan paling tinggi dalam kari ayam RTE (210 MPN/g), manakala *S.* Enteritidis dikesan paling tinggi dalam dada ayam (93 MPN/g).

Dalam bab yang berikutnya, PCR-ELISA telah berjaya dibangunkan dan dibandingkan dengan PCR untuk mengesan *S.* Enteritidis dalam daging ayam mentah dan sosej ayam. Had pengesanan (LOD) oleh PCR-ELISA ialah 9.4 CFU/mL dalam daging ayam mentah dan 2.46 CFU/mL dalam sosej ayam, manakala PCR mengesan 9.4×10^1 CFU/mL dan 2.46×10^1 CFU/mL masing-masing, menunjukkan bahawa PCR-ELISA adalah 10 kali lebih sensitif berbanding PCR konvensional. LOD sebenar PCR-ELISA yang dikira menggunakan GraphPad Prism adalah 15.23 CFU/mL untuk sosej ayam dan 3.28 CFU/mL untuk daging ayam mentah.

Prevalens dan kuantiti *Salmonella* spp., *S.* Typhimurium and *S.* Enteritidis dalam sampel yang ditemui dalam kajian ini menunjukkan bahawa sampel ayam yang tercemar mendatangkan risiko kepada pengguna kerana ia mengandungi kandungan tahap pencemaran yang tinggi yang mana beberapa daripadanya melebihi dos jangkitan (10^3 CFU/mL) *Salmonella* kepada manusia. Sementara itu, PCR-ELISA menawarkan asai alternatif dan ditambahbaik berbanding PCR dan PCR masa-nyata untuk pengesanan *S.* Enteritidis secara semi-kuantitatif dan jumlah sampel yang besar. Kaedah PCR, MPN-mPCR dan PCR-ELISA yang dibangunkan dalam kajian ini boleh digunakan secara selektif sebagai alat saringan untuk pemantauan pencemaran patogen bawaan makanan supaya tindakan yang bersesuaian dapat diambil oleh pihak yang berkaitan.

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I certify that a Thesis Examination Committee has met on 15 November 2017 to conduct the final examination of Mohd Afendy bin Abdul Talib on his thesis entitled "Prevalence of *Salmonella* spp. in Chicken Meat and its Products, and Development of PCR-ELISA for Detection of *Salmonella* Enteritidis" 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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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

AOAC	Association of Official Analytical Chemists (AOAC)
ATCC	American Type Culture Collection
BAM	Bacteriological Analytical Manual
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BPW	Buffered peptone water
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
EDTA	Ethylene-diamine-tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FDA	United States Food and Drug Administration
FoodNet	Foodborne Diseases Active Surveillance Network
FSIS	Food Safety and Inspection Service
GALT	Gut-associated lymphoid tissue
G	Gram
h	hour
min	minute
$MgCl^2$	Magnesium chloride
mL	mililiter
ISO	International Organization for Standardization
LIA	Lysine iron agar
LOD	Limit of Detection
MPN-mPCR	Most probable number-multiplex polymerase chain reaction
OD	Optical density
PCR	Polymerase chain reaction
PCR-ELISA	Polymerase chain reaction-enzyme-linked immunosorbent assay
RTE	Ready-to-eat
s	second
SC	Selenite cystine
spp.	species
Ta	Annealing temperature
T_m	Melting temperature
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA
TSA	Trypticase soy agar
TSI	Triple sugar iron agar
TSB	Tryptic soy broth
TT	Tetrathionate broth
US	United States

U	Unit
µL	microliter
USDA	United States Department of Agriculture
UPM	Universiti Putra Malaysia
UV	Ultraviolet
V	Volt
WHO	World Health Organization
x g	Gravity unit
XLD	Xylose lysine deoxycholate

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Foodborne illnesses are defined by World Health Organization (WHO) as diseases, usually either infectious or toxic in nature, caused by agents that enter the body through the ingestion of food. Foodborne diseases could be caused by a wide range of biological and chemical agents or hazards resulting varying degrees of severity, ranging from mild indisposition to chronic or life-threatening illness, or both (Kaferstein, 2003). These agents include bacteria, viruses, protozoa, helminthes, and natural toxins, as well as chemical and environmental contaminants.

