UNIVERSITI PUTRA MALAYSIA

PATHOGENICITY OF MALAYSIAN ISOLATES OF SALMONELLA ENTERITIDIS PHAGE TYPES IN SPECIFIC PATHOGEN-FREE CHICKENS FOR VACCINE DEVELOPMENT

AMANULLAH AKHTAR

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By

AMANULLAH AKHTAR

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

May 2012

DEDICATIONS

Dedicated to my wife Munazza Aman and, children Muhammad Munam Aman, Muhammad Mussab Aman, Sumayya Huda and Muhammad Mueed Aman.

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

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Chairman: Profesor Mohd Hair-Bejo, PhD

Faculty: Veterinary Medicine

Salmonella enteritidis (SE) is the major and leading cause of food-borne illnesses associated with the consumption of SE contaminated chicken meat, eggs and poultry products. These ever increasing SE outbreaks have leaded the poultry industry and public health agencies to control the SE in commercial poultry. Vaccination against the disease has reduced the SE infection in poultry and subsequently the burden of foodborne illnesses. The objectives of the study were to determine the pathogenicity of Malaysian isolates of SE PTs in specific pathogen-free (SPF) chickens for the development of inactivated vaccine against the disease.

The pathogenicity of SE PTs 6A, 7, 3A and 35 was determined in three separate experiments. In experiment 1, one-day-old SPF chicks were divided into sacrificed groups (A1, B1 and C1) of 30 chicks each and mortality groups (MA1, MB1 and MC1) of 20 chicks each. The chicks in groups A1 and MA1 and, B1 and MB1 were inoculated orally with 0.1mL (10^8cfu/mL) of SE PT6A (UPM-0527)

and SE PT7 (UPM-0530), respectively. The non inoculated groups C1 and MC1 served as controls. Clinical signs and mortality were observed twice daily. On days 1, 3, 5, 7, 14 and 21 post inoculations (pi), five chicks were sacrificed from each sacrificed group. Before sacrificed chicks were individually weighed and, cloacal swab and blood samples were collected. On necropsy gross lesions were recorded and samples were collected for bacteriology and histology. The MA1, MB1 and MC1 served to determine the mortality. The same experimental design model was used for experiment 2, where groups A2 and B2 represented the sacrificed groups inoculated with SE PT3A (UPM-0541) and SE PT35 (UPM-0525), respectively. The same experimental design model was also used for experiment 3, except that in this experiment only SE PT6A isolate (UPM-0791) was used, and the chicks were sacrificed as early as 3, 6 and 12 hrs pi and samples were also collected for electron microscopy examination. The clinical signs of depression were 63%, 40%, 40% and 27%, anorexia were 50%, 40%, 30% and 27%, ruffled feathers were 50%, 30%, 40% and 40%, vent pasting were 40%, 37%, 23% and 20% and, diarrhoea were 33%, 20%, 7% and 7% on day 1 pi in SE PT6A (A1), PT7 (B1), SE P3A (A2) and PT35 (B2) inoculated chicks, respectively. Moreover, inability to move was only observed in experiment 1. Diarrhoea was the only clinical sign observed on days 3, 4, 5, 6 and 7pi and intermittently till day 14 pi in experiment 3. Mortality of 20%, 10% and 5% was observed in chicks inoculated with SE PT6A (MA1), SE PT3A (MA2) and SE PT35 (MB2), respectively. The significant ($p<0.05$) body weight gain difference was recorded in experiment 1. On day one pi, SE isolation was 100% from faecal swab, mid-gut contents, caecal tonsils and caecal contents, 80% from spleen and liver and, 60% from the blood in group A1 (SE PT6A). The PT6A showed highest

isolation throughout the experiment followed by PT7, PT3A, PT35 and SE PT6A isolate of ducks. Gross lesions of unabsorbed yolk, airsaculitis, fibrinous pericarditis, fibrinous perihepatitis, enlarged kidneys, splenomegaly were recorded in about 15% of chicks in group A1. Whereas 10% gross lesions were observed in groups B1 (SE PT7), A2 (SE PT3A) and B2 (SE PT35). Mild inflammation was observed in majority of tissues showing lesions. Degeneration and necrosis were observed in spleen, liver and bursa of Fabricius. Electron microscopy showed the *Salmonella* present in different organs as early as 6 hrs pi. The similar changes as observed in histopathology were noted in electron microscopy. Overall, SE PT6A isolate of chicks was more pathogenic than other PTs studied.

