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# Production and immunogenicity of selected proteins of *Salmonella* Enteritidis

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SUMMARY

Over the past years, *Salmonella* Enteritidis (SE) has become the most prevalent serovars isolated in Canadian patients. Most cases in humans are associated with consumption of chicken meat, raw egg and related products. For controlling *Salmonella* transmission and infection in poultry, available commercially killed vaccines poorly stimulate mucosal immunity, while the use of live vaccines remains controversial. Therefore an oral subunit vaccine may be a solution. Five bacterial proteins were chosen as potential candidates and identified as Glyceraldehyde-3-phosphate dehydrogenase, Enolase, Lipoamide dehydrogenase, DNA protection during starvation protein and Elongation factor-Tu. Our objectives were to produce and purify these proteins and study their immunogenicity. The proteins genes were amplified and cloned into pQE-30 vector, then transformed into *Escherichia coli* M15 for expression. Purification was performed using FPLC. SPF laying hens were separated into 6 groups and injected intramuscularly 3 times at 16, 20 and 28 weeks of age. Five groups were injected with a single protein respectively while the sixth group was injected with PBS as control. Eggs were collected during the duration of the experiment and blood was collected when hens were sacrificed at 36 weeks of age. IgY was extracted from egg yolk and serum and IgA from egg white. Immunodot, westernblot and ELISA were used to evaluate the immunogenicity of proteins and antibody levels they induced. We found that these five proteins could stimulate production of specific antibody *in vivo*. GAPDH, Enolase and DPS induced higher antibody titer than LpdA and Ef-Tu.

Key words: SE, ST, vaccine, hen, IgY, IgA, ELISA

RÉSUMÉ

Au cours des dernières années, *Salmonella* Enteritidis est devenus les sérotypes les plus souvent isolés chez les patients canadiens, les cas étant liés à la consommation de viande de poulet et d'œufs crus. Les vaccins tués commercialement disponibles pour la volaille, stimulent mal l'immunité mucosale, tandis que l'utilisation de vaccins vivants reste controversée. Par conséquent, un vaccin sous-unitaire par voie orale peut être une solution. Cinq protéines bactériennes ont été choisies comme candidates potentielles et identifiées, soit Glyceraldehyde-3-phosphate dehydrogenase, Enolase, Lipoamide dehydrogenase, DNA protection during starvation protein et Elongation factor-Tu. Notre objectif a été de produire et de purifier ces protéines et de démontrer leur immunogénicité. Les gènes des protéines ont été amplifiés et clonés dans le vecteur pQE-30 pour expression dans *Escherichia* coli M15. La purification a été effectuée par FPLC. Des poules pondeuses SPF ont été séparées en 6 groupes et injectées par voie intramusculaire à different âges avec une des 5 protéines, ou le PBS chez le groupe témoin. Les œufs ont été ramassés pendant l'expérience et du sang a été prélevé à 36 semaines d'âge. Les anticorps IgY ont été extraits à partir du jaune d'oeuf et du sérum, et les IgA à partir du blanc d'oeuf. Des immunodots, westernblots et ELISA ont évalué l'immunogénicité des protéines et les niveaux d'anticorps induits . Nous avons constaté que ces cinq protéines pourraient stimuler la production d'anticorps spécifiques *in vivo*. GAPDH, Enolase et DPS ont induit des titres d'anticorps plus élevés que LpdA et EF-Tu.

Mots-clés : SE, ST, vaccin, poule, IgY, IgA, ELISA

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# ABBREVIATIONS

APC: Antigen presenting cell

ATP: Adenosine triphosphate

CD: Cluster of differentiation

CDC: Centers for Disease Control and Prevention

CFU: Colony forming unit

CHEP: Canadian hatching egg producer

CHEQ: Canadian hatching egg quality

CIPARS: the Canadian Integrated Program for Antimicrobial Resistance Surveillance

CMI: Cell-mediated immunity

CTL: Cytotoxic T cell

DC: Dendritic cell

DNA: Deoxyribonucleic acid

Dps: DNA protection during starvation prtein (DNA protecting protein)

EFSA: the Europe Food Safety Authority

EF-Tu: Elongation factor thermo unstable

ELISA: Enzyme-linked immunosorbent assay

FAE: Follicle associated epithelium

FPLC: Fast protein liquidchromatography

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

HACCP: Hazard analysis and critical control points

HRP: Horseradish peroxidase

IEL: Intraepithelial lymphocyte

IFN: Interferon

Ig: Immunoglobulin

IL: Interleukin

LpdA: Lipoamide dehydrogenase

LPS: Lipopolysaccharide

M cell: Microfold cell

MHC: Major histocompatibility complex

MLN: Mesenteric lymph nodes

NAD: Nicotinamide adenine

NESP: National Enteric Surveillance Program

NK: Natural killer cell

OD: Optical density

OFFSAP: On-farm food sfety assurance program

OMP: Outer membrane protein

PBS: Phosphate buffered saline

PHAC: Public health agency of Canada

PMN: Polymorphonuclear neutrophil

PT: Phage type

RNA: Ribonucleic acid

SCFA: Short-chain fatty acids

SCV: *Salmonella* containing vacuole

SPI: Salmonella Pathogenicity Island

T3SS/TTSS: Type three secretion system

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# INTRODUCTION

*Salmonella* spp. is one of the major causes of foodborne illnesses in humans, which remains a worldwide problem. Over the past years, the most prevalent serovars isolated in Canadian patients were *Salmonella* Enteritidis (SE) (CIPARS, 2007-2009). Human SE cases are most commonly associated with the consumption of contaminated eggs, egg products and more recently with poultry meat. For Salmonella infection in chickens, we have observed an increase in the prevalence of SE in samples from abattoir, retail meat and animal clinical isolates (CIPARS, 2007-2009). The Canadian egg industry has over the past years developed several programs to detect and limit SE contamination. However, even with good biosecurity protocols and various controlling programs, breeder and layer flocks still become infected with SE and can transmit it horizontally and vertically. In order to prevent SE contamination, vaccination has been suggested and various killed vaccines are commercially available. Although they were able to induce an immune response, these vaccines did not protect SE challenged hens from excreting the bacteria and even laying positive SE eggs at 55 and 65 weeks of age (Tran *et al.*, 2010). In fact, the cell-mediated response appears to play an important role in the resolution of *Salmonella* infection, presumably because *Salmonella* is an intracellular facultative pathogen. Unfortunately, commercially available killed vaccines poorly stimulate mucosal immunity, and live vaccine use remains controversial due to risk of virulence recovery and food safety concerns. Another vaccine strategy is the development of sub-unit vaccine orally administered using protein antigens. Thus subunit vaccine may then induce efficient stimulation of mucosal immune system to protect birds from *Salmonella*.

For this project, five candidate proteins have been chosen in our lab from previous work, based on their immune reactions to SE and ST whole-cell antigens as well as well

conservation in both *Salmonella* serovars. Their cell-surface expression and immunogenicity were also reported in other different species in different precious researches. These 5 proteins were identified as Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Enolase, Lipoamide dehydrogenase (LpdA), DNA protection during starvation protein (Dps) and Elongation factor-Tu (EF-Tu). In this project, based on biological and immunological characters of these selected proteins, we have the hypothesis: these proteins are immunogenic and able to induce immune responses in laying hens and antibody production in both sera and eggs (yolk and white). Our main objectives are to produce and demonstrate the immunogenicity of these immunoreactive proteins, with the ultimate objective of eventually developing a new subunit vaccine against *Salmonella* Enteritidis and *Salmonella* Typhimurium in laying and breeder hens. For the protein production, we need to use an optimal method to purify these proteins effectively once they are overexpressed in *E.coli* M15 cells, in order to ensure the satisfactory quality and purity of produced proteins. After production of these selected proteins, we will test if each protein is immunogenic i.e., able to induce specific antibody *in vivo* (as measured in eggs and sera) after immunization laying hens with each of them. If these proteins are demonstrated to be immunogenic to be capable of inducing specific antibody production, we will continue to have a closer look at specific antibody titer levels and changes post vaccination and maintenance of blood, egg yolk and egg white antibody titers.

# LITERATURE REVIEW

# 1 GENERAL INTRODUCTION OF *SALMONELLA*

Salmonellosis is an important zoonotic infection, and human salmonellosis causes widespread morbidity and economic loss. In recent years, some countries have observed a marked increase in the number of human cases (Hendriksen *et al.*, 2011). Much of this increase has been associated with poultry meat and table eggs consumption (Foley *et al.*, 2011). The predominance of *Salmonella* Enteritidis as a human pathogen has overshadowed other *Salmonella* serovars, many of which are capable of causing serious illness such as *Salmonella* Typhimurium DT 104 (Bohaychuk *et al.*, 2006; Foley *et al.*, 2008; Foley *et al.*, 2011).

The economic losses associated with human salmonellosis are not only associated with the cost of investigations, treatment, and prevention of illness but also may affect the whole chain of food production. Estimated annual costs for salmonellosis might have reached billions of dollars in the United States and Canada in the 1990s only (Sockett, 1991; Clark *et al.*, 2001).

Although primarily an intestinal bacteria, *Salmonella* is widespread in the environment and is commonly found in farm effluents, human sewage, and in any material subject to fecal contamination. Salmonellosis has been recognized in all countries, especially in the areas of intensive animal husbandry, such as poultry and swine production (Berends *et al.*, 1996; Akkina *et al.*, 1999). Although disease can affect all species of domestic animals, young and gestation animals are the most susceptible. However, most infected animals are asymptomatic carriers i.e. are infected without showing any sign of illness. The challenge in the food animal industry resides not only in the detection of these asymptomatic carriers to avoid

contamination of the food chain, but also mostly in preventing animals to becoming infected. Vaccination could therefore be an important tool in a prevention and control program.

## 1.1 Classification of *Salmonella*

*Salmonella* is a genus of rod-shaped, Gram-negative, non-spore-forming, predominantly motile enterobacteria with diameters around 0.7 to 1.5 μm, lengths from 2 to 5 μm, and flagella which grade in all directions (i.e., peritrichous). They are chemoorganotrophs, obtaining their energy from oxidation and reduction reactions using organic sources, and are facultative anaerobes. Most *Salmonella* produce hydrogen sulfide that is important for bacteriological isolation (Clark and Barrett, 1987). They are found worldwide in cold- and warm-blooded animals (including humans), and in the environment (Yue, 2012).

Genus *Salmonella* includes two species: *Salmonella* bongori and *Salmonella* enterica. *Salmonella enterica* is subdivided into 6 subspecies (*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*). *Salmonella enterica* subspecies *enterica* has 2610 different serotypes and *Salmonella* Enteritidis belongs to this subspecies, The serotypes are characterized by three surface antigens: the flagella "H" antigen, the oligosaccharide "O" antigen and the polysaccharide "Vi" antigen (found in Typhi and Paratyphi serotypes) (Bronze and Greenfield, 2005).

*Salmonella* serovars can be divided into two groups, those that are host adapted and others that are not. Host-adapted serovars are able to cause diseases in specific hosts, including *S.* typhi

and the paratyphoid *Salmonellae* (*S.* paratyphi A, *S.* paratyphi B, and *S.* paratyphi C) for human, and *S.* Choleraesuis (swine), *S.* Dublin (cattle), *S.* Abortusovis (sheep), *S.* Pullorum (poultry) and *S.* Gallinarum (poultry) for animals (Barrow, 1992; Selander *et al.*, 1992; Bolton *et al.*, 1999; Uzzau *et al.*, 2001). On the other hand, non-host-specific *Salmonella* serovars cause salmonellosis in humans and a wide variety of animal hosts as well. *Salmonella enterica* serovar Enteritidis (SE) belongs to this group, and have presented serious continuous challenge in food safety and poultry industry over the past years being responsible for significant health problems in human (Mølbak *et al.*, 2006).

