UNIVERSITI PUTRA MALAYSIA

EVALUATION OF RAPID METHODS FOR ISOLATION AND CHARACTERIZATION OF *SALMONELLA* SEROVARS ISOLATED FROM RAW POULTRY AND VEGETABLES

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FSMB 2003 24

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By

NOORZALEHA BINTI AWANG SALLEH

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

August 2003

UPM

Dedicated to my loving husband, Ahmad Idzam and lovely daughters, Wanis, Hajar and Akmal

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

EVALUATION OF RAPID METHODS FOR ISOLATION AND MOLECULAR CHARACTERIZATION OF *SALMONELLA* SEROVARS ISOLATED FROM RAW POULTRY AND VEGETABLES

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August 2003

Chairman: **Professor Gulam Rusul Rahmat Ali, Ph.D.**

Faculty: **Food Science and Biotechnology**

Cases of salmonellosis in humans have increased in recent years. Poultry, eggs, meat and dairy products are the most commonly implicated foods in salmonella food infection. The widespread increase in salmonellosis is of major health concern especially in the developing countries. The emergence of *S.* Typhimurium DT104 has further worsen the problem because of its known multidrug-resistance. For the past three decades, *S.* Typhimurium was the most frequently isolated serotype in Malaysia. However, in recent studies *S.* Weltevreden were reported to be the most predominant serovar present in foods. The present study examines the incidence of salmonellas in poultry and four types of vegetables. However, the main focus of the study is to evaluate different rapid methods for isolation and subsequently characterize the isolates obtained using various molecular typing tools. The vegetables chosen for the study include 'selom' (*Oenanthe stolonifera*), 'pegaga' (*Centella asiatica*), 'kesum' (*Polygonum minus*) and 'kangkong' (*Ipomoea aquatica*).

Three hundred and sixty one *Salmonella* isolates were isolated from 157 samples of raw poultry and the four types of vegetables. The study demonstrated that recoveries of *Salmonella* were higher in poultry than in vegetables. Samples enriched in Rappaport Vassiliadis (RV) broth and incubated at 42°C gave higher recoveries compared to RV broth at 37°C, Mannitol Selenite Cysteine broth (SC) at 42°C and 37°C. Selective enrichment in RV broth incubated at 42°C and subsequent plating on Hektoen Enteric Agar (HEK) gave the highest number of *Salmonella* isolation from poultry and vegetables samples. More *Salmonella* serotypes were isolated from samples enriched in RV than from SC media while there was no obvious difference among Hektoen Enteric Agar, Rambach Agar, Xylose Lysine Deoxycholate Agar and Bismuth Sulphite Agar used for the recovery of *Salmonella* spp.

The conventional cultural method gave highest recoveries of *Salmonella* followed by enzyme-linked immunosorbent assay (ELISA) and immunomagnetic separation (IMS). 46.6% (34/73) of poultry and vegetable samples were positive for *Salmonella* by conventional method, 26% (19/73) by ELISA and 17.8% (19/73) by IMS. Mixed growth of diverse flora was observed on Rambach Agar, Hektoen Enteric Agar, Xylose Lysine Deoxycholate Agar plates from IMS while growth of typical colonies of *Salmonella* were observed on plates using conventional method.

Antibiotic susceptibility test was carried out on 103 and 109 *Salmonella* isolates from raw poultry and vegetable samples respectively. *Salmonella* isolates from poultry produced 30 antibiotic resistance patterns while isolates from vegetables displayed only

17 patterns. Majority of the *Salmonella* isolates were resistant to more than one antibiotic. Isolates from poultry exhibited different resistance patterns from those of vegetables. Comparatively, poultry isolates were resistant to a large number of antibiotics than vegetables isolates. All isolates of *S.* Weltevreden showed resistance to sulfamethoxazole while *S.* Agona exhibited resistance to doxycycline HCl, erythromycin, sulfamethoxazole, streptomycin and tetracycline. On the other hand, *S.* Hadar showed more diversified pattern of resistance and all the isolates were resistant to more than three antibiotics.

Out of 23 serotypes screened, only 13 harbour plasmids while 16 others were plasmid-free. The plasmid sizes ranged from 0.6 to 58 MDa. Some of these plasmids could be responsible for the antibiotic resistance and virulence of *Salmonella.* However, these two properties were not determined in this study.