The safety and efficacy of inactivated single SE PTs 1, 3A, 6A, 7 and 35 and, their five different combinations were determined in two separate trials. Each SE PT was propagated and fermented individually in the bioreactor at 150rpm, pH 7 and temperature 37°C for 20 hrs. Purity test and plate count was performed. The harvest was collected for single SE PTs and combination of SE PTs, inactivated with 0.7% formaline and kept at 37 °C for 24 hrs. After sterility test adjuvant was added individually in all inactivated single SE PTs and the combinations and, kept for 48-72 hrs. For safety and efficacy, a group of 20 chicks was inoculated subcutaneously with 0.1mL of 10^{10} cfu/mL inactivated single SE PT or combination being studied. The uninoculated chicks served as controls. Clinical signs and mortality was observed. On day 14pi, 4 chicks from each group were sacrificed after weighing and, collection of blood and cloacal samples. Eight chicks were challenged by orally inoculating with 0.2mL of 10^{10} cfu/mL of SE PT6A (UPM-0527) on day 14 pi. On days 7 and 14 pc, 4 chicks from each group were sacrificed after weighing and, collection of blood and faecal samples. On

necropsy samples were collected for bacteriology and histopathology. In both trials, there was no significant difference ($p>0.05$) in body weight gain among all the groups. Clinical signs of depression, anorexia and diarrhoea were observed in 10% of chicks in challenged control group (CV0C) on day 3 to 5 post challenged (pc) in the first experiment (single) and almost similar in the second experiment (combination). No post mortem lesions were observed in any of the groups, except that bursa of Fabricius was swollen at 2 weeks pi in the CV0C. On 4 week pi, two serum samples from CV3AC group (inactivated SE PT3A) showed antibody titer of 2407 and 2842. Whereas, from combinations only a serum sample of CV673C group (combination of SE PTs 6A+7+3A) showed antibody titer of 601. The bacteriology results indicated that inactivated SE PTs have the ability to reduced fecal shedding and bacterial isolation from different organs when compared to control challenged chicks. The inactivated single SE PT6A and combination of SE PTs 6A, 3A and 7 were more effective among all the tested PTs.

In conclusion, young chicks are more susceptible to SE infections. SE PT6A (A1) is more pathogenic than other PTs studied. The different SE PTs can colonize the intestine and lead to systemic infections on oral inoculation. Different isolates of the same PTs may vary for their pathogenicity. The inactivated single and combination SE PTs are safe and effective to control the disease in chickens. These are able to reduce but are unable to completely eliminate the SE in the host.

Key words: *Salmonella* enteritidis, phage type, pathogenicity, vaccine, SPF chicks.

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

PATOGENISITI JENIS PHAGE *SALMONELLA* ENTERITIDIS ISOLAT MALAYSIA DALAM AYAM BEBAS-PATOGEN KHUSUS UNTUK PEMBANGUNAN VAKSIN

Oleh

AMANULLAH AKHTAR

Mei 2012

Pengerusi: Profesor Mohd Hair-Bejo, PhD

Fakulti: Perubatan Veterinar

Salmonella enteritidis (SE) adalah punca utama dan penyakit bawaan makanan yang dikaitkan dengan penggunaan ayam daging, telur dan produk ayam tercemar oleh SE. Peningkatan wabak SE ini menjadi dorongan kepada industri ternakan dan agensi kesihatan awam untuk mengawal SE dalam ternakan komersil. Vaksinasi terhadap penyakit ini telah mengurangkan jangkitan SE pada haiwan ternakan dan seterusnya beban penyakit bawaan makanan. Walaubagaimanapun, kelazimannya jenis phage (PT) SE adalah berbeza di lokasi geografi yang berbeza dan variasi dalam kebisaannya mendorong untuk pencirian isolat tempatan, interaksinya dengan perumah untuk digunapakai dalam strategi kawalan terhadap organisma ini. Objektif kajian ini adalah untuk menentukan patogenisiti SE PTs diasingkan dari Malaysia dalam ayam bebas-patogen khusus (SPF) bagi pembangunan vaksin tidak aktif terhadap penyakit ini.

Patogenisiti SE PTs 6A (UPM-0527), 7 (UPM-0530), 3A (UPM-0541) dan 35 (UPM-0525) yang diasingkan dari ayam dan SE PT6A (UPM-0791) dari itik