## 1.2 Prevalence of *Salmonella*

### 1.2.1 Prevalence in human

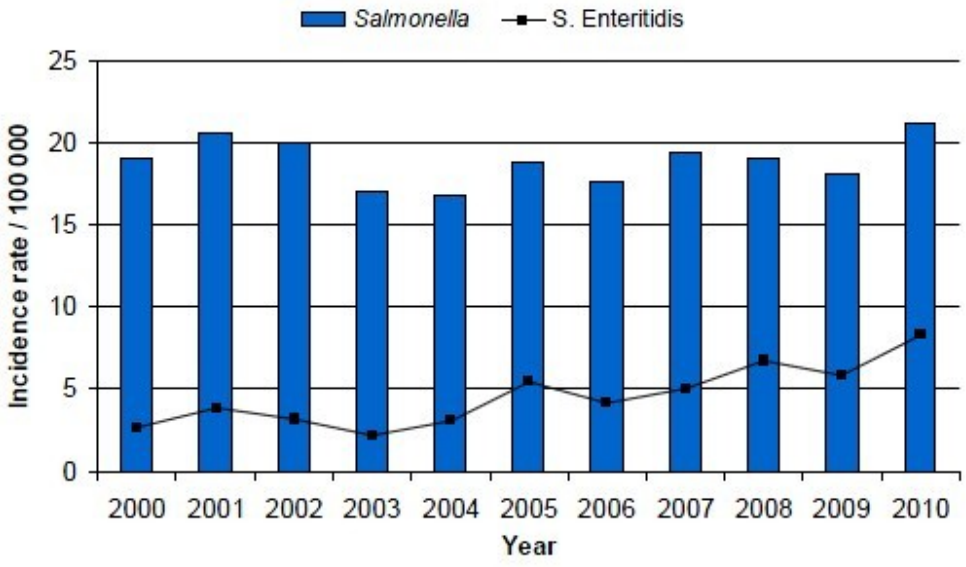
*Salmonella* is considered as one of the most common foodborne illness etiologic agent in human. In a recent CDC (Centers for Disease Control and Prevention) summary of USA, *Salmonella* Enteritidis (SE) was described as the most prevalent serotype responsible for 17.5% of all human salmonellosis (CDC, 2009). In the EU, SE is the serovar most frequently associated with human illness. In 2008, a total of 131,468 confirmed cases of human salmonellosis (notification rate 26.4 per 100,000 populations) were reported from 27 European countries. The total number of reported human salmonellosis cases in the EU has decreased steadily by several thousand cases annually since 2004, from 195,947 cases in 2004 to 133,258 cases in 2008 (EFSA, 2008). In 2009, the number of salmonellosis cases in humans decreased by 17.4 %, compared to 2008, and the statistically significant decreasing trend in

the European Union continued for the fifth consecutive year. In total 108,614 confirmed human cases were reported in 2009 and in particular, human cases caused by SE decreased markedly. It is assumed that the observed reduction of salmonellosis cases is mainly attributed to successful implementation of national *Salmonella* control programs in poultry (EFSA, 2009).

In Canada, from 2005 to 2010, *Salmonella* accounted for the most frequent cases in human when comparing with other foodborne pathogens, which include *Campylobacter*, *E. coli*, *Shigella*, *Vibrio*, etc. Based on the latest NESP (National Enteric Surveillance Program) annual report (2010), in all provinces of Canada, including Quebec, *Salmonella* caused much more human cases than other select major organism groups as (NESP Annual Summary, 2010). Among all the serovars of *Salmonella*, SE was one of the most important public health concerns. From 2005 to 2010, the ranking among the top three serovars remained unchanged with SE being the most frequently reported, followed by ST and *Salmonella* Heidelberg, (NESP Annual Summary, 2010). PT8 and PT13 were the predominant phage types found in human SE (CIPARS Annual Report, 2008).

In 2010 a record high of 2827 SE isolations were reported to NESP. SE was the most prevalent cause of human salmonellosis in Canada representing approximately 39% of all human *Salmonella* isolates reported in 2010. The proportion of salmonellosis cases attributed to SE has been steadily increasing over time, from 14% in 2000 to 39% in 2010 (Figure 1).

**Figure 1. Incidence rate of *Salmonella* spp. and SE as reported to NESP from 2000 to 2010 (NESP Annual Summary, 2010).**




1.2.2 Prevalence in chickens

In United States, the presence of SE was identified in 35% of layer flocks (end of laying period) from cecal sample collected in Northern US slaughterhouses between 1991 and 1995 (Ebel and Schlosser, 2000). The prevalence of environmental contamination in SE was 7.1% for 200 laying hens' farms in 15 states (Garber *et al.* 2003). Meanwhile, the high percentage of samples positive for SE was detected in ground chickens (0.46%, 8/1722 samples) and broilers (0.26%, 124/47090 samples) (White *et al.* 2007). In 2010, CDC investigated a multistate outbreak of SE infections in the United States, and a total of 3,578 cases in humans were identified from May 1 to November 30, 2010. According to the data from the investigations conducted by public health official in 11 states, the shell eggs were a likely source of this huge outbreak. Certain egg suppliers and farms conducted a nationwide egg recall after the outbreak was reported (CDC, 2010). This was the largest egg recall in American history. Throughout

the whole country, more than 500 million eggs were involved in the nationwide recall (FDA, 2010). Total costs to American shell-egg producers is not clear yet, however, the negative media attention produced a drop in prices that cost the shell-egg industry over $100 million in September 2010 only (Capturing Recall Costs Measuring and Recovering the Losses. 2011.).

In Europe, SE was one of the most predominant serotypes detected in laying hens and their eggs in 2008. Over the past 20 to 25 years, SE has been frequently isolated from environmental samples of egg-laying flocks when compared to other isolates of other serotypes (EFSA, 2007). The prevalence of SE and ST in laying flocks has increased from 0.5% (2005) to 2.3% (2006) and 3.2% (2007) (EFSA. 2009), even with the nations control programs in place. The EU has passed legislation that requires member states to actively work to reduce the presence of *Salmonella* in poultry flocks at all levels of production by setting up national control programs that must target specific *Salmonella* serovars (most regulations currently only cover SE and ST). This has resulted in a significant decrease in the prevalence of SE in broiler, layer and breeding flocks since 2008 (Keery, 2010).

In Canada, SE has become the third most important *Salmonella* serovar from chicken sources including samples from abattoir and retail meat, while the isolation rate, which was less than 1% in 2002, increasing to 7% in 2006 (CIPARS annual report, 2006; PHAC, 2007). SE isolated from chicken fecal samples has also increased sharply over the past five years, going from just less than 1% in 2002 to 13% in 2006 in the samples collected at slaughter (PHAC, 2007). In parallel, the increase has also been observed in broilers with successive increases observed over the last 3 years (2006: 20% 2007: 30% 2008: 40% percentage cultures positive)

(Middleton, 2009). In Canada, in 2008, in an abattoir surveillance program, *Salmonella* isolates were recovered from 27% (234/851) from chicken cecal samples, in which the SE was the second most common serovar (19%, 45/234) following *Salmonella* Kentucky (40%, 93/234). In Canadian retail meat surveillance, *Salmonella* isolates were recovered from 40% (382/960) of retail chicken samples, in which Kentucky (31%, 120/382), Heidelberg (20%, 78/382) and Enteritidis (16%, 62/382) were most frequent serovars. Also in 2008, in the province of Quebec, *Salmonella* was present in 42% (120/287) of retail chicken samples, and *Salmonella* Enteritidis was the third most prevalent (16%, 62/382) compared to all other serovars. Regarding surveillance of animal clinical isolates, including layer hens, broiler chickens, and their environment, the most common *Salmonella* serovar was Enteritidis, accounting 47% of all the 209 *Salmonella* isolates (CIPARS, 2008).

## 1.3 Source of *Salmonella*

### 1.3.1 Sources of animal infection

Poultry may acquire *Salmonella* infection from various sources, including parent birds, feedstuffs, rodents, wild birds, and other vehicles.

Wild animals provide a *Salmonella* reservoir, and are consequently a potential source for transmission of infection to domestic animals. Birds of all species, rodents, foxes, badgers, and other animals have been shown to be sources of *Salmonella* (Edel *et al.*, 1976; Euden, 1990; Evans and Davies, 1996). Insects, such as litter beetles and flies, have been observed to be risk

factors for the re-introduction of *Salmonella* into poultry houses after depopulating, cleaning and restocking the premises (Davies and Wray, 1995).

Henzler and Opitz (1991) demonstrated that the most important vectors of *Salmonella* transmission are rodents, especially mice. In 1992, they tested 2103 environmental samples and 715 mice and rats from five of the farms were rated as clean of SE and five as contaminated based on culture results of environmental samples for SE. On contaminated farms, SE was isolated from 24.0% of the mice and 7.5% of the environmental samples, which represented 75.3% of all *Salmonella* isolations from mice but only 18.0% of *Salmonella* isolations from environmental samples on these farms. SE was not detected in mice on clean farms. Rodents can be long-term sources of *Salmonella* infection: it was found that 3-week-old chicks can acquire infection via mice artificially infected with SE 2 and 5 months previously. Artificially and naturally infected rodents were found to excrete $10^4$–$10^6$ cfu/g in some individual droppings; while their droppings can be contaminated for up to 3 months post infection (Meerburg and Kijlstra, 2007). So it suggested that rodents should be included in all epizootiological studies of poultry production facilities as a risk factor.

*Salmonella* can contaminate feed, which is a potential source of *Salmonella* in animals entering the food chain (Guard-Petter 2001). When the feed is contaminated with the bacteria, the birds will get infected and potentially introduce *Salmonella* to the whole flock or their offspring. When infected birds defecate in open water drinkers, they will contaminate the drinking water. The open water suppliers are also easy to be contaminated by dust, rodents and other wild animals when they contact the water source.

Humans (such as farm staff, veterinarians, and visitors) and domesticated animals (such as cats and dogs) can also serve as vectors of *Salmonella* introduction into food animal flocks (Kinde *et al.*, 2005; Hoelzer *et al.*, 2011). Moreover, *Salmonella* may also be transmitted by airborne spread of aerosols from, for instance, manure, human waste dumps and contaminated water (Hardman *et al.*, 1991; Zongo *et al.*, 2010; Henrigues *et al.*, 2013). Moreover, contaminated working cloths and boots, cages and vehicles will cause potential cross contaminations between flocks, hatchery, and even chicken farms.

In addition, the presence of large amount of dust in poultry houses may also be a risk. Indeed dust has been recognized as a vehicle of transmission of *Salmonella* when large numbers of organisms are present and it could cause infection in flocks (Harbaugh *et al.*, 2006).