Specific PCR with genus specific primers, ST11 and ST15 were used to reconfirm the *Salmonella* isolates. All of the 42 serotypes of *Salmonella* examined, possessed the 429 bp fragments, which is specific for *Salmonella.*

RAPD and PFGE are the two commonly used methods for epidemiological studies. For RAPD analysis, primer GEN1-50-02 was used throughout the study for differentiation of 44 isolates of *S.* Weltevreden, 23 of *S.* Agona and 14 of *S.* Hadar isolates. In molecular typing for epidemiological studies, the percentage level of similarity is arbitrarily taken

based on the clustering of the strains analyzed. The results of RAPD and PFGE analysis were interpreted based on the 70% similarity level.

For RAPD analysis, primer 2 (GEN1-50-02) was used to discriminate the strains. *S.* Weltevreden produced a major band at 1200 bp while *S.* Agona bands were at 600 bp position and *S.* Hadar produced amplified fragments at ~ 1700 bp location. At 70% similarity level, *S.* Weltevreden isolates generated 7 clusters and 4 single isolates while *S.* Agona produced 4 clusters and *S.* Hadar just 5 clusters. Clusters obtained by RAPD and PFGE showed some disagreements between their results in discriminating the strains.

Out of 44 isolates of *S.* Weltevreden, only 39 were typeable. At 70% similarity level, they generated 7 clusters and 14 single isolates. The *F* values were in the range of 0.67 to 1.00. At the same level of similarity, 10 typeable isolates of *S.* Agona only produced 1 cluster and 8 single isolates with *F* values range from 0.82 to 1.00. From the dendrogram constructed *S.* Hadar isolates generated 2 clusters and 7 single isolates with *F* values range from 0.66 to 1.00.

As a conclusion, good recoveries of *Salmonella* require enrichment regardless of the methods used. For conventional method it is recommended to use at least two selective enrichment broths with several combination of plating media. Likewise, a combination of several typing methods will ensure reliable and reproducible results.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

EVALUASI KAEDAH PENGASINGAN RAPID DAN PENCIRIAN BAGI *SALMONELLA* SEROVAR YANG DIPENCIL DARI AYAM DAN SAYUR-SAYURAN MENTAH

Oleh

NOORZALEHA BINTI AWANG SALLEH

Ogos 2003

Pengerusi: **Profesor Gulam Rusul Rahmat Ali, Ph.D.**

Fakulti: **Sains Makanan dan Bioteknologi**

Sejak akhir ini, kes-kes salmonellosis telah meningkat dengan begitu ketara. Ayam, telur, daging dan hasil tenusu merupakan makanan yang seringkali dikaitkan dengan infeksi *Salmonella*. Peningkatan yang meluas ini telah mendapat perhatian umum terutama dari negara-negara yang nembangun. Keadaan ini lebih membimbangkan dengan kemunculan bakteria *S.* Typhimurium DT104 yang mempunyai multikerintangan. Bagi tempoh 30 tahun yang lalu *S.* Typhimurium adalah serotip yang kerap dipencil tetapi dalam pengajian ini, *S.* Weltevreden merupakan serotip yang paling dominan dipencilkan dari sampel-sampel yang dikaji.

Penyelidikan ini mengkaji insiden *Salmonella* dari sampel ayam dan sayuran ulam seperti 'selom' (*Oenanthe stolonifera*), 'kesum' (*Polygonum minus*), 'pegaga' (*Centella asiatica*) dan 'kangkong' (*Ipomoea aquatica*). Walau bagaimana pun focus kajian adalah

mengevaluasi kaedah-kaedah rapid bagi pemencilan dan pencirian molekular bagi pencilan bakteria *Salmonella.*

Sebanyak 361 pencilan *Salmonella* telah dipencilkan dari 157 sampel ayam dan ulam. Hasil kajian menunjukkan bakteria *Salmonella* banyak terdapat pada ayam dari ulam. Sampel yang diperkaya dalam kaldu RV dan diinkubasi pada suhu 42°C lebih banyak menghasilkan *Salmonella* berbanding RV pada suhu 37°C, kaldu SC pada suhu 37°C dan 42°C. Tidak ada perbezaan yang ketara diantara bilangan pencilan dari plat HEK, RAM dan XLD.