ditentukan dalam tiga eksperimen berasingan. Dalam eksperimen-1, anak ayam SPF berumur 1 hari telah dibahagikan kepada kumpulan dikorbankan (A1, B1 dan C1) sebanyak 30 ekor setiap kumpulan dan kumpulan kematian (MA1, MB1 dan MC1) sebanyak 20 ekor setiap kumpulan. Anak ayam SPF dalam kumpulan A1 dan MA1 dan, B1 dan MB1 masing-masing disuntik melalui mulut dengan 0.1mL (10^8cfu/mL) SE PT6A dan SE PT7. Ayam yang tidak disuntik dalam kumpulan C1 dan MC1 mewakili kumpulan kawalan. Tanda klinikal dan kematian diperhatikan sekurang-kurangnya dua kali sehari. Lima anak ayam diambil dari kumpulan dikorbankan (A1, B1 dan C1) pada hari 1, 3, 5, 7, 14 dan 21 pos-inokulasi (pi). Setiap anak ayam ditimbang dan dikorbankan selepas sampel darah dan najis diambil. Semasa nekropsi lesi mata kasar direkodkan dan sampel diambil untuk pengasingan dan pengenalpastian bakteria. Sampel hati, limpa, ileum, cecum, caecal tonsils dan bursa Fabricius juga diambil untuk pemeriksaan histopatologi. Anak ayam dari kumpulan kematian (MA1, MB1 dan MC1) digunakan untuk penentuan kematian. Kaedah eksperimen yang sama dalam eksperimen 1 telah digunakan di dalam eksperimen 2, di mana kumpulan A2 dan B2 masing-masing mewakili kumpulan dikorbankan yang diinokulasi dengan SE PT3A (UPM-0541) dan SE PT35 (UPM-0525). Kaedah eksperimen yang sama dalam eksperimen 1 juga digunakan dalam eksperimen 3 kecuali dalam eksperimen 3 hanya satu isolat SE PT6A (UPM-0791) yang digunakan, anak ayam telah dikorbankan seawal 3, 6 dan 12 jam selepas pi dan sampel diambil untuk pemeriksaan mikroskop electron. Hasil kajian masing-masing menunjukkan tanda klinikal kemurungan 63%, 40%, 40% dan 27%, tiada selera makan 50%, 40%, 30% dan 27%, bergolak bulu 50%, 30%, 40% dan 40%, buntut menampal 40 %, 37%, 23% dan 20%, dan cirit-birit 33%, 20%, 7% dan 7% pada hari 1 pi

bagi anak ayam disuntik SE PT6A (A1), PT7 (B1), PT3A (A2) dan PT35 (B2). Tambahan pula, ketidakupayaan untuk bergerak hanya dilihat di dalam eksperimen 1. Cirit-birit dilihat sekali sekala pada hari 3, 4, 5, 6 dan 7 sehingga hari 14 pi pada isolat SE PT6A daripada itik yang telah diinokulasi pada anak ayam. SE PT6A (MA1), PT3A (MA2) and SE PT35 (MB2) masing-masing menyebabkan 20%, 10% dan 5% kematian. Tiada kematian diperhatikan di eksperimen 3. Perbezaan kenaikan berat badan ketara ($p < 0.05$) dicatatkan dalam eksperimen 1. Pada hari pertama pi, pemencilan SE adalah 100% daripada najis, kandungan usus tengah, tonsil caecal dan kandungan caecal , 80% daripada limpa dan hati dan, 60% daripada darah dalam kumpulan A1 diinokulasi dengan A1 (SE PT6A). PT6A (kumpulan A1) menunjukkan pemencilan tertinggi menerusi eksperimen ini dan telah diikuti dengan PT7, PT3A, PT35 dan isolat SE PT6A daripada itik. Lesi matakasar telur kuning yang tidak diserap, airsaculitis, perikarditis fibrinus, perihepatitis fibrinus, ginjal dan limpa bengkak telah dicatatkan pada lebihkurang 15% daripada anak ayam yang dijangkiti dengan kumpulan A1 dan 10% anak ayam dalam kumpulan B1 (SE PT7), A2 (SE PT3A) and B2 (SE PT35). Bengkak dan infiltrasi hetrophil diperhatikan pada majoriti tisu yang menunjukkan lesi. Degenerasi dan nekrosis diperhatikan dalam hati, limpa, dan bursa Fabricius. Mikroskop elektron dalam eksperimen 3 menunjukkan *Salmonella* hadir di dalam organ yang berbeza seawal 6 jam pertama pi. Perubahan yang sama diperhatikan pada histopatologi dapat dilihat dibawah mikroskop elektron. Keseluruhannya, SE PT6A daripada anak ayam lebih patogenik di kalangan semua PTs bawah kajian.