Once chickens get infected with the organisms in the surrounding environment, in addition to contaminate other birds, the bacteria will be excreted with feces and the infection will exist in reproductive organs and tracts as well. Egg might be infected either via its formation process in reproductive tracts or via penetration of bacteria in the feces through the egg shell, and the infection in the egg will be transmitted to offspring at last (Gantois *et al.*, 2009). Environmental contamination in hatcheries also can be a key factor of egg contamination (Skov *et al.*, 1999). Nest boxes, hatchers or hatchery trucks can lead to outer shell contamination (Schoeni *et al*, 1995). Contaminated eggshells have long been thought to lead to the spread of *Salmonella* in the hatchery (Cox *et al.*, 2000). Cox *et al.* (1990, 1991) found that breeder and broiler hatcheries were highly contaminated with *Salmonella*. In the broiler

hatchery, this contamination was detected on 71% of eggshell fragments, 80% of chick conveyor belt swab samples, and 74% of samples of pads placed under newly hatched chicks to gather feces (Cox et al., 2000). The presence of chicken manure and other moist organic materials facilitate the survival and growth of *Salmonella* by providing the required nutrients and physical protection (Gantois 2009).

### 1.3.2 Sources of human infection

Human most commonly gets *Salmonella* via ingestion of contaminated food. Food products derived from raw or undercooked eggs and poultry meat are the most important sources for human infection with *Salmonella*. Chicken carcasses may contain *Salmonella* either because animals are infected or because they were in contact with contaminated feces as well as possible cross-contamination from the slaughter or transport equipment during processing (*Salmonella* Surveillance: Annual Summary, 2006; Rasschaert *et al.*, 2008).

Unpasteurized milk and beef can be the common vehicles of food-borne infection too, and other foods cross-contaminated during preparation, storage or serving may be involved as well. An increase numbers of infections have also occurred following ingestion of contaminated uncooked vegetables, fruits, etc (Barak *et al*, 2005). Pork and pork products are also increasingly recognized as an important source of human salmonellosis (Jansen et al., 2007). Moreover, direct or indirect contact with animals colonized with *Salmonella* is considered to be another source of infection, including contact during visits to petting zoos and farms (Friedman *et al.*, 1998). Recently, turtles were reported as an important source of

*Salmonella* in humans, especially in children, and have caused several outbreaks in USA in 2012 (CDC, 2013). This has led to stricter regulations regarding the sales of these reptiles in pet shops. In the EU, Human SE cases are reported most commonly associated with the consumption of contaminated eggs and poultry meat (EFSA, 2008).

## 1.4 Transmission of *Salmonella*

*Salmonella* can be spread by horizontal transmission to other hosts including humans as well as vertical transmission, via an egg-associated (trans-ovarian) transmission to progeny.

### 1.4.1 Horizontal transmission

Chickens can get infected by *Salmonella* via various vectors in their surrounding environment and then horizontally transmit it to others. These vehicles include feed and water, as well as wild animals. In one flock, *Salmonella* can be transmitted via contaminated feces to individuals. Contaminated chickens become intestinal carriers, shedding the microorganism through their feces for long periods of time. In a study by Nakamura *et al.* (1993), shedding of SE persisted for more than 28 weeks after infection of newly hatched group-housed chicks in a seeder bird model. In the study of Van Immerseel *et al.* in 2004, both high dose ($10^9$ cfu) and very low dose ($10^2$ cfu) of SE resulted in persistent excretion for at least 18 weeks in chickens. Contamination will also occur between flocks as contaminated transport vehicle, introduction of the contaminated birds or flocks, and so on, may infect birds. Moreover, wild birds, mammals, rodents, insects etc. are generally regarded as the main reservoir for *Salmonella* in

the environment (Meerburg and Kijlstra, 2007).

### 1.4.2 Vertical transmission

The vertical transmission occurs when reproductive organs are infected with *Salmonella* by direct contamination of the yolk, albumen, eggshell membranes or eggshells before oviposition. This route was believed to be important in the large number of egg-associated outbreaks (Okamura *et al.*, 2001a, b; Gantois, 2009).

*Salmonella* is introduced to the egg from infected ovaries or oviduct tissue before the hen lays the egg (Keller *et al.* 1995). *Salmonella* can gain access to the peritoneal cavity, ovary and oviduct areas of an adult hen with resulting contamination of the structures of the egg, such as the yolk, membranes and shell, during ovulation and egg formation within the oviduct (Keller *et al*, 1995). Adult laying hens infected may carry the organism in their large intestines and shed it in their feces, which may lead to contamination of the eggshell surface then the contents of eggs. The eggshell structure provides several readily accessible sites, including shell surface, shell pore and the outer and inner shell membranes, for *Salmonella* to reside, often as a result of environmental contamination. This will lead to the laying of contaminated eggs, and these infected chicks will grow up to become pullets and subsequently lay contaminated eggs (Sanchez *et al*, 2002).

However, research has shown that 0 to 0.6% of the eggs with infected contents laying by hens having contaminated reproductive tract, which indicated that there might be factors within the

eggs that control the pathogen before the eggs are laid (Barrow and lovell, 1991; Keller *et al.*, 1995; Gast, 1994; Gantois, 2009). While it the proportion of infected eggs laid by infected hens could be greatly different when layers got experimental infected, which implied that oral-exposure doses of Salmonella Enteritidis for laying hens can significantly affect both the frequency and location of deposition of this pathogen inside eggs (Gast et al., 2013). Once in the egg, the albumin is not ideal to bacterial survival. The reproductive tract produces and incorporates into the albumen antimicrobial components that are growth restricting for *Salmonella*. The most well known are lysozyme and ovotransferrin. Lysozyme may affect integrality of the cell wall of Gram-negative bacteria by forming pores, and ovotransferrin may create an iron-deficient environment for bacteria to inhibit their growth and interact with the membrane and interfere with biological functions of the bacterial cytoplasmic membrane (Gantois *et al.*, 2009).

## 1.5 Infections of *Salmonella*

### 1.5.1 Infections in human

Most people are probably exposed to *Salmonella* from time to time, either from contaminated foods or from environmental sources. The incidence of *Salmonella* is especially high in infants and young children, elderly, and patients suffering chemotherapy and immunodeficiency, and this is because of either an immature immune protection or suppressed immune function (Sirinavin and Garner, 2000; Kendall *et al.*, 2003). Under certain conditions, this exposure leads to clinical infection, subclinical infection or asymptomatic carriage.

When human are infected with non-typhoid *Salmonella*, they will have diarrhea (sometimes bloody). Stomachache, fever, nausea, and vomiting are the most classic symptoms of gastroenteritis. In most of cases, salmonellosis symptoms will develop within 24-48 hours after exposure. In some other situations, patients may be asymptomatic and symptoms develop as late as 10 days after exposure (Mølbak and Neimann, 2002; Onwuezobe *et al.*, 2012).

### 1.5.2 Infection in chickens

Several factors can affect the susceptibility of poultry to *Salmonella* colonization. Young chicks are more susceptible to *Salmonella* infection and gut colonization from hatching to 96 hours of age because of immature immune system (Bohez et al., 2007). The stress from environment, transport and other diseases also may result in weak resistance to *Salmonella* infection. In other cases, some feed additives, such as antimicrobials and anticoccidials, may interfere with the inner-balance by killing gut microflora without purpose, which intern to destroy the integrity of intestinal mucosal protection. Salmonella would then take place of these microflora and get chance to colonize on the intestinal wall to facilitate the infection (Bailey, 1988; Foley, 2011).

Mortality rates in poultry infected with SE PT4 were 2% in broilers during the first 48 hours of life, with a cumulative mortality and morbidity rate of 6% and 20% respectively, at 5 days of age (McIlroy *et al.*, 1989). Affected young chicks may exhibit symptoms including anorexia, adypsia, depression, ruffled feathers, huddling together in groups, reluctance to

move, drowsiness, somnolence, dehydration, white diarrhea and stained or pasted vents (Baskerville *et al.*, 1992). Laying flocks are often clinically normal, despite the isolation of SE from fecal droppings, dust and litter (Hinton *et al.*, 1989; McIlroy *et al.*, 1989). However, clinical signs are sometimes found in laying hens. Salmonellosis in broilers due to ST infection has been characterized by growth retardation, blindness, twisted necks, lameness and mortality and cull rates that varied between 1.7% and 10.6% in flocks during the first 2 weeks of age (Padron, 1990).

Post-mortem lesions seen in chicks affected with salmonellosis may consist of dehydration, emaciation, an unresorbed or poorly resorbed yolk sac, and infection of the yolk sac with sometimes presence of necrotic debris. Lesions related to septicemia such as splenomegaly, hepatomegaly, necrotic foci and petechiation in the liver and spleen, various serositis such as airsacculitis, perihepatitis, pericarditis and peritonitis have been described in all type birds as well as lesions to the ovary and oviduct in mature birds. Watery intestinal contents and reddened areas of the mucosal surface of the duodenum, ileum and colon, typhlitis, with or without bloodstained or inspissated cecal cores have been reported, (Wray and Wray, 2000).

In newly hatched chicken, SE can cause diarrhea and septicemia with invasion and infection of a variety of internal organs including liver, spleen, peritoneum, ovary and oviduct (Lutful Kabir, 2010). When the animals become infected with SE, extensive interstitial edema of the lamina propria and the submucosa of the intestines can be observed within one day of infection, followed by a rapid influx of granulocytes and macrophages (Desmidt *et al*, 1996).

In this regard, the course of SE infection in young chicken resembles that in susceptible humans.

## 2 PATHOLOGY AND VIRULENCE OF *SALMONELLA*

*Salmonella* is orally taken up by the hen and enters the intestinal tract. Bacteria possess different strategies to attach and colonize the intestinal lumen are able to invade the intestinal epithelial cells. As a consequence, immune cells, more specifically macrophages, are attracted to the site of invasion and enclose the *Salmonella* bacteria. This allows the bacteria to survive and multiply in the intracellular environment of the macrophage. These infected macrophages migrate to the internal organs such as the reproductive organs.

### 2.1 Pathogenesis

#### 2.1.1 Getting to the intestinal gut

*Salmonella* is usually orally taken by hosts. *Salmonella* first need to pass the acidic environment of the proventriculus so that it can move into the small intestine. Thus bacterial resistance against these acidic conditions plays an important role in infection (Kwon and Ricke, 1998; Marcus *et al*, 2000). Two types of acid-tolerance systems have been described: one is activated on exposure to an acidic environment during log phase growth, which is regulated by the ferric uptake regulator (Fur) (Foster and Hall, 1996) and is activated by exposure to pH 5 with short life about 20 to 40 minutes (Rychlik and Barrow, 2005), and the

other one develops during stationary phase, which depends on the alternate sigma factor RpoS (Fang *et al*, 1992; Seshadri and Samuel, 2001). The log phase rpoS-dependent acid resistance also can be observed after at least 60 minutes of adaptation period (Rychlik and Barrow, 2005). Besides, PhoPQ and OmpR are also pH-response regulators (Bang *et al*, 2002). PhoPQ is a two-component signal transduction system present in *Salmonella*. The phoPQ-dependent acid tolerance response mainly protects *Salmonella* in inorganic acid environment. OmpR is central to the stationary phase-inducible acid tolerance, and its activity is induced to protect *Salmonella* survival in organic acid environment (Rychlik and Barrow, 2005).

### 2.1.2 Adhesion

After its successful passage through the stomach and upon entering the gut, *Salmonella* has to counterbalance the intestinal peristalsis and to ensure its adhesion for gut colonization. Adhesion to host tissues is a crucial step during pathogenesis: the first tight contact between host and microbe is a prerequisite for triggering distinct processes like biofilm formation or protein translocation that may then be followed by entry into the host cell and later systemic dissemination.