Kaedah konvensional telah menghasilkan pencilan yang tertinggi diikuti oleh kaedah ELISA dan IMS. Sebanyak 46.6% (34/73) dari sampel ayam dan sayur-sayuran telah dikenalpasti sebagai positif , 26% (19/73) menerusi kaedah ELISA dan 17.8% (13/73) melalui kaedah IMS. Terdapat pelbagai jenis pertumbuhan koloni diatas plat HEK, RAM dan XLD dengan kaedah IMS. Sebaliknya kaedah konvensional telah menghasilkan satu jenis pertumbuhan koloni sahaja yang tipikal *Salmonella.*

Sebanyak 103 pencilan dari sampel ayam dan 109 pencilan dari sampel sayur-sayuran telah diuji kerintangan antibiotik. Majoriti dari pencilan *Salmonella* menunjukkan kerintangan terhadap lebih dari satu antibiotik. Sampel ayam menghasilkan 31 paten kerintangan sementara pencilan dari sampel sayur-sayuran menunjukkan 16 paten sahaja. Pencilan dari ayam menunjukkan kerintangan terhadap lebih banyak antibiotik berbanding pencilan dari sayur-sayuran. Semua pencilan *S.* Weltevreden mempunyai

kerintangan terhadap antibiotik sulfamethoxazole sementara *S.* Agona merintang terhadap antibiotik doxycycline HCl, erythromycin, sulfamethoxazole, streptomycin dan tetracycline. Pencilan *S.* Hadar mengambarkan paten rintangan yang lebih kepelbagaian dan semua pencilan merintang lebih dari tiga antibiotik.

Hanya 13 serotip sahaja yang didapati mempunyai plasmid yang bersaiz dalam linkungan 0.6 hingga 58 MDa. Kehadiran plasmid boleh menyebabkan bakteria mempunyai kerintangan antibiotik atau virulen. Walau bagaimana pun kajian lanjut mengenai kedua faktor ini tidak dilakukan dalam studi ini.

ST11 dan ST15 adalah dua primer yang digunakan untuk mengesahkan kesahihan bakteria *Salmonella* diperingkat genus. Kesemua 42 serotip *Salmonella* didapati mempunyai band dilokasi 429 bp. Fragmen ini adalah spesifik bagi genus *Salmonella.*

Amplikasi rawak polimorpik DNA dan gel elektroforesis medan denyut adalah dua teknik molecular yang telah digunakan untuk mendiskriminasikan 44 pencilan *S.* Weltevreden, 23 pencilan *S.* Agona dan 14 pencilan *S.* Hadar. Tingkat keserupaan dalam teknik molecular taiping seperti RAPD dan PFGE untuk kajian epidemiologi adalah didasarkan pada kebangkalian analisis kluster. Dalam kajian ini, semua keputusan analisis RAPD dan PFGE telah dibuat berasaskan tahap 70% keserupaan.

Dalam analisis RAPD, penggunaan primer 2 (GEN1-50-02) telah menghasilkan band 1200 bp bagi *S.* Weltevreden, *S.* Agona mempunyai band pada lokasi 600 bp sementara *S.*

Hadar ditempat ~ 1700 bp. Pada tahap 70% keserupaan, pencilan *S.* Weltevreden menghasilkan 7 kluster dan 4 isolat tunggal, *S.* Agona 4 kluster dan *S.* Hadar hanya menghasilkan 5 kluster sahaja.

Dalam analisis PFGE, hanya 39 pencilan *S.* Weltevreden sahaja yang boleh ditaip dari keseluruhan 44 pencilan. Pencilan ini telah menghasilkan 7 kluster dan 14 isolat tunggal pada tahap 70% keserupaan (nilai *F* diantara 0.6 hingga 1.00). Pada tahap yang sama, pencilan *S.* Agona pula menghasilkan hanya1 kluster dan 8 isolat tunggal dengan nilai *F* diantara 0.82 hingga 1.00. Bagi *S.* Hadar , terdapat 2 kluster dan 7 isolat tunggal sahaja.

Kesimpulannya, tanpa mengira jenis kaedah yang digunakan langkah pengkayaan amat perlu bagi menghasilkan bilangan bakteria yang memuaskan. Bagi kaedah konvensional, disyorkan penggunaan kaldu pengkayaan lebih dari satu serta kombinasi pelbagai media plat. Begitu juga bagi kaedah taiping molekular. Kombinasi dari pelbagai teknik taiping yang berbeza dapat memberi gambaran serta diskriminasi yang lebih baik dan tepat.

ACKNOWLEDGEMENTS

In the name of Allah, The Most Gracious and The Most Merciful

First and foremost, I would like to express my heartfelt thanks to Almighty for giving me the will, strength and the perseverance to pursue and complete my Ph.D.

I would like to express my sincere gratitude and appreciation to the chairman of my supervisory committee, Professor Dr. Gulam Rusul Rahmat Ali for his invaluable guidance, dedicated efforts, supervision and continuous support throughout the study.