Keselamatan dan keberkesanan SE PTs 1, 3A, 6A, 7 dan 35 dalam ketidakaktifan tunggal dan, lima kombinasi berbeza telah ditentukan dalam dua kajian

berasingan. Setiap SE PT telah dikembangbiakkan dan difermentasikan secara individu di dalam bioreactor pada 150rpm, pH 7 dan suhu 37 °C selama 20 jam. Ujikaji keaslian dan kiraan plat telah dibuat. Tuaian tersebut telah dibahagi kepada SE PTs tunggal dan satu lagi kombinasi SE PTs, dinyahaktifkan dengan 0.7% formalin dan disimpan pada suhu 37 °C selama 24 jam. Selapas ujikaji steril, adjuvant telah ditambahkan secara berasingan di dalam semua SE PTs tunggal yang tidak aktif dan dalam kombinasi SE PTs, dan disimpan selama 48-72 jam. Dalam menentu keselamatan dan keberkesanan bagi setiap SE PT yang tidak aktif, kumpulan ayam SPF yang terdiri daripada 20 ekor setiap kumpulan telah diinokulasi secara di bawah kulit masing-masing dengan 0.2mL 10^{10} cfu/mL SE PT tunggal yang tidak aktif dan dalam kombinasi SE PTs. Kumpulan yang tidak diinokulasi bertindak sebagai kawalan. Ayam telah dipantau untuk tanda klinikal dan kematian. Pada hari ke14 pi, empat ayam daripada setiap kumpulan telah dikorbankan selepas berat ditimbang dan sampel darah dan najis diambil. Lapan ayam telah dicabar dengan menginokulasi melalui mulut 0.2mL 10^{10} cfu/mL SE PT6A (UPM-0527) pada hari ke 14 pi. Pada hari ke 7 dan 14 pi, empat ayam daripada setiap kumpulan telah dikorbankan selepas berat ditimbang dan sampel darah dan najis diambil. Sampel diambil untuk ujian bakteria dan histopatologi. Dalam kedua-dua percubaan, tiada perbezaan yang ketara ($p>0.05$) pada berat ayam bagi semua kumpulan. Tanda klinikal kemurungan, tiada selera makan dan cirit-birit diperhatikan dalam 8-10% anak ayam dalam kumpulan kawalan yang dicabar (CV0C) pada hari 3-5 selepas dicabar (pc) dalam eksperimen pertama (tunggal) dan hampir sama dalam eksperimen kedua (kombinasi). Tiada lesi pos-mortem yang dapat diperhatikan di mana-mana kumpulan kecuali bursa Fabricius yang bengkak pada 2 minggu pi dalam CV0C. Pada 4 minggu pi, dua sampel

serum daripada kumpulan CV3AC (SE PT3A tidak aktif) menunjukkan titer antibodi 2407 dan 2842. Manakala dari kombinasi hanya sampel serum CV673C (kombinasi SE PTs 6A+7+3A) menunjukkan titer antibodi 601. Keputusan bakteriologi menunjukkan bahawa SE PTs tidak aktif mempunyai keupayaan untuk mengurangkan pengasingan bakteria daripada najis dan organ yang berlainan berbanding untuk anak ayam kawalan yang dicabar. SE PT tunggal 6A dan kombinasi SE PTs 6A, 3A dan 7 adalah lebih berkesan di antara semua PT yang dikaji.

Kesimpulannya, anak ayam muda lebih terdedah kepada jangkitan SE. SE PT6A (A1) adalah isolat yang lebih patogenik di kalangan semua PTs dikaji. SE yang berbeza boleh menjajah dan menceroboh usus dan menyebabkan jangkitan sistemik melalui inokulasi melalui mulut. Isolat berbeza dari SE yang sama mungkin berbeza untuk patogensitinya. SE PTs tidak aktif secara tunggal atau dalam gabungan berkesan dan selamat untuk mengawal penyakit pada ayam. Ini dapat mengurangkan walau tidak dapat menghapuskan secara keseluruhannya SE yang ada pada perumah.

Kata kunci: *Salmonella* enteritidis, jenis phage, pathogenisiti, patogenesis, vaksin, anak-anak ayam SPF.

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I certify that an Examination Committee has met on 31 May 2012 to conduct the final examination of Amanullah Akhtar on his Doctor of Philosophy thesis entitled "Pathogenicity of Malaysian isolates of *Salmonella* enteritidis phage types in specific pathogen-free chickens for the development of vaccine against the disease." in accordance with Universiti Pertanian Malaysia (Higher Degree) act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the student be awarded the degree of Doctor of Philosophy

Members of the Examination Committee were as follows:

TENGKU AZMI BIN TENGKU IBRAHIM, PhD
Professor**,** Dato' Dr
Faculty of Veterinary Medicine,
Universiti Putra Malaysia,
(Chairman)

ABDUL RANI BN BAHAMAN, PhD
Professor**,** Dato' Dr
Faculty of Veterinary Medicine,
Universiti Putra Malaysia,
(Internal Examiner)

JASNI BIN SABR, PhD
Associate Professor, Dr
Faculty of Veterinary Medicine,
Universiti Putra Malaysia,
(Internal Examiner)

PRIYA MOHN DAS, PhD
Professor, Dr
Faculty of Veterinary Science,
Bangladesh Agriculture University, Bangladesh
(External Examiner)

SEOW HENG FONG, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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

Mohd Hair-Bejo, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Zunita Zakaria, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

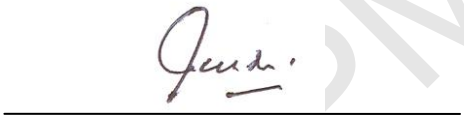
Abdul Rahman Omar, PhD
Professor
Institute Bioscience
Universiti Putra Malaysia
(Member)

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.