The various adhesion systems present in *Salmonella* have been organized into different categories. Fimbrial and non-fimbrial adhesins are the two major groups of adhesive structures (Soto and Hultgren, 1999), with the latter group including two adhesins (SiiE and BapA) from the type I secretion system (T1SS) and autotransported adhesins from the type V secretion system (T5SS). Additionally, several surface structures of *Salmonella*, whose main functions

are primarily not involved in adhesion, also contribute in part to the attachment and colonization of host tissues. These structures include flagellum, the Type III Secretion System (TTSS) and LP*S*. These factors will be described in the next section.

### 2.1.3 Invasion

*Salmonella* must be able to adhere to and invade the epithelial cell layer lining the intestine in order to cause enteritis and/or systemic disease.

The primary sites of invasion are the Peyer's patches, which contain specialized membranous epithelial cells (M cells) in the follicle-associated epithelium (FAE) that overlay aggregation of lymphoid cells. Experiments in mice suggest that bacterial entry and destruction of M cells play a major role in the invasion process in order to reach the Peyer's patches (Jones *et al*, 1994; Monack *et al*, 1996; Jensen *et al*, 1998). The M cells facilitate host colonization and are able to sample the bacteria from the lumen content and present it to the immune system. The FAE facilitating uptake of bacteria is helped by four features: low quantities of mucus which is associated with the absence of goblet cell, low concentration of secretary IgA (sIgA) since lacking of polymeric immunoglobulin receptors, and an inner glycocalyx as well as an irregular brush border (Jepson and Clark, 2001). *Salmonella* is capable of invading enterocytes as well as M cells, and dendritic cells (DC) and microphages have also been implicated in the transfer of *Salmonella* across the intestinal epithelium.

Invasion of the intestinal mucosa results in an extrusion of the infected epithelial cells into the intestinal lumen and a destruction of microvilli, which leads to a loss of absorptive surface. The invasion of epithelium causes structural damage to intestinal wall cells (Burkholder and Bhunia, 2009). Bacterial invasion of the M cells or enterocytes also elicits an acute inflammatory response in the host intestinal epithelium, characterized by the production of the proinflammatory cytokines, which stimulate the influx of polymorphonuclear leukocytes into the infected mucosa (Eckmann *et al*, 1993; Saarinen *et al*, 2002.). In addition, invasion of the M cells and enterocytes and the following inflammation may bring adverse consequences. Cell death and sloughing of the FAE provide new opportunities for bacteria to invade the submucosal tissues (Jepson and Clark. 2001).

The molecular mechanisms by which *Salmonella* modulates the intracellular trafficking remain largely to be defined, but it has been demonstrated that *Salmonella* can invade non-phagocytic cells through its type III secretion system (T3SS-1), which induces a Trigger entry process (Jantsch *et al*, 2011), which is characterized by dramatic cytoskeletal rearrangements and the apparition of large membrane ruffles at the bacterial entry site (Velge et al., 2012). However, it was demonstrate that SPI-1 facilitates systemic infection but is not essential for invasion and systemic spread of the organism in chickens (Desin et al., 2009), since Salmonella is able to induce Zipper entry system via outer membrain protein Rck to invade cells (Rosselin et al., 2010). The Rck invasin expressed on *Salmonella* outer membrane interacts with its receptor on the host cell membrane, leading the invading bacteria are tightly bound to the host cell membrane, and only minor cytoskeletal protein rearrangements are initiated by specific contact between bacterial ligands (invasin) and host cell surface receptors

(Velge et al., 2012). Zipper entry system is a T3SS-independent invasion mechanism, which implys that SPI-1 is not unique factor for Salmonella cell invasion any more (Rosselin et al., 2010).

### 2.1.4 Infection/dissemination

The bacteria can be taken up by macrophages, survive and then be carried as engulfed bacteria to systemic sites through the lymphatic system (Richter-Dahlfors *et al*, 1997). Survival of *Salmonella* within macrophages is generally considered to be essential for the translocation of bacteria from the gut-associated lymphoid tissue to the liver and spleen (Gantois *et al*, 2009).

Once *Salmonella* has breached the epithelial barrier, it comes into contact with cells of the reticuloendothelial system, in particular resident macrophages that are intimately associated with M cells. *Salmonella* can proliferate in epithelial cells and non-activated macrophages. The bacteria are demonstrated to primarily replicate in macrophages, as it is found in the lymphatic tissues and organs during systemic infection (Jantsch *et al*, 2011).

It has reported that SPI-2 is essential for the intracellular survival and replication of the bacteria. SPI-2 carries genes that encode for a second Type III Secretion System (TTSS-2) that is structurally and functionally distinct from the TTSS that is encoded by SPI-1 mediating invasion (Jantsch *et al*, 2011). Via TTSS-2, *Salmonella* may deliver proteins into *Salmonella*-containing-vacuoles or through the vacuolar membrane into the host cytosol. This process

influences intracellular trafficking and contributes to the intracellular survival of *Salmonella* in macrophages (Uchiya *et al*, 1999).

After host cells invasion, *Salmonella* can survive and replicate within a modified phagosome known as the *Salmonella*-containing vacuole (SCV) with an active modification (Steele-Mortimer, 2008; Jantsch *et al*, 2011). It is documented that the avoidance of phagolysosomal fusion is unlikely to be a major pathogenic strategy of *Salmonella*. Studies in various cell types also demonstrated that the vacuole acidifies; however, depending on the mechanism of host cell entry, vacuolar acidification may be delayed in both macrophages and epithelial cells (Jantsch *et al*, 2011). In addition, the ability of *Salmonella* to survive exposure to lysosomal contents is mediated by its resistance to antimicrobial peptides, nitric oxide, and oxidative killing, and these features are important for its survival within macrophages and to virulence (Jantsch *et al*, 2011).

### 2.1.5 Metabolic adaptation

During the various stages of an infection, *Salmonella* encounters a variety of environmental challenges, such as nutrient starvation, oxidative stress and digestive enzymes. *Salmonella* is equipped with a series of adaptive mechanisms that enable it to survive these challenges. Apart from the various tightly controlled *Salmonella* pathogenicity islands (SPI) that function at various stages of the infection, a sophisticated regulation of bacterial metabolism appears to exist. One group of genes that play a role in metabolic adaptation is the starvation stress response genes, called “starvation-stress response genes” (Spector, 1998). These genes encode

for metabolic functions that are required for *Salmonella* to survive in the starving host environment during infection and thus can be considered as determinants of virulence. The identification of genes that are required for bacterial survival at a certain stage of infection deserves more attention as they may provide opportunities to develop attenuated strains with vaccine potential.

## 2.2 Virulence factors

### 2.2.1 *Salmonella* Pathogenicity Island (SPI)

Many virulences of *S. enterica* are encoded by genes on *Salmonella* Pathogenicity Island (SPI). At present, 12 different SPI have been described (Hensel, 2004). The major SPIs of SE include SPI-1, SPI-2, SPI-3, SPI-4 and SPI-5. SPI-1 and SPI-2 have been studied most frequently. Salmonella pathogenicity islands 1 and 2 (SPI-1 and SPI-2) each encode a specialized type III secretion system (T3SS) that enables Salmonella to manipulate host cells at various stages of the invasion/infection process (Winer et al., 2010). The SPI-1 encoded T3SS can induce cytoskeletal rearrangements resulting in the uptake of *S. enterica* even by host cells so that it is required for the transport of *S. enterica* proteins across the cytoplasmic membrane of a host cell into its cytosol (Kaniga et al., 1995). The effector proteins encoded within SPI-1 are translocated into the host cell cytoplasm through the secretion apparatus. In particular, AvrA, SipABCD, SopE, SopE2, SopB, and SopD are translocated into the host enterocyte by the secretion machinery encoded by SPI-1. These effector proteins orchestrate the cytosol changes that result in uptake of *Salmonella*. It is believe that SPI-1 is essential for

Salmonella invasion host cells until recent that some studies demonstrate that SPI-1 is not necessary for the cell invasion anymore but can facilitate the rapid host cell invasion (Desin et al., 2009). SPI-2 encoded T3SS is required for the transport of *S. enterica* proteins across the phagosomal membrane (Cirillo et al., 1998; Hensel et al., 1998) and increase intracellular survival (Hensel *et al*, 1995; Vazquez-Torres and Fang, 2001). Hensel *et al.* (1998) and Cirillo *et al.* (1998) have demonstrated that SPI-2 is required for survival in host phagocytes. Subsequent research by Vazquez-Torres *et al.* (2000) suggests that SPI-2 may interfere with trafficking of the NADPH oxidase to *Salmonella*-containing vacuoles thereby preventing phagocyte-dependent oxidative killing. It has been also recently suggested that SE SPI-2 T3SS facilitates invasion and systemic spread in chickens, although alternative mechanisms for these processes appear to exist for the systemic spread levels of SPI-2 mutants could match that of the wild-type strain (Winer et al., 2010). SPI-3 genes are involved both in gut colonization due to MisL-dependent fibronectin binding and intracellular survival due to high-affinity magnesium transport encoded by *mgtABC* (Smith et al., 1998; Dorsey et al., 2005). SPI-4 genes are required for the intestinal phase of disease by coding for non-fimbrial adhesin (Morgan et al., 2004), and the genes localized in SPI-5 are co-regulated with either SPI-1 or SPI-2 genes and therefore code for effector proteins transported by either of these T3SS (Knodler et al., 2002). However, the vast majority of this information has been obtained in a mouse model and ST, and much less data are available for SE or poultry although poultry in particular represent major reservoirs of SE.

### 2.2.2 LPS

LPS of *Salmonella*, which is made of lipid A, the core oligosaccharide chains and O polysaccharides, is a major component of the outer membrane and an important toxin that interacts with the host immune system to induce inflammation and produce septic shock, fever, and death.

*Salmonella* can modulate the structure of the O-antigen as a means of dampening host innate immune responses, and an action that presumably enhances the microorganism's ability to persist and survive in the host (Ernst *et al*, 2001). The lipid A has a potential biological activity that is able to cause pathophysiological effects, tells the endotoxic shock, pyrogenicity, complement activation, coagulation changes and hemodynamic changes. Lipid A contributes to the pathogen or toxic activity of *Salmonella*. LPS is considered a component that has the capacity to stimulate cytokine synthesis (Henderson *et al*, 1996).

### 2.2.3 Flagellin

Flagellin composes protein subunits of flagella. Flagellin is typically diphasic in *Salmonella*. The availability of two genetic systems (genes distantly located on the chromosome) expressing different flagellins could help the microorganism to survive the host's defenses.

Flagella exist in two forms termed antigenic phase 1 and phase 2 and H antigens are then two-phase (Popoff, 2001). SE antigen H is called a single-phase (phase 1: g, m), while ST has both type of antigen H (phase 1: i and phase 2: 1,2). Research has shown that flagellin g, m is highly antigenic. This protein is extracted in relatively pure form from the surface of

*Salmonella*. Flagellin (g, m) is used commercially for the serological analysis of serogroup identification because the production of this antigen is relatively easy. This distinguishes the SE infection and those of other strains of *Salmonella* (McDonough *et al*, 1998).