Foodborne illness or disease that caused by foodborne pathogen occurred every year both in developed and developing countries throughout the world. The incidence of foodborne disease is difficult to be estimated globally but it was reported that an estimate of 600 million or almost 1 in 10 people in the world fall ill after eating contaminated food and 420,000 die every year. Centers for Disease Control & Prevention, US in 2011 (Scallan, et al., 2011) estimated that roughly one of six Americans or 48 million people get sick, 128,000 are hospitalized, and 3,000 die of foodborne diseases. Among these, children under 5 years of age carry 40% of the foodborne disease burden, with 125,000 deaths occur every year (WHO, 2015).

Diarrhea where considerable proportion comes from foodborne is a main cause of deaths in susceptible person such as young children, elderly and infants causing 550 million people to fall ill and 230,000 deaths every year. Mead et al. (1999) estimated that foodborne diseases may result in 76 million illnesses, 325,000 hospitalizations and 5000 deaths each year in the U.S. In Australia, about 5.4 million cases, 15,000 hospitalizations and 120 deaths of foodborne disease reported each year (Astridge et al., 2011). Meanwhile, in England and Wales, about 1.3 million cases, 21,000 hospitalizations and 500 deaths reported each year (Adak et al., 2002).

In Malaysia, no statistics report published on the numbers of hospitalizations and deaths. However, according to MOH Health Facts report 2013, 2014 and 2015, food poisoning incidence is escalating from 44.93 cases per 100,000 population in 2012, 47.79 in 2013 and increased 22.72% to 58.65 per 100,000 population in 2014. Based on 30.6 million Malaysia population in 2014, there

were 17,946 people involved in the food poisoning incidence which should be looked seriously as this reported figure probably represents only a fraction of true incidence since most of the cases are unreported or undiagnosed. This is often true when as most individual regard mild and diarrhea as a common illness and transient discomfort rather than a symptom of disease and hence may not consult the doctor (Soon et al., 2011).

Foodborne illnesses can be divided into two major groups of which are foodborne pathogens and unspecified agents. The foodborne pathogens comprise of 31 pathogens known to cause foodborne illness and many of these pathogens are tracked by system developed by public health sector that tracks and monitor diseases and outbreaks (Scallan, et al., 2011). While most foodborne illness cases go unreported to health departments, and are thus of unknown origin, the CDC estimates that 9.4 million of the illnesses are caused by 31 known foodborne pathogens and about 90% of foodborne illnesses, are caused by 8 known pathogens which account for the vast majority of illness, hospitalizations and deaths in US namely *Salmonella* (non-typhoidal), Norovirus, *Campylobacter* spp., *Toxoplasma gondii*, *E. coli* (STEC) O157, *Listeria monocytogenes*, *Clostridium perfringens* and *Staphyloccus aureus* (Scallan, et al., 2011).

Salmonella continues to be a major foodborne pathogen for animals and humans worldwide (Olsen et al., 2000; Humphrey, 2000) and it is the leading cause of foodborne outbreaks and infections in many countries (Wallace et al., 2000). Foodborne Diseases Active Surveillance Network (FoodNet), US released their morbidity and mortality weekly report (MMWR) reported that in 2014, most commonly diagnosed and reported illness caused by *Salmonella* continues to be a challenge as incidence of *Salmonella* infections per 100,000 population is the highest at 15.45 or 7,452 cases followed by *Campylobacter* and *Shigella* at 13.45 and 5.81 respectively (Crim et al., 2014). Among 6,565 of *Salmonella* serotype isolates in 2004, the top three isolates was Enteritidis (1,401), Typhimurim (806) and Newport (724). Thus, *S.* Enteritidis and *S.* Typhimurium was chosen as the candidate foodborne pathogens in this study.