AMANULLAH AKHTAR

Date: 31 may 2012

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SPF	Specific pathogen-free
pi	Post inoculation
pc	post challenged
mL	Milliliter
g	Gram
SD	Standard deviation
M	Mean
SEM	Standard error of means
CFU	Colony forming unit
SE	*Salmonella* enteritidis
CE	Competitive exclusion
Ab	Antibodies
mμ	Micrometer
HE	Hematoxylin and eosin
PT	Phage type
SEM	Scanning electron microscope
TEM	Transmission electron microscope
LPS	Lipopolysaccharide
MRHA	Mannose-resistant hemagglutinin
SAT	Slide agglutination test
ELISA	Enzyme linked immuneosorbent assay
UPM	Universiti Putra Malaysia
G-ve	Gram stain negative
G+ve	Gram stain positive

MDa	Megadalton
DOC	Day old chick
GIT	Gastrointestinal tract
BA	Blood agar
RV	Rappaport vassilidase
BG	Brilliant green
XLD	Xylose lysine deoxycholate
CMI	Cell mediated immunity
TSI	Triple sugar iron
hrs	Hours

CHAPTER 1

INTRODUCTION

Salmonella is a genus of Gram-negative non-spore forming rod shape bacteria which belongs to family *Enterobacteriaceae*. In the genus, there are only two species, *Salmonella enterica* and*Salmonella bongori*. The species are further classified into serotypes by Kaufmann-White scheme. Presently, there are over 2500 serotypes determined on the basis of somatic (O), flagellar (H) and capsular (Vi) antigens (Adams and Moss, 2008). Whereas, on the basis of pathogenesis and host adaptability*Salmonella*serotypes can be divided into two major groups, the host adopted and the wide-host range serotypes. The host adopted serotypes have specific hosts and only cause systemic diseases in their respective hosts. In contrast, the wide-host range serotypes infect humans and a variety of animal species. This group is the principal concern of foodborne diseases in humans (Uzzau *et al.*, 2000; Barrow and Wallis, 2000). The two wide-host-range serotypes, *Salmonella enterica serovar Enteritidis* (SE) and *Salmonella enterica serovar Typhimurium* (ST) are most prevalent worldwide (Galanis *et al.*, 2006; EFSA, 2007). However, recently SE has replaced ST as a primary etiologic agent of *Salmonella* infections as well as the leading serotype responsible for foodborne human salmonellosis in most parts of the world (Fisher, 2004; Patrick *et al.*, 2004; CDC, 2006; Galanis *et al.*, 2006).

The SE serotypes are further classified into phage types (PTs) on the basis of their susceptibility to the lytic bacteriophages (Ward *et al.*, 1987). The different phage types differ in their geographic distribution. Also, the occurrence of

various phage types in a geographic location changes over time that may change the status of the prevalent as well as the predominant phage types in a geographical location (Fisher, 2004a).

*Salmonella*can cause infections in humans and a wide range of animal species. *Salmonella* are not only public health concern because of typhoid fever but mainly being the cause of the foodborne infections (Adams and Moss, 2008; Bhunia, 2008; EFSA, 2009). While estimating the global burden of non-typhoidal *Salmonella* gastroenteritis, it was estimated that 93.8 million cases of gastroenteritis due to *Salmonella* species occur globally each year, with 155,000 deaths. Out of these 80.3 million cases were foodborne (Majowicz *et al.,* 2010). Non-typhoidal *Salmonella* being the second most common bacterial cause of gastrointestinal infection in England and Wales caused 116,000 cases of illness, 3,400 hospitalisations and 268 deaths in 1995 (Adak *et al.,* 2002). Only in the USA, 95%of human salmonellosis cases were associated with the consumptionof contaminated food products. They caused 1.4 million illnesses, 600 deaths and economic loss of $464 million to $2.3 billion (Frenzen *et al.,* 1999; Mead *et al.,* 1999).