### 2.2.4 OMP

OMPs interface the cell with the environment, thus representing important virulence factors with a significant role in the pathobiology of gram-negative bacteria and bacterial adaptation (Hamid and Jain, 2008). The OMPs of gram-negative bacteria are immunologically important because of their accessibility to the host defense system. OMPs (82.3 and 75.6 kDa) were shown to be involved in attachment of *Salmonella* Enteritidis to intestinal epithelial cell lines (Fadl *et al.*, 2002). SE membrane usually contains three major proteins of the outer membrane: OmpC (36 kDa), OmpF (35 kDa) and OmpA (33 kDa). A Canadian study (Poppe *et al*, 1993) with 318 SE isolates primarily from poultry and their environment showed 35 of 36 strains had the same profile of OMPs (42, 40 and 37 kDa). The expression of OMPs in SE can be significantly influenced by conditions of growth of the bacterium (Chart *et al*, 1993). Chart et al. (1993) found that the expression of iron regulated OMPs: 74, 78 and 81 kDa was induces when SE is growing in trypticase soy broth containing ovotransferin. Recently, outer membrane protein of SE Rck was reported can induce a Zipper enter system to help the bacterial invasion to host cells (Rosselin et al., 2010).

## 3 CHICKEN IMMUNE RESPONSES

Chicken immune system is divided into two types of immunity – innate and adaptive. Innate immunity such as physical barriers and chemical barrier prevents the entry of pathogens. Meanwhile, adaptive immunity takes over when innate immunity fails to stop an invading pathogen. Adaptive immunity involves targeted recognition of specific molecular features on the surface of a pathogen, resulting in a series of events intended to eliminate that pathogen and establish protection to subsequent challenges. Although innate immunity is effective, this response is normally unable to fight against pathogens and prevent disease completely. On the other hand, acquired immunity not only can protect birds against pathogens, but also will provide more rapid and effective protections when the host gets infected with the same pathogens again. Adaptive immunity can be further divided to humoral and cell-mediated immunity.

## 3.1 Innate immune responses

Innate responses are considered important in the earliest phases of microbial invasion, to rapidly limit the spread of the pathogen very rapidly until adaptive responses become mobilized to clear the infection. Also, the innate and adaptive responses are highly integrated. The earliest pathogen recognition events that occur in the body lead to recruitment and enhancement of innate responses, as well as activation of the adaptive immune system (Davison *et al.*, 2008).

### 3.1.1 Constitutive barriers

Innate immune responses are important in controlling the early phases of infection with *Salmonella*. When *Salmonella* is infected orally, it will enter gastrointestinal tract to try to attach and colonize the epithelial cells and in turn to cause further infection. In the gastrointestinal tract mucosa, there are several physical and chemical defenses to help to stop the bacteria.

There is also mucus lining on the intestinal epithelium. It is a mixture of glycoproteins produced by goblet cells whose viscous slimy consistency can trap bacteria and prevents them from reaching the surface of the epithelial cells. In addition, mucosal cells are constantly being replaced and old cells are ejected into the lumen, which is also helpful to stop bacteria reaching and colonizing the intestinal epithelia (Salyers *et al.*, 2011). Mucus also possesses proteins that have a certain antibacterial activity. One example is the lysozyme, which can digest the Gram-negative cell wall if breaches in the outer membrane are made by membrane-disrupting substances, such as the bile salts found in the intestine. Another example is the lactoferrin, an iron-binding protein that sequesters iron and deprives bacteria of this essential nutrient.

The epithelium lining the intestinal tract consists of tightly packed cells which are attached to each other by protein structures called tight junctions. The tight binding of epithelial cells to one another prevents bacteria from transiting through the epithelial layer. To get through the epithelium, bacteria must either take advantage of breaches caused by wounds or be capable of invading epithelial cells, passing between them or passing through them to get to underlying tissue.

Antimicrobial peptides (AMP) are important components of the natural defenses and have been isolated from most living organisms. They will form pores in the membrane of bacteria and fungi leading to cell death to eliminate the further infection. Defensins are toxic peptides isolated from most living organisms, and only β-defensins exist in chickens (Xiao *et al.*, 2004). They kill bacteria by forming pores in their membranes and collapsing the proton motive force that is essential for bacteria survival. In the crypts of the intestinal mucosa, defensins offer presumably protection to the intestinal stem cells, which divide constantly to replenish the intestinal mucosa to eliminate bacterial adhesion (Salyers *et al.*, 2011). It was also reported that β -defensin antimicrobial peptides might play a role in intestinal epithelium and vagina immune responses against *Salmonella* Enteritidis (Derache et al., 2009; Anastasiadou et al., 2013).

### 3.1.2 Heterophils

Heterophils are the avian Polymorphonuclear leukocytes (PMN) that are an essential component of the innate immune system. Heterophils actually contribute to host resistance against *Salmonella* infection (Swaggerty *et al.*, 2005). Heterophils in poultry are equivalent to the neutrophils in mammals and are important mediators of natural resistance during bacterial infections (Davison *et al.*, 2008). Because of their early response and their ability to kill pathogens, heterophils are considered a biomarker for assessing the competence of innate immunity in poultry (Swaggerty, 2003). In chickens, heterophils accumulate in the propria mucosae of the caeca within 18 hours after an experimental infection with a SE field strain

(Van Immerseel, 2002). During infection, *Salmonella* can be rapidly detected and killed by the various functions of heterophils (Kogut *et al.*, 1994; Kogut, 2001). Detection of bacterial Toll-like receptors stimulates heterophil phagocytosis and oxidative burst (Kogut et al. 2001; Farnell et al. 2003) and induces expression of pro-inflammatory cytokines (Kogut et al. 2005). Antimicrobial substances contained in heterophil granules can be released through degranulation to kill phagocytized bacteria (He et al. 2005). It has also been implied that heterophils play a central role in protecting the host against colonization and invasion of intestine mucosa (Kogut *et al.*, 1994; Van Immerseel, 2002).

### 3.1.3 Phagocytes

Phagocytes include immature dendritic cells, monocytes and heterophils that ingest and kill bacteria. They are able to defend the blood and tissue once the bacteria breach the epithelial surface successfully. When bacteria encounter the phagocyte, they are first engulfed by endocytosis into a phagosome. Fusion of phagosomes and lysosomes to form the phagolysosome releases toxic lysosomal enzymes and proteins that kill most bacteria. Debris from dead bacteria is then released by exocytosis (Salyers *et al.*, 2011). The oxidative burst of phagocytosis activate products are reactive oxygen (ROI) and reactive derivatives of nitrogen (RNI) such as chloramines, hydrogen radicals, and the hydrogen peroxide. ROI are necessary to protect against *Salmonella*. These compounds kill bacteria very efficiently within macrophages (Raupach and Kaufmann, 2001).

Assessing the ability of heterophils and monocytes in phagocytosis revealed that heterophils phagocytose more SE than monocytes do (Stabler *et al.*, 1994). In addition, heterophils can kill intracellular *Salmonella*, while the majority of non-opsonized *Salmonella* survive within monocytes. Therefore, heterophils are capable of killing bacteria more effectively than monocytes (Stabler *et al.*, 1994; Swaggerty *et al.*, 2005).

## 3.2 Adaptive immune responses

### 3.2.1 Antigen presentation

B lymphocytes express surface immunoglobulin (Ig) molecules with great specificities for antigens, and T lymphocytes recognize processed antigens on antigen presenting cells (APCs). Upon binding of an antigen to B cells expressing surface immunoglobulin (Ig), cell division and clonal expansion ensue and Igs with identical antigen specificity are secreted from the differentiated B cells. In contrast, T cells only recognize small fragments of antigens in association with MHC molecules that have been processed by APCs (Lillehoj and Trout, 1996).

Immunization with antigens through the gut induces the production of local antibody and cellular responses. The nature of the antigen influences the mode of antigen uptaking, processing, presenting, and the type of APC. Dendritic cells, macrophages, and epithelial cells are representative of APCs in the gut. In addition, Peyer's patches are critical to initiate antigen-specific immune response to pathogens capable of penetrating M cells, which may

pinocytose and phagocytose both soluble and particulate (e.g., viruses and bacteria) antigens in the lumen of the gut (Cerutti and Rescigno, 2008). The M cells can present antigen to underlying lymphoid cells, leading to the sensitization of lymphoid cells present in distinct T and B-cell zones in the PP (Lillehoj and Trout, 1996).

### 3.2.2 Humoral immune response

The humoral immunity mediated by antibodies produced by B cells. There are three classes of antibodies that are produced in the chicken after exposure to a pathogenic organism: IgM, IgY (IgG), and IgA (Lipman *et al.*, 2005). IgY is detected after 5 days following exposure, peaks at 3 to 3 1/2 weeks, and then slowly decreases (the method of antibody detection was not mentioned, while ELISA is widely used for antibody analysis) (Esatu et al., 2012). IgYs are the most important protective sera antibody in the chicken and is measured by most serological test systems. Tran et al. (2010) found that high titers of serum IgY could not be associated with reduction of intestinal SE burden after an experimental challenge. This suggested that during *Salmonella* infection, IgY protection might not as important as IgA protection. IgA and IgM are the predominant Igs in the local intestinal mucosa. IgM appears after 4-5 days following exposure to a disease organism and then disappears by 10-12 days. It is effective in elimination of microbes (Lillehoj and Trout, 1996). IgA appears after 5 days following exposure. Secretary IgA can prevent environmental antigen influxing into internal body compartments, neutralize viruses and microbial toxins, and prevent microbial pathogens adhering and colonizing mucosal surfaces, as well as facilitate antigen catch by binding to M cells (Lillehoj and Trout, 1996; Cerutti and Rescigno, 2008).

The B cells respond by producing antibodies after day 5 following bacteria exposure via macrophages. The lag period occurs because the B-cells must be programmed and undergo clonal expansion to increase their numbers. If the chicken is exposed a second time to the same disease, the response is quicker and a much higher level of antibody production occurs (memory immune responses) (Chalghoumi *et al.*, 2009) which is the concept for vaccination. Antibodies do not have the capability to kill bacteria directly. To respond to bacterial infection, antibodies in serum need to activate the complement to mediate their antibacterial effect, which are opsonophagocytosis and direct bacterial lysis. In the mucosal surface, on the other hand, since IgA cannot activate the complement system, its major role is neutralization of antigens by binding to bacterial surface antigens and preventing the cells from attaching to their targets on epithelial cells.

Because *Salmonella* is intracellular bacteria, antibodies are unlikely to protect the host against the intracellular stage of infection (Lillehoj et al., 1996). Cell-mediated response is therefore necessary to lyse infected cells. Only when the bacteria are released into the extracellular environment, the antibodies can then they participate in the elimination of bacteria (Erf, 2004). So, to fight *Salmonella* infection, cell-mediated immune response is more important.

### 3.2.3 Cell-mediated immune response

For endogenous antigens or intracellular pathogens, the cell-mediated immunity is the functional aspect of the avian immune system that works to destroy the infected cell to expose pathogens then to kill them.

Examples of actions by cell-mediated responses include activation of macrophages, cell lysis by cytotoxic T lymphocytes and natural killer (NK) cells, all mediated by cytokines released by T helper cells or other cells (Lillehoj and Trout, 1996). Cytokines are chemical messengers that coordinate the interactions between immune cells as one of their extensive functions. Cytokines are crucial stimulators of the initiation and maintenance of the immune response and play a role as effector molecules themselves to impact the duration and strength of the response (Kogut, 2000).