Salmonella is the leading causes of foodborne illness and is common foodborne pathogen infecting humans and animals (Tirado & Schmidt, 2001). It has been estimated that in the United States alone there are 1.4 million *Salmonella* infections per year (Voetsch et al., 2004). An infection by *Salmonella* bacteria is called salmonellosis it is a major cause of many food poisoning cases, thus regarded as high public health concern (Aarestrup et al., 2007). Predominantly, there are two types of salmonellosis, which are typhoidal salmonellosis and non-typhoidal salmonellosis. Typhoidal salmonellosis caused enteric fever which is a severe illness and patients depend on antibiotic treatment and supportive theraphy to cure the infection. While on the other hand, non-

typhoidal salmonellosis is a major cause of human gastroenteritis worldwide (Kornschober et. al., 2009) and is the cause of the most foodborne illness worldwide. *S.* Enteritidis and *S.* Typhimurium are under non-typhoidal salmonellosis. It was estimated that approximately 400 persons die each year with acute salmonellosis.

Children are the one that most likely to get salmonellosis. The rate of diagnosed infections in children less than five years of age is higher than the rate in other persons (Rudas et al., 2015). Severe infections were most likely to be seen in young children, the elderly, and those with impaired immune systems. Most persons infected with *Salmonella* develop diarrhea, fever, and abdominal cramps 12 to 72 hours after infection. The illness usually lasts 4 to 7 days, and most persons recover without treatment. However, in some persons, the diarrhea may be so severe that the patient needs to be hospitalized. In these patients, *Salmonella* infection may spread from the intestines to the blood stream and/or the lymphatic system which can overcome the host's natural defence system (D'Aoust, 1997) and can cause death unless the person is treated promptly with antibiotics.

The source of salmonellosis as indicated by the most outbreaks are caused by the food contamination not limited to dairy, poultry and meat products. Chicken, eggs and their derivative products are particularly at high risk. Most of foodborne disease cases were due to lack of cleanliness in food preparation and unhygienic handling of food (Griffith, 2000; Chen et al., 1997). Human infections resulted from eating raw or undercooked eggs (Chousalkar & McWhorter, 2017; Martelli & Davies, 2012), poultry and meat (Yoon et al., 2014; Akbar & Anal, 2013), raw milk and dairy products (Ahmed & Shimamoto, 2014; Lubote et al., 2014) seafood (Nguyen et al., 2016; Mus et al., 2014), spices (Kara et al., 2015) and; fruits and vegetables (Vestrheim et al., 2016; Callejón et al., 2015) have been reported. With the recurring and increasing incidences of salmonellosis outbreaks that have been reported worldwide, it has been of great interest of various parties in controlling these outbreaks lead through the surveillance and control system before any action plan can be devised and employed. Generally, it is an important task for food regulators, manufacturers, restaurants and the food industry sector, as well as for legislative body in controlling the outbreaks of foodborne pathogens in particular, *Salmonella*. The surveillance and control requires reliable and/or rapid method in the detection and identification of *Salmonella* spp. Detection of *Salmonella* is therefore of outmost importance in food safety (Thornton et al., 1993).

Great attention has been given to the development of methods for detecting these pathogens including culture isolation, deoxyribonucleic acid (DNA)

probes and polymerase chain reaction (PCR) assays. Although conventional culture methods deemed as the gold standard for detection of *Salmonella* spp. in food, they are time-consuming, taking up to 4-7 days to get results which involve pre-enrichment in non-selective medium, followed by plating onto selective agar and isolation of presumptive colonies for biochemical and serological tests. On the other hand, nucleic acid amplification technologies such as PCR provide rapid and sensitive tools for detection of *Salmonella*. Successful amplification to detect *Salmonella* using *invA* gene has been reported with diagnostic potential (Rahn et al., 1992). *Salmonella* detection has been improved with greater sensitivity and permits quantification by the application of real-time PCR (Zhang et al., 2015). A PCR requires primer sequences unique to the pathogen of interest and it is crucial for the detection to be specific towards targeted pathogen. Target genes in designing primers specific to *Salmonella* detection are mostly associated with their virulence genes such as *invA, fimA, spv, stn, hilA,* and *fliC*. In this study, *in silico* PCR technique were used to search for *Salmonella* spp., *S.* Typhimurium and *S.* Enteritidis specific PCR primers and probes through the prediction of probable PCR products and search of potential mismatching location of the specified primers or probes. New primer sequences for those three pathogens were designed and subsequently used in multiplex PCR assay for prevalence studies.