My special thanks and appreciation goes to my co-supervisor, Associate Professor Dr. Son Radu for his tremendous help in teaching me to write proper scientific papers. His advices, suggestions and consistent encouragement have kept me going without which Ph.D would probably be just a dream.

Sincere thanks are also extended to my other co-supervisors, Dr. Zaiton Hassan and Dr. Abdul Reezal Abdul Latiff for their suggestions, advices and supports.

I am indebted to my dear friend, Cheah Yoke Kqueen for his willingness and constant assistance and help in time when I was having difficulties with my molecular works. Words cannot express my gratitude for his patience and support.

My sincere gratitude also goes to all the staff of the Faculty of Food Science and Biotechnology, Universiti Putra Malaysia, who has contributed one way or another throughout the course of study.

I am grateful to my friends, Zainuri, Wai Ling, Rozila, Sushil, Samuel, Lesley, Gwen, Yin Tze, Vicky, Irene, Daniel, Jurin, Tung, Maya, Liha and Halifah. I shall cherish and treasure the joy and the happy moments we shared together in the lab. Thank you for making my student life an interesting and a delightful one.

Lastly, I would like to express my sincere and immeasurable thanks to my dear, beloved husband, Ahmad Idzam, my three beautiful and loving daughters, Wanis, Hajar and Akmal for their endurance, patience and encouragement. I love you all.

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LIST OF ABBREVIATIONS

AE	Elution buffer
AL	Lysis buffer
APHA	American Public Health Association
ATL	Tissue lysis buffer
AW	Wash buffer
BAM	Bacteriological Analytical Manual
BGSA	Brilliant green sulpha agar
BSA	Bismuth sulphite agar
CDC	Center for Disease Control and Prevention
CSE	Chromogenic ester medium
DCA	Deoxycholate citrate agar
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FDA	Food Drug Association
GET	Glucose-EDTA-Tris
HEK	Hektoen enteric agar
IMS	Immunomagnetic separation
LB	Luria Bertoni broth
LIA	Lysine iron agar
LPS	Lipopolysaccharide
MAR	Multiple antibiotic resistance index
MKTBG	Muller Kauffmann tetrathionate brilliant green broth
MLCB	Mannitol lysine crystal violet brilliant green agar
MM	Miller Mallison agar
NC	Negative control
NCCLS	National Committee for Clinical Laboratory Standards
PBS-T	Phosphate buffer saline with Tween 20
PC	Positive control
PCR	Polymerase chain reaction
%	Percentage
PFGE	Pulsed field gel electrophoresis
RAM	Rambach agar
RAPD	Random amplified polymorphic DNA
REA	Restriction enzyme analysis
RV	Rappaport Vasilliadis broth
SBG	Bacto sulpha enrichment broth
SC	Selenite cystine broth
SS	Salmonella shigella agar
SDS	Sodium dodecyl sulphate
TAL	Thin agar layer
TBE	Tris-Boric-EDTA

TSI	Triple sugar iron agar
UV	Ultra violet
VRI	Veterinary Research Institute
WHO	World Health Organisation
XLD	Xylose lysine deoxycholate agar
XLT4	Xylose lysine tergitol 4

CHAPTER 1

GENERAL INTRODUCTION

Salmonellae have long been recognized as an important cause of human food-borne disease. The typhoid bacillus was first isolated in 1884, when the German microbiologist Gaffkey obtained *S.* Typhi from human spleen (Scherer and Miller, 2001). Subsequently, *S.* Choleraesuis was isolated from the intestines of pigs infected with hog cholera in 1885 by the veterinary pathologist Daniel Salmon. The generic term *Salmonella* was given to the bacteria by Lignieres in 1900, in the honour of Dr. E. Salmon (Edwards and Ewing, 1986). Today, there are 2463 serovars that have been identified (Popoff and Le Minor, 1997; Brenner *et al.*, 2000; Popoff *et al.*, 2000).

Despite global improvement in public health facilities, salmonellosis remains a major problem in many parts of the world. The incidence in European countries shows currently a 20-fold increase during the last 10-15 years (WHO, 1997). In early 1990, increasing incidence of *S. enterica* serotype Enteritidis was observed in the Southeast Asia region (Chunsuttiwat, 1995), Europe and North America (Tauxe, 1991). In the United States, salmonellae are one of the most prevalent food-borne pathogens. They are estimated to cause approximately 1.5 million cases of infection, 15,000 hospitalizations and 500 deaths annually (Mead *et al.*, 1999). It is estimated that the reported cases only represent 1-10% of the real incidence of the disease (Oosterom, 1991). Food-borne illness statistic