T lymphocytes are the antigen specific cells in the cell-mediated immunity（CMI） response, capable of recognizing a wide range of pathogens. T lymphocytes are subclassified by surface markers and receptors. All T cells express a CD3 complex on their cell surface, independent of the T cell receptor presenting. T helper cells are typically identified by CD4 surface markers, serving primarily a regulatory role in adaptative immunity, both cell-mediated and humoral. T helper cells function to activate macrophages by secretion of cytokines and stimulate B cell growth and differentiation. Cytotoxic T lymphocytes (CTLs) can be identified by typically having CD8 on their surface and are important in lysis of intracellular pathogen infected cells and tumor cells (Moser and Leo, 2010). CTLs recognize foreign antigens in the context of MHC class I molecules and initiate the cyctotoxic effects, whereas helper T cells recognize antigens in association with MHC class II molecules and differentiate to Th1 and Th2 with the

effects of different cytokines, then in turn to stimulate the cytotoxic activation of macrophage and CTLs, and secrection of antibodies, respectively (Lillehoj and Trout, 1996; Tizard, 2009).

NK cells have been postulated to play an important role as a primary host defense mechanism against tumors, bacteria, and viruses, as well as in the homeostasis of normal tissues (Herberman *et al.*, 1978). The observation that chicken intestinal intraepithelial lymphocyte (IEL) contain NK cells that mediate spontaneous cytotoxicity (Chai and Lillehoj, 1988) suggests that NK cells may play an important role in local defense (Lillehoj and Trout, 1996).

### 3.3 Local immune response for *Salmonella*

The local immune system comprises T cells, a large number of B and plasma cells. The mucosa-associated lymphoid tissue (MALT) has special structures responsible for the first line of defense to the mucosal surface. With MALT pathogens are limited to adherence to the epithelium and intestinal colonization. Peyer's patches (PP) are more inductive site for IgA responses to pathogens and antigens ingested in the gastrointestinal tract (Lillehoj et al., 1996).

The initiation of *Salmonella* infection appears originally in the mucosal surface where a humoral immune response usually occurs after infection. The immunoglobulin (Ig) is the predominant local secretory IgA, although the responses of IgY and IgM can also be observed. IgA is synthesized locally by plasma cells and is secreted through the mucous membranes and are present in secretions (Tizard, 2009). IgA acts like inhibiting the adhesion of

microorganisms to the surface of mucosal cells, thereby preventing the entry of microorganisms in body tissue (Basset, 2003).

Ovary and oviduct are responsible for vertical transmission of pathogens. SE can be isolated from the mucosal surface of the oviduct (Hoop and Pospischil, 1993) and persists in reproductive tissues of naturally or experimentally infected chickens (Hoop and Pospischil, 1993; Keller et al., 1995). Direct bacterial contamination of egg yolk or albumen Salmonella before shell formation following reproductive organs' infection and bacterial penetration through the freshly laid eggshell (Barrow et al., 1991) were observed. It is interesting that the SE infection significantly induces the proliferation of B and $IgA^+$ cells increased rapidly secretary IgA in the reproductive organs (ovary and oviduct) (Withanage et al., 1998; Withanage et al., 1999). A correlation between the increase of specific antibodies to SE and decrease the number of bacteria in the ovary and oviduct was observed (Withanage et al., 1999) and the antibody titers of the oviduct titles were the same as those of the serum. Therefore, the local immunity in the oviduct is also very important. It has been reported oviduct is the site where maternal antibodies are transferred to eggs and the presence of IgA and IgM (egg white) and IgG (yolk) (Kovacs-Nolan *et al.*, 2012). These antibodies could therefore provide newborns temporary protections before their own immune system fully developed.

# 4 CONTROL AND PREVENTION PROGRAMS FOR *SALMONELLA*

## 4.1 Controlling programs

### 4.1.1 The European Union (EU)

The European Union (EU) has required member states to actively work to reduce and eliminate the presence of *Salmonella* in poultry flocks at all levels of production by setting up national control programs (European Union, 2008). These national programs target specific *Salmonella* spp. currently including SE, ST, *S.* Virchow, *S.* Infantis and *S.* Hadar (European Commission, 2010). Programs are unique to each state and to be all-inclusive, covering primary production, animal feed production and processing and preparation of foodstuffs for human consumption. The National Control Program (NCP) was introduced in the United Kingdom (UK) in 2007 and began in February 2008. The goal for breeders was to have no more than 1% of all breeding flocks test positive for *Salmonella* of public health significance (SE, ST, *S.* Virchow, *S.* Hadar and *S.* Infantis) by the end of 2009, and for broilers is to have less than 1% prevalence of SE or ST in all broiler flocks by the end of 2011 (DEFRA, 2008). Any samples testing positive for SE or ST in chickens must be reported and submitted for serotyping to the National Reference Laboratory (DEFRA, 2007a and 2008). Sampling protocols vary significantly for the different NCP programs. Chicken breeding flocks are tested three times before going into lay, and boot swabs or fecal samples are then collected every two weeks while in the flock is in lay. Official sampling occurs three times during the lay cycle or when a suspect positive case is detected. Any breeder flock testing positive for SE or ST is destroyed along with any eggs at the hatchery, and the hatchery is inspected to ensure that no spread of bacteria has occurred. If, however, the test is positive, the producer must make a plan with a veterinarian on how to reduce or eliminate the infection, but no chickens or

eggs are destroyed. Table egg chickens have to be tested once as day old chicks, once as pullets two weeks before they go into lay, and again at 22-26 weeks of age (DEFRA, 2007b). The hens are then tested every 15 weeks while in lay. If SE or *S.* Typhimurium is detected and confirmed in a flock, the eggs produced are required to be heat treated, and they will not be sold in the table egg market.

### 4.1.2 The United States

In the United States, the FDA produced a final rule for SE control in table eggs coming effective in July of 2009 (FDA, 2010). The prevention measures include buying pullets from SE monitored facilitie. SE monitored is defined as either a flock that is certified as US SE Clean or a flock between 14 and 16 weeks of age from which barn environmental samples are negative (FDA, 2010). The SE testing protocol for table eggs involves testing pullets at 14 to 16 weeks of age, taking environmental samples from hen barns at 40 to 45 weeks of age and testing the environment of any flock that is molted 4 to 6 weeks after the molt is completed (FDA, 2009). Current policy for testing procedures involves producers submitting swabs of manure from each row/bank of each barn (FDA, 2008). If any of the samples tested positive, the producer is required to review the control protocols for any shortcomings and then must decide between diverting eggs to the breaker for the rest of the lifespan of the flock or submitting eggs for testing (FDA, 2010). Egg testing involves submitting a sample of at least 1,000 eggs at two-week intervals for serological testing. If four consecutive tests come back negative no further testing must be done. If an egg sample in one test is positive, it is mandatory to divert all production to the breaker, with a producer only being allowed to return

to the table egg market provided four egg tests in a row are negative. Even then the producer must submit eggs for testing once a month for the duration of the life of that flock.

### 4.1.3 Quebec

In Canada, the controlling programs are varies among different provinces. The program in Quebec is considered unique in Canada, even in the entire North America. After a SE PT4 outbreak in Northwest Quebec in 1996 which caused more than 150 patients to be infected and 2 patients to die, the Quebec Egg Board decided to take steps to develop a comprehensive Food Safety Program involving all partners from the industry and various governmental agencies. To lower the risk of contamination, the producer must contract the services of professional exterminator to eliminate rodents or any other potential vector from the laying barn and must supply proof that the replacement pullets are SE negative, and must agree to have the environment of his laying barns tested by the Quebec Egg Board at intervals as determined in the program. Environmental samples are taken between 2-5 weeks and between 12-16 weeks of age, and four tests during lay period of SE-free flock (environmental samples) are to be done. If a flock tests positive for SE, all the eggs will be destroyed and the flock will be depopulated. This SE surveillance program is developed and managed by producers and recognized by governmental agencies. It has the highest sampling frequency in North America. All these factors make the SE surveillance program in Quebec effective and unique in Canada and North America.

### 4.1.4 Other provinces in Canada

To control *Salmonella* infection, all other provinces in Canada uses CHEQ program and SC-SC (Start Clean, Stay Clean), SC-SCP (Start Clean, Stay Clean: Pullets) programz. CHEQ program (Canadian Hatching Egg Quality) is created by The Canadian Hatching Egg Producers (CHEP), which focuses primarily on biosecurity and proper egg handling and storage in broiler breeder operations as well as controlling pests, poultry health and cleaning and disinfecting protocols. "Start Clean, Stay Clean" (SC-SC) is the On Farm Food Safety Assurance Program (OFFSAP) developed for the Canadian egg industry. The stated aim of the SC-SC™ program is to prevent or reduce chemical and biological contamination in the production unit environment, the pullet, the hen and the egg. The SC-SC™ program works to mitigate food safety risks and focuses on preventative measures that can be applied to all egg production system scenarios. "Start Clean, Stay Clean: Pullets", or SC-SCP for short, is an OFFSAP program that is based on HACCP principles. The program focuses on good management practices and critical control point issues associated with inputs and process steps in pullet production. In addition to these common programs, some provinces have their own unique features. In British Columbia, mandatory industry led biosecurity program for all poultry producers. Alberta has emergency response plan for all poultry producers in case of a disease outbreak. In Manitoba, egg farmers have egg quality program. In Ontario, Egg Farmers of Ontario (EFO) require vaccination of replacement pullets going to farms previously positive for SE and all SE positive pullet flocks to be slaughtered and voluntary supply flock testing program with follow up protocols including treatment and depopulation.

With increased consumer demands for safer products, the poultry meat industry should consider such a program and design strategies at all levels of production to suit this request. All steps of production must participate in a *Salmonella* control program if reduction is to be achieved.

## 4.2 Biosecurity methods

Good biosecurity measures are key components of *Salmonella* control programs to avoid the introduction of this pathogen onto the farm or to reduce or eliminate infection pressure. The importance of preventing the introduction of *Salmonella* through the purchase of animals cannot be overemphasized enough which is why the focus is placed on cleaning up and keeping *Salmonella* free breeding flocks. Hygienic measures should take into account animals, housing, management, pest control, vehicle disinfection, hand washing, and clothes and boot cleaning after each use (Snow *et al*., 2010; Berge and Wierup, 2012).

The importance of cutting off introduction of *Salmonella* from the environment needs to be emphasized. This however extends beyond a fence, shower block and controlling the movement of people. The poultry house should be built to be easy for cleaning, immediate surroundings should be clear of vegetation and objects. Houses and hatchery area must be properly bird proofed and be able to keep rodents and other animals away from the houses. All feed used in poultry houses should be made in feedmills with good manufacturing or Hazard Analysis and Critical Control Point (HACCP)-based practices and monitored for *Salmonella* to minimize the risk of introducing *Salmonella* through contaminated feed. Drinking water

should come only from regularly tested sources treated to ensure purity. New and/or well-disinfected equipments for eggs and newborn birds are also essential (Berge and Wierup, 2012).

Feed plays a crucial role in the control of *Salmonella* on two levels. First, the feed may potentially be an important vector to introduce *Salmonella* onto the farm. Proper control programs must, therefore, be in place at any feed manufacturing site, as well as on the farm to avoid ingestion of *Salmonella* by the animals, including preventing contamination by purchasing ingredients only from suppliers with a proven record of accomplishment with respect to *Salmonelle* control as well as control of dust, wild animals and moisture in fee manufacturing environment (Jones, 2011). Second, when animals are exposed to *Salmonella* through feed and other sources, feed composition, texture and supplements can be used to additionally minimize the risk of colonization and shedding of *Salmonella*. Thus, feed additives are recognized as a useful tool in prevention program (Berge and Wierup, 2012).