Salmonella Enteritidis is motile, facultative anaerobic *Salmonella* serotype that led the *Salmonella*to become the major and primary cause of bacterial foodborne infections in humans worldwide (Galanis *et al.,* 2006; CDC, 2006). SE accounted for 85% of *Salmonella*cases in Europe and 38% in Asia (Galanis *et al.,* 2006). Only in USA during the period 1985-1998 SE outbreaks caused 28644 illnesses, 2839 hospitalizations and 79 deaths (Patrick *et al.,* 2004). Only SE accounted about $870 million of $3.5 billion total annual costs of medical care and lost

productivity resulting from foodborne*Salmonella* infections of humans in the USA (USDA, 1998).

The first laboratory confirmed reported *Salmonella*foodborne outbreak was also caused by SE in 1888, in Germany that resulted in death of 58 people (Topley and Wilson, 1929). It could not get attention due to rare cases for a long time. However, after about 100 years, the number of SE outbreaks dramatically increased throughout the world (Rodrigue *et al.,* 1990; Cogan and Humphrey, 2003). Since then, it increased over time. It was 25.6% in 1990, 36% in 1995 and 65% in 2002 worldwide (Herikstad *et al.,* 2002; Galanis *et al.,* 2006). In Malaysia from 1983 to 1992SE increased by 760% (Rohani *et al.,* 1997). Recently, the incidence of SE infections in USA was about 25% higher while the overall incidence of human salmonellosis was lower in 2005 than in the mid-1990s (CDC, 2006).

Food originating from animals, especially from poultry are implicated an important and main source of these foodborne infections (Kimura *et al.,* 2004; Adak, 2005; Huneau-Salaün *et al.,* 2009). Among the poultry, chicken is the main reservoir of SE. Hence, the consumption of SE contaminated chicken meat, eggs and their products are the major source for foodborne infections worldwide (Guard-Petter, 2001; Oslen *et al.,* 2001: Pieskus *et al.,* 2006). Nearly 80% of SE outbreaks with a known food source were implicated only to eggs and egg-containing foods (Patrick *et al.,* 2004). Also, the consumption of chicken is reported a significant risk factor for SE infections in humans (Kimura *et al.,* 2004).

There is epidemiological connection between poultry and humans as SE PTs commonly present in poultry have been also isolated from humans in a geographical location (Van Duijkeren *et al.,* 2004; Akhtar *et al.,* 2010). SE has been frequently isolated from broilers and layers in Europe and has been the most common serotype in humans. It has been reported in layers (57.7%), layer breeders (63%), broiler breeders (42%) and table eggs (72.9%) in Europe (EC, 2004; EU, 2007a;b) and accounted for 85% of *Salmonella*cases in the same region (Galanis *et al.,* 2006).

Poultry can become infected horizontally through infected litter, faeces, feed, water, dust, insects, equipment, fomites, infected chicks and rodents contaminated with *Salmonella* (Poppe, 2000). They can also be transmitted by other animals, wild birds and personnel. *Salmonella* may contaminate developing embryo before hatch through ovarian transmission or penetration of the egg-shell after the egg has been laid (Gast, 2003; De Reu *et al.,* 2006). The horizontal transmission from *Salmonella*-contaminated eggs to *Salmonella*-free eggs during incubation results in hatching of infected chicks (Maryam *et al.,* 2010). Of the so many sources for SE infection in chicken, it mostly occurs after the ingestion of contaminated feed or water.

The ingested bacteria proceed through alimentary canal to reach the intestinal tract which is used as a portal of entry during infection (Amy *et al.,* 2004). The bacteria interact with intestinal mucosal surface to adhere and subsequently colonize. The colonization of the intestinal tract occurs if not cleared by the host defence system. After colonizing the intestine, bacteria interacts with and translocates across the intestinal epithelium by active invasion of enterocytes,

invasion into specialized epithelial cells called microfold cells (M cells) or through dendritic cells (Desmidt *et al.,* 1997; Vazquez-Torres *et al.,* 1999; Niess *et al.,* 2005). The bacteria may proceed through intestinal wall into deeper tissues to reach the reticulo-endothelial system and disseminate to other tissues such as liver, spleen and reproductive tract causing systemic infection. SE is clever enough to invade, survive, multiply in macrophages and use them as carrier for the dissemination to various tissues in contrast to the defined defence role of macrophages (Desmidt *et al.,* 1997; Barrow and Wallis, 2000; Gast, 2003; Kramer *et al.,* 2004). During the journey from ingestion to infection, SE face acidic environment, competition with normal intestinal micro-biota and other host defences which decides its fate (Chappell *et al.,* 2009). As a result, SE can colonize as symptomless carrier, can cause disease or cleared by host.

Mostly, SE infection in poultry is symptomless and unnoticed but can cause clinical disease and mortality under certain circumstances such as newly hatched chicks and stress conditions (Barrow and Wallis, 2000; Gast,2003). In newly hatched chicken SE can cause severe morbidity and high mortality whereas the older chicken may remain symptomless even with intestinal colonization and systemic dissemination (Barrow, 1991; Desmidt *et al.*, 1997).