Although PCR is considered as an alternative to conventional culturing, this procedure allows limited number of samples that can be analysed during one electrophoresis run since traditional PCR required amplification in a thermocycler, followed by post-PCR analysis such as gel electrophoresis or by hybridization with a probe. In this perspective, conventional PCR method could turn out to be inadequate in fulfilling demands for sectors such as food industry and regulatory bodies with routine and large sample screenings of foodborne pathogens.

Polymerase Chain Reaction-Enzyme Linked Immunosorbent Assay (PCR-ELISA) format is a high-throughput detection system and viable alternative to real-time PCR methods. PCR products are labelled (eg. by biotin) during amplification and a capture probe specific to the PCR amplicon is used to immobilize the amplicon to a well of microtiter plate (Elizabeth and Anne, 2005). Biotinylated PCR method was demonstrated to provide a simple, accurate, high sensitivity and specificity comparable to commercial system at around one-fourth the cost (Yam et al., 2004). Due to the low cost and high throughputness of this system, PCR-ELISA method was established and studied for the detection of *S*. Enteritidis.

With the high and growing demands of Malaysia population towards chicken meat and meat-based products, this study will gather the prevalence and estimated quantity of *Salmonella* covering raw chicken meat, process chicken meat products and chicken-based ready-to-eat food samples. In addition, this study is also aimed to develop and evaluate the detection of these samples by PCR-ELISA and compared with the conventional PCR to evaluate its sensitivity and specificity.

1.2 Problem statements

Increased cases of *Salmonella* infection in Malaysia and worldwide has urged great attention to the development of detection methods for this foodborne pathogen. This has encouraged the development and use of evolving techniques such as PCR and PCR-ELISA which has faster analysis time and better sensitivity than the conventional culture method. So far, there has no published data on the prevalence and estimated quantities of *Salmonella* spp., *S.* Enteritidis and *S.* Typhimurium in the chicken samples covering the raw and processed chicken and RTE chicken-based food by MPN-mPCR and MPN-plating. Hence, the MPN-mPCR and MPN-plating methods were used in this study to gather these data to have an insight of the *Salmonella* contamination in the samples and the risks associated with the microbial load towards human health.

Similarly, only a few studies to date reported on *Salmonella* detection by PCR-ELISA and this study will be the first to perform PCR-ELISA for *S.* Enteritidis detection in raw chicken and chicken sausage. Food matrices can influence the detection of *Salmonella* (Ryan et al., 2015) in a sample and therefore, this study will also focus on the detection of *S.* Enteritidis which was spiked in two different food matrices that have two distinct characters. One is non-processed matrix (raw chicken meat) and highly processed matrix (chicken sausages) were the reason of choosing these two samples in this study with purpose to discover the matrices effect towards PCR-ELISA performance. It is anticipated that results from these works will generate new knowledge and improve current detection method to benefit the scientific community.

1.3 Objectives

The objectives of this study were as follows:

1. To design specific PCR primers to detect *Salmonella* spp., *Salmonella* Enteritidis and *Salmonella* Typhimurium.

2. To develop and optimize multiplex PCR conditions for the simultaneous detection of *Salmonella* spp., *S.* Enteritidis, *S.* Typhimurium.

3. To determine prevalence and estimated quantity of *Salmonella* spp., *S.* Enteritidis and *S.* Typhimurium in raw chicken and chicken-based products from hypermarket and stalls by MPN-mPCR.

4. To establish PCR-ELISA assay to detect *S.* Enteritidis artificially-contaminated raw chicken meat and chicken sausage samples as study model and perform comparison with conventional PCR.

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