Organic acids may have dual functions in reducing *Salmonella* contamination. First, they may reduce *Salmonella* load in the feed and, second, they may reduce the potential for infection and shedding in the animal. Short-chain fatty acids (SCFA), such as formic, acetic, propionic and butyric acids, have all been shown to down-regulate expression of invasion genes in Salmonella spp. (Van Immerseel *et al.*, 2006). Research also showed that individual acids vary in their effect on *Salmonella* but, in general, medium-chain fatty acids are more effective than short-chain fatty acids (Johny *et al.*, 2009)

Probiotics and prebiotics are also used as feed additives to help the young birds to combat the pathogenic enteric bacteria. The benefit actions of probiotics include regulation of intestinal microbial homeostasis, stabilization of the gastrointestinal barrier function (Salminen et al., 1996), enzymatic activity inducing absorption and nutrition (Hooper et al., 2002; Timmerman et al., 2005), interference with the ability of pathogens to colonize and infect the mucosa (Gill, 2003). Microorganisms used as probiotics in animal feed are mainly bacterial strains belonging to the genera *Lactobacillus, Enterococcus, Pediococcus,* and *Bacillus*, as well as microscopic fungi such as *Saccharomyces.* A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confers benefits upon host well being and health and it is well-established positive impact on the intestinal microflora (De Vrese and Schrezenmeir, 2008). Most identified prebiotics are carbohydrates and oligosaccharides; dietary carbohydrates such as fibers, are candidate prebiotics, but most promising are nondigestible oligosaccharides (NDOs) (Gaggla et al., 2010)

Competitive exclusion (CE) cultures are a form of probiotic culture that is composed of a mixture of non-pathogenic bacteria typically found in the gastro-intestinal tract of adult birds. This treatment is generally used as a prophylactic measure aimed at increasing the resistance of young chicks to *Salmonella* (Mead, 2000), but it also can be used after antibiotic therapy to restore the normal microbiota (Seo *et al.*, 2000). CE was initially developed and used in poultry production, where newly hatched chicks could be protected from subsequent *Salmonella* infections by accelerating the establishment of a complex, protective microflora (Nurmi *et al.*, 1992; Schneitz *et al.*, 1992)

Oral administration of chicken egg yolk immunoglobulin (IgY) has attracted considerable attention as a means of controlling *Salmonella* in poultry (Xu *et al.*, 2011). It has been shown that specific IgY against *Salmonella* Enteritidis or *Salmonella* Typhimurium whole cells inhibits bacterial growth in liquid medium (Lee *et al.*, 2002). In the study by Rahimi *et al.* (2007), 3-day-old chicks administered with purified yolk immunoglobulin (IgY) in drinking water and challenged with *Salmonella* Enteritidis, showed significantly lower fecal shedding and lower levels of *Salmonella* Enteritidis in the caecum. Non-immunized egg yolk powder has also been effective for eliminating and preventing *Salmonella* colonization in poultry. It is not clear the precise mechanism by which the egg yolk component prevents or eliminates infection; however, it can be attributed to its ability to prevent *Salmonella* from attaching and invading the intestinal epithelial cells, the first required site for infection (Kassaify and Mind, 2004).

## 4.3 Vaccines

The criteria for an ideal vaccine against *Salmonella* infections in production system should include: (1) effective protection against both mucosal and systemic infection; (2) non-pathogenic to animals and man; (3) efficacy in reducing intestinal colonization, and thus reducing environmental contamination, and egg infection; (4) compatibility with biosecurity measures; and (5) cost-effective application (Pritchard et al., 1978; Barrow, 1999).

### 4.3.1 Inactive vaccines

Killed vaccines have been used to control host nonspecific *Salmonella* infections in poultry with very varying success. The protective immunity provided by killed pathogen vaccines is inferior because the killed pathogen stimulates mainly antibody production and is rapidly destroyed and eliminated from the host immune system, and relevant antigens are destroyed during vaccine preparation (Barrow, 2007). Killed vaccines generally fail to induce cytotoxic T cells (Foster and Spector, 1995) and secretory IgA responses, which are important for mucosal surfaces protection (Baay and Huis in 't Veld, 1993). In a previous studies in our lab, two commercial killed SE bacterin vaccines have been evaluated, and we have demonstrated that even if the humoral response and antibody (serum IgY and mucosal IgA) titers persisted throughout the production period after immunization, they were not sufficient to protect SE challenged hens from excreting the bacteria and these challenged hens still kept laying positive SE eggs at 55 and 65 weeks of age (Tran *et al.*, 2009, not published).

### 4.3.2 Attenuated live vaccines

Attention has been paid to the development of avirulent vaccine strains of *Salmonella.* There is some evidence that such *Salmonella* strains are more immunogenic in mice and in poultry than killed or subunit vaccines are (Collins, 1974; Zhang-Barber *et al.*, 1999). Live vaccines have been shown to be more effective in increasing lymphocyte proliferation in response to *Salmonella* Enteritidis antigens in laying hens. Although a number of different live *Salmonella* strains have been tested for their efficacy in experimental or semi-field studies, only a few are registered and commercially available for use in poultry in Europe (Vandeplas *et al.*, 2010).

There are some important safety facts for Salmonella live vaccines, such as the investigation of reversion to virulence of attenuated vaccines or the potential that antibiotic resistance genes can be transferred to other microorganisms (EFSA, 2004). Information on the biological properties of the vaccine strains have to be given, the probability of recombination or genomic re-assortment with field or other strains should be considered. Genetic stability should also be considered as an important aspect of the safety of live Salmonella vaccines (Barbezange et al, 2000)

### 4.3.3 Subunit vaccines

Protein-based subunit vaccines against SE have been studied in poultry. There are a lot of researches about OMPs as subunit vaccine candidates. Vaccines based on OMPs of SE induce a stronger antibody response than vaccines made from whole bacteria, which may indicate that OMPs contain the major immuno-dominant proteins of SE (Meenakshi *et al*, 1999). Hamid and Jain (2008) found that an OMP of ST with an apparent molecular mass of 49 kDa was highly immunogenic, which was capable to induce humoral and cell-mediated immune responses, and conferred 100% protection to immunized rats against challenge with very high doses (up to 100 times the 50% lethal dose) of ST. In another study, shedding of *Salmonella* Enteritidis in chickens decreased after immunized 9-week-old chickens with two outer membrane proteins (75.6 and 82.3 kDa) subcutaneously, followed by two boost immunizations with time intervals of 2 weeks interval (Meenakshi *et al.*, 1999; Khan *et al.*, 2003). Immunization of either of the outer membrane proteins decreased cecal colonization about 1000-fold when the animals were orally infected with $8\times10^8$ CFU virulent SE strain

(Khan *et al.*, 2003). Okamura et al. (2012) reported that after chickens were immunized with OmpA, serum IgY was induced even though while no decrease in cecal excretion and tissue colonization after SE challenge was observed. There have been a few studies involving Salmonella pathogenicity island (SPI)-1 and SPI-2 (Wisner et al., 2011; Desin et al., 2011). It has showed that a significant humoral immune response was induced after chickens were immunized with SPI-1 and SPI-2 proteins subsequently. Production of serum IgY and their transferring to egg yolk were observed. After a SE challenge, a reduction in the SE levels in the liver but not in the spleen was noticed. In addition, FliC, the flagellin antigen of *Salmonella* Enteritidis, also could induce a high IgY level in chickens after immunization. However, neither the proliferative response nor the interferon-gamma secretion of splenic cells upon stimulation with rFliC was induced (Okamura et al., 2012). In all these studies, protein antigens were given to chickens subcutaneously or intramuscularly. Although induction of serum IgY was generally observed, local mucosal IgA increase was hardly evaluated and protections against systemic bacterial colonization after orally SE challenge were various as well. Protections were not strictly correlated to level of serum IgY level which implies that the local immune response (IgA) plays an important role in protection against *Salmonella* infection. This might be because the initial site of entry and attachment of *Salmonella* is usually the mucosal surface once the bacteria are orally taken. The results could be different if these proteins were orally administered and delivered to the intestinal mucosa directly. Unfortunately, there has been very little work based on orally- administered protein subunit vaccine against SE in chickens.

In this project, five proteins have been chosen to be potential candidates for the new subunit vaccine against *Salmonella* Enteritidis and *Salmonella* Typhimurium in layers and breeders. They have been identified to be Glyceraldehyde-3-phosphate dehydrogenase, (GAPDH) Enolase, Lipoamide dehydrogenase (LpdA), DNA protection during starvation protein (Dps) et Elongation factor-Tu (EF-Tu). These proteins were selected from a previous study in our lab because that they are well conserved at the genetic level and well conserved with highly identical in both SE and ST. The surface expression of all these proteins has been reported, and the immunological functions of these proteins are already known in other species.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (35.6 kDa)

GAPDH is present in the outer membrane and cytoplasm and is highly antigenic. It is considered to be a strong vaccine candidate for bacterial infections (Park *et al*., 2011). GAPDH is a glycolytic enzyme catalyzing the oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate in the presence of cofactor nicotinamide adenine (NAD) and of an inorganic phosphate molecule. Binding of surface GAPDH to host proteins or tissues was studied in bacterial and fungal species suggesting that adhesion of GAPDH with plasminogen and plasmin is probably part of the mechanisms contributing to bacterial invasion (Modun and Williams, 1999; Jobin et al., 2004; Egea et al., 2007). Bacterial surface GAPDH, expressing on the surface of pathogenic bacteria in general, has been observed mostly in Gram-positive species including *Streptococcus* species (Pancholi and Fischetti, 1992), *Staphylococcus* species (*S.* Epidermidis and *S.* Aureus) (Modun and Williams, 1999) and mycobacteria (Bermudez et al., 1996). Observation of surface GAPDH in enteropathogenic

*Escherichia coli* (EPEC) by Kenny and Finlay (1995) was the first report indicating that gram-negative bacterial GAPDH is also secreted outside the cell. Surface expression of GAPDH in *Neisseria Meningitidis* and *N. Lactamica* has also been reported (Grifantini et al., 2002). Cell-wall associated GAPDH also exist in fungal pathogens such as *Candida albicans* (Gil-Navarro et al., 1997) and *Kluyveromyces marxianus* (Fernandes et al., 1992).

There is an increasing number of reports of immunogenic GAPDH from bacterial species published. Immunization with DNA vaccine encoding GAPDH of facultative intracellular bacteria *Brucella abortus* decreased spleen bacterial load of challenged mice (Rosinha *et al.*, 2002). Intra-peritoneal administration of purified recombinant surface GAPDH of Gram negative *Edwardsiella tarda* induced humoral protective response in Japanese flounders from disease and increased their survival rate (Liu *et al.*, 2005). In *Candida albicans*, GAPDH is a highly immunogenic protein capable of inducing increased IgM responses in patients with systemic candidiasis or neutropenic patients (Gil-Navarro et al., 1997). Our laboratory has also already demonstrated the immunogenicity of ST glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in experimentally and naturally infected pigs. Immunization with GAPDH encapsulated in PLGA microsphere could protect pigs against clinical signs associated with experimental infection by ST, and pigs were hardly showing fever and diarrhea after being challenged by $10^8$ cells of ST (Quessy *et al*., 2007).