The SE infection in poultry and its outcome depends upon so many factors related to pathogen, host and environment. Among the pathogen factors, SE phage type itself is one of factors that affect the pathogenicity and pathogenesis of SE infection. There is variation among the various phage types of SE in their virulence for chickens as well as variation in virulence exists among various isolates of the same SE phage types (Barrow, 1991; Alisantosa *et al.,* 2000). The

virulence of a phage type also varies with the variation in source of isolates and location of isolates (Poppe *et al.,* 1993).

Generally the severeity of SE infection in poultry has been assessed in terms of abnormal clinical signs, mortality, effects on growth and production of host, bacterial isolation, invasion and lesions in various organs of the host (Alisantosa *et al.,* 2000; Dhillon *et al.,*2001; Ahmad *et al* ., 2008). Clinical signs of infection include anorexia, depression, ruffled feathers, diarrhoea, dehydration, vent pasting, laboured breathing (Alisantosa *et al.,* 2000; Dhillon *et al.,* 2001). The mortality for different SE PTs infections in chicken may vary from zero to 96% (Barow, 1991; Dhillon *et al.,*2001). The SE has been isolated from the cloacal swabs, caecal and intestinal contents, caecal tonsils, liver and spleen (Alisantosa *et al.,* 2000; Dhillon *et al.,* 2001; Ahmad *et al.,* 2008).

The pathological changes and lesions caused by SE infection in poultry include severe enteritis, enlarged congested spleen, liver and kidneys, perihepatitis, peri-carditis, air sacullitis, degeneration and necrosis of different tissues including intestine, liver and spleen (Alisantosa *et al.,* 2000; Dhillon *et al.,*2001; Gast, 2003; Ahmad *et al*., 2008).

Despite its global economic impact and public health importance little is known about the interactions between the SE and the chicken from different phage types. Mostly the research has been conducted on SE PT4. There are few studies for a limited number of other than SE PT4 such as PT1, PT8, PT13A, PT14 and PT23. The SE PT4 has been reported the most pathogenic in poultry but it is not true in all cases (Barrow, 1991; Gast and Benson, 1995; Alisantosa *et al.,* 2000; Dhillon *et al.,* 2001; Ahmad *et al.,* 2008).

The colonization of intestinal and reproductive tract in poultry has been a serious food safety concern in addition to spreading infection in other flocks and the progeny. On one hand, the symptomless infection leads to a chronic carrier state in poultry. These carrier birds may contaminate meat and eggs for human consumption and subsequently result in foodborne infections. They can also transmit disease horizontally and vertically (Desmidt *et al.,* 1997; Gast, 2003). On the other hand, the clinical disease may cause loss in the form of mortality, decreased growth and production, compulsory disposal of birds or eggs, and loss of consumer and market confidence (USDA, 2010).

It is interesting to note that SE PTs behaving about commensally in chicken cause food borne diseases in humans. The chicken may harbour SE without showing any clinical signs of disease and any decline in growth, production or performance. The consumption of the same chicken's meat or eggs may result in foodborne salmonellosis in humans. This salmonellosis may result in abdominal pain, cramps, vomiting, diarrhoea and even death in humans.

Therefore, there is a strong need to introduce effective measures for controlling *Salmonella*infection especially SE in poultry (WHO, 2009; Majowicz *et al.*, 2010). The primary aim of *Salmonella*control in poultry is to prevent entering these organisms in food chain via meat or eggs.

Raising poultry under *Salmonella*-free conditions would be one of the best strategies to control SE contamination and subsequently food borne infections (Cox, 1995). However, it is difficult even at high management and production costs due to its wide range of reservoirs, ability to survive for long time in environments and high potential to spread through various sources of

transmission (Poppe, 2000; Zamri-Saad and Saleha 2006; Marryum *et al.,* 2010). The successful past experiences and research suggests the vaccination and competitive exclusion as possible measures to control *Salmonella* infection in poultry (EFSA, 2004; Zamri-Saad, 2006; Gast, 2007). Today, vaccination is considered more important option while the use of antibiotics is criticized and banned in most parts of the world. Many *Salmonella* especially SE has developed resistance to commonly used antimicrobials and is a serious public health concern (Dias de Oliveira *et al*. 2005).

A variety of vaccines including live and inactivated vaccines have been developed against *Salmonella* in poultry that are available in the global markets (EFSA, 2004). Presently, poultry producers vaccinate the breeders (layers and broiler breeders) and layer flocks to control *Salmonella* infection in many countries worldwide (EFSA, 2004).

The live vaccines have the advantage to induce a protective response which mimics the natural one and could be ideal. However, the risk for reversion of vaccine candidate *Salmonella* and its possibility of spreading in environment or to humans contradicts the basic purpose of vaccination. The inactivated *Salmonella* vaccines have no such safety issues. The use of only inactivated vaccine in layers has been recommended by the experts to avoid risk of spreading vaccine strains to eggs (EFSA, 2004). The inactivated *Salmonella* vaccines can reduce *Salmonella* colonization in organs and the faecal shedding as well (Neto *et al.,* 2008**;**Toyota-Hanatani *et al.,* 2009). These vaccines not only protect the recipient birds, but also the progeny. No doubt the inactivated vaccines are time consuming and laborious but, ensure the vaccination

ofeachbird. Moreover adjuvant is used to enhance the immunogenic potential of vaccines**.** In this regard, the very recent report for the alum adjuvant may be a new break through. It has been reported that alum adjuvant triggers an ancient pathway of innate recognition of crystals in monocytes and triggers them to become dendritic cells (Lambrecht *et al.,* 2009).

In Malaysia SE cases has been continuously increasing. The alarming increase of 760% during the period 1982-1992 has been reported (Rohani *et al.*, 1997). The isolation of SE from poultry (55.3%), poultry meat (21.5%), processing plants(83.0%), carcasses of poultry (79.9%) and, hatchery and poultry environment (7.6%) reveals the high threat level at all stages of poultry production and processing in Malaysia (Rusul *et al.,* 1996; Zamri-Saad and Saleha., 2006). At the same time, an alarming increase in human food poisoning associated with *Salmonella* species has been reported. SE accounted for more than 30% of human salmonellosis since 1993 (Rohaini *et al.,* 1995). Moreover, the SE caused human salmonellosis in Malaysia is not only increasing in frequency, but with more systemic involvement (Yasin *et al.,* 1998).

Poultry has been reported a potential vector for *Salmonella*in Malaysia (Rusul *et al.,* 1996). Therefore, the high prevalence of SE in poultry could be a reason for increase in human salmonellosis. Malaysia has one of the highest per capita consumption of chicken (35 kg) and eggs (280) per year (USAD, 2005; 2006).This increase in meat and egg consumption makes an increased potential for exposure to *Salmonella*through these foods. Moreover, the increasing trends to eat out of home in Malaysia may enhance the chances of chicken born

salmonellosis as commercially prepared foods, especially chicken, are significant risk factors for SE infections (Kimura *et al.,* 2004).

The Malaysian poultry industry is very well developed and has contributed 86% of livestock production and 75% of the ex-farm value of livestock industry (Hair-Bejo, 2010). It has shown growth of 51.4% during the period 2000 to 2008 and has been forecasted for the growth of 19.7% in 2012. Moreover, the Malaysian vision to become the halal food hub for about 2 billion Muslims around the globe and targeting an estimated US$547 billion per year halal food market will provide the new opportunities for the growth of poultry industry (MIDA, 2009).

All it urges for the effective control measures to control SE in poultry. It is obvious that any reduction in food borne diseases would depend upon an effective control of chicken contamination (Adak *et al.,* 2005). Recently SE has declined in England and Wales due to the control of *Salmonella* in chicken flocks (Gillespie and Elson, 2005). Also anymore growth of poultry industry including its international trade requires the *Salmonella* free poultry production (Majowicz *et al.,* 2010). Therefore, the control of SE in Malaysian poultry would play a vital role for the food safety, public health and bright future of poultry industry in the country.

The control and eradication programme of *Salmonella* in poultry has been started since 1992. It has been limited to good management practices and monitoring of *Salmonella*. The detailed study for the prevalence of various SE phage types and their behaviour in chicken is basic requirement to intervene the infection. In Malaysia very little is known about the behaviour of local SE phage types in chicken. So far no such study has been conducted to develop SE vaccines in

Malaysia. The research described was design to address the pathogen host interactions of various SE phage types isolated from Malaysian commercial poultry to provide valuable insights for future research work and develop the first local SE vaccine against the disease in poultry. This may serve as breakthrough in *Salmonella* control strategies and a milestone for future research on the subject.

The hypothesis of this study was:there are low and highly pathogenic SE phage types (SE PTs) isolated from commercial chickens in Malaysia with the highly pathogenic PTs have better ability to colonize and cause disease than the low pathogenic PTs, and the combination of inactivated SE PTs can give better protection against SE infections as compared to the single PT.

The objectives of this study were to:

1. determine the pathogenicity of different SE phage types isolates in SPF chicks.
2. determine the safety and efficacy of different inactivated single SE phage type in SPF chicks.
3. determine the safety and efficacy of the inactivated SE phage types isolates combinations in SPF chicks.

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