Enolase (45.6 kDa)

Enolase is found in surface of bacterial cell and catalyses the interconversion of

phosphoenolpyruvate and 2-phospho-D-glycerate during glycolysis. It is now clear that enolase is a multifunctional protein (Pancholi, 2001). One important property of eukaryotic and prokaryotic enolases is to bind plasminogen with great affinity (Miles et al., 1991; Redlitz et al., 1995; Pancholi and Fischetti, 1998). As a bacterial virulence factor, an important role of surface-expressed pneumococcal enolase in direct plasminogen-binding ability of *S.* pneumoniae has been found (Bergmann et al., 2001). As an immunogenic plasminogen receptor of *Borrelia burgdorferi*, Enolase released in outer membrane vesicles could be responsible for external proteolysis in the pericellular environment and have roles in nutrition and in enhancing dissemination of the bacteria (Toledo et al., 2012). Surface expression of Enolase has been previously found in different bacteria, including *Streptococcus* species (Bergmann et al. 2001), *Aeromonas hydrophila* (Sha et al., 2003), *Borrelia burgdorferi* (Toledo et al., 2012). Immunogenicity of Enolase in Streptococcus pneumonia and *Candida albicans* were demonstrated by its immuno-recognition with anti-enolase antibodies present in diseased patients' sera (IgG) (Sandini et al., 1999; Whiting et al., 2002). Enolase also has been reported as an immunodominant antigen in the cell walls of *Pneumocystis carinii* (Fox and smullan, 2001).

DNA protection during starvation protein (DPS) (18.7 kDa)

The DNA-binding protein Dps that is generally regarded as cytoplasmic, and is found in many eubacterial and archaebacterial species, protecting DNA under a variety of stress conditions. It appears to protect cells from oxidative stress and/or nutrient-limited environment. Dps has been shown to accumulate during the stationary phase, to bind to DNA non-specifically, and

to form a crystalline structure that compacts and protects the chromosome. In a recent study, Dps was found significantly enriched in outer membrane fraction of *Salmonella* when bacteria were incubated under a phagosome-mimicking condition. This was the first report demonstrating that this protein is outer-membrane-localized in *Salmonella* (Brown et al., 2012). The positive role of Dps-like protein in adhesion and virulence during bacterial infections such as *Salmonella* Typhimurium was observed by Haikarainen and Papageorgiou (2010). Dps is a known virulence determinant of *Salmonella* (Haikarainen and Papageorgiou, 2010), but how it translocates to the outer membrane and its role(s) at the cell surface remains to be investigated. It also has been shown that Dps protects *Salmonella* from iron-dependent killing by hydrogen peroxide, promotes *Salmonella* survival in murine macrophages and enhances *Salmonella* virulence (Halsey *et al.*, 2004). Interestingly, Dps was recently observed on the cell surface of *Escherichia coli*, where it may play a role in attachment to abiotic surfaces (Goulter-Thorsen et al., 2011). Dps also has been identified as an outer membrane protein involved in *E. coli*'s response to pH change and it was induced in acid condition (Wu *et al*., 2008).

Lipoamide dehydrogenase (LpdA) (50.6 kDa)

LpdA is a cytoplasm membrane-associated and classically involved in the conversion of 2-oxo acids to their respective acyl-CoA derivatives. It is also present in organisms that do not contain 2-oxoacid dehydrogenase complexes. For instance, in *Escherichia coli,* LpdA stimulates ATP-binding cassette transport of several carbohydrates, and ubiquinone-mediated transport of amino acids implicated in virulence (Hakansson *et al.*, 2007; Smith *et al.*, 2002).

In ST, it is reported that LpdA was induced (up to 11-fold) upon addition of bactericidal/permeability-increasing protein from human neutrophils (Qi *et al.*, 1995). Recently, LpdA was identified as novel surface-exposed virulence factor of *P.earuginosa* for its contribution to survival of *P. aeruginosa* in human serum (Hallstrom et al., 2012). In *Neisseria meningitidis*, LpdA constitutes an immunogenic surface antigen (Exposito Raya *et al.*, 1999).

Elongation factor-Tu (EF-Tu) (43.3 kDa)

EF-Tu is a cytoplasmic protein, which is involved in polypeptide elongation during protein synthesis, but EF-Tu has recently been reported to be surface-associated in *Streptococcus pyogenes* (Severin *et al.*, 2007) and *N. meningitidis* (Kolberg *et al.*, 2006; Williams *et al.*, 2007). Its roles as a bacterial adhesion and invasion for *Pseudomonas aeruginosa*, *Mycoplasma pneumonia* and *Francisella tularensis* have been reported (Kunert et al., 2007; Balasubramanian, et al., 2008; Barel, et al., 2008). Moreover, using serological proteome analysis, EF-Tu was identified as a seroreactive protein of *Bacillus anthracis* (Chitlaru *et al.*, 2007). EF-Tu also recently reported as a novel vaccine immunogen against *Burkholderia* infection since it is membrane-associated, secreted in outer membrane vesicles (OMVs), and immunogenic during bacterial infection in the murine model of melioidosis (Nieves et al., 2010).

Therefore, based on all the information obtained from previous researches, we assume that SE-derived GAPDH, Enolase, Dps, LpdA and EF-Tu are immunogenic and able to induce

immune responses and production of specific antibodies in eggs after immunizing hens with them. We will produce and purify these proteins and demonstrate their immunogenicities, in turn to prove that they are interesting to be further studied as potential candidates of a new subunit vaccine against *Salmonella* Enteritidis and *Salmonella* Typhimurium in laying and breeder hens.

# MATERIALS AND METHODS

**Construction of protein expression vector**

The construction of protein expression vector was done at Laval University. Genes of interest, coding the selected *Salmonella* proteins i.e. GAPDH, Enolase, LpdA, Dps and EF-tu, were amplified by polymerase chain reaction (PCR) using genomic DNA from the field isolate *Salmonella* Enteritidis SHY-04-1540 as a template. Design of the primers was based on the already known complete genome of *Salmonella* enterica subsp. enterica serovar Enteritidis str. P125109 (NCBI Reference Sequence NC_011294.1), as the selected proteins were known to be well conserved among the various strains of *Salmonella* Enteritidis. For convenient cloning into the multiple cloning site of pQE-30 expression vector (Qiagen, Cat. 32149), two restriction sites were introduced in the primers, BamHI and HindIII, respectively in 5' and 3' of the genic sequences. Finally, in order to obtain fusion proteins in frame with N-terminal 6XHistidine of pQE-30, the initiation codon of the gene sequences was removed. These recombinant plasmids allow the expression of fusion proteins, which then can be easily purified by nitrilotriacetic acid (NTA) affinity chromatography. The integrities of cloned sequences were checked by DNA sequence analysis.

**Transformation of *E. coli* M15 cells**

Back to our lab, *E.coli* M15 cells (QIAexpressionist, QIAGEN) were transformed with each of the five recombinant pQE30 vectors (conferring ampicillin resistance) according to the manufacturer's instructions (QIAGEN).

High-level expression of proteins is possible in this system due to the presence of the T5 promoter/lac operator transcription-translation for expression in *E.coli*. *E.coli* M15 cells contain the low-copy plasmid pREP4 that confers kanamycin resistance and constitutively expresses the *lac* repressor protein.

**Expression of the recombinant proteins**

Single colonies of the transformants were picked and transferred into 50 ml of LB media containing both ampicilin (100 μg/ml) and kanamycin (25 μg/ml) (Sigma-Aldrich Corporation, St. Louis, MO, USA). One extra culture was inoculated to serve as a non-induced control. The cultures were grown overnight at 37℃. Five hundred ml of pre-warmed medium (including antibiotics) were inoculated with 25 ml of the overnight cultures at 37℃ with vigorous shaking. When the absorbance at 600 nm was 0.6, IPTG (Isopropyl β-D-1-thiogalactopyranoside) (Invitrogen Corporation, Burlington, ON, Canada) was added to a final concentration of 1 mM to induce expression of recombinant proteins. After the cultures were grown for an additional 4.5 h, cells were harvested by centrifugation at 5 000 g for 20 min at 4℃, and the pellet was stored in -20℃.

**Purification of recombinant proteins**

The cell pellet was thawn for 15 min on ice and resuspended in 10 ml of lysis buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole (Fisher Scientific, Fair Lawn, NJ, USA), pH 8.0, filtered by 0.22 μm syringe filter), and lysozyme (Sigma-Aldrich Corporation, St. Louis, MO,

USA) was added to a final concentration of 1 mg/ml to destroy the bacterial cell wall and expose the proteins. After the lysozyme was dissolved completely, 60 µl Benzonase Nuclease (EMD Millipore Corporation, San Diego, CA, USA) was added and the contents were incubated on ice for 90 min with shaking. The lysate was then centrifuged at 10 000 g for 20 min at 4℃, and the cleared supernatant was collected and filtered through a 0.22 µm syringe filter before applying it to affinity purification. For the following steps, two different methods were used to purity the proteins; Ni-NTA superflow resin from Qiagen, and Fast protein liquid chromatography (FPLC)

1. Purification with QIAexprss Kit Type IV (Qiagen, Catalog No. 32149)

Four milliliter of the supernatant obtained above was mixed with 1 ml the 50% Ni-NTA slurry by gentle shaking at 4℃ for 1 h. the supernatant/Ni-NTA mixture was loaded into a column, and the column flow-through was collected. Then, the column was washed twice with 4ml of wash buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 20 mM imidazole, pH 8.0), and the wash fractions were kept for SDS-PAGE analysis. The 6xHis-tag recombinant protein was eluted from the resin four times with 0.5 ml elution buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 500 mM imidazole, pH 8.0). The recombinant protein was analyzed by SDS-PAGE as described later in this chapter.

2. Purification by Fast protein liquid chromatography (FPLC)

Ni/NTA affinity purification was performed on an AKTA Xpress FPLC system using 1 ml HisTrap HP columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Columns were equilibrated with lysis buffer, the lysate loaded and the columns washed by lysis buffer until

the absorption of post-column flowthrough returned to base levels. Bound proteins were eluted with a linear gradient of imidazole elution buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 1 M imidazole, pH 8.0, filtered by 0.22 μm syringe filter), from 1% to 50% elution buffer, i.e. from 10 to 500 mM imidazole. Weakly bound contaminating proteins typically eluted at 30-60 mM imidazole, the peak maximum of 6xHistidine tagged proteins was between 80 and 150 mM imidazole. Samples were taken at various time points and loaded on SDS-PAGE gels to evaluate the purity of target recombinant proteins.

**Immunization of laying hens**

Eighteen Specific Pathogen Free (SPF) White Leghorn hens from the Canadian Food Inspection Agency Ottawa Laboratory (Ottawa, ON, Canada) were received at 14-week of age. They were separated into 6 groups (n=3 birds/group), individually wingtagged for identification purposes, put in separate cages and fed *ad libitum* a *Salmonella* free commercial feed (first growth then layer diets). Feces samples of all birds before immunization were collected weekly for bacteriological examination to confirm *Salmonella*-free status of laying hens. They received 3 intramuscular injections in the breast muscle at 16, 20, and 28 weeks of age, respectively. Group 1 was treated as the control group and injected (IM) with 0.5 ml PBS and 0.5 ml FIA (Freund's incomplete adjuvant) (Sigma-Aldrich Corporation, St. Louis, MO, USA) each time, and groups 2 to 6 were injected (IM) with 50 μg of each recombinant protein in 0.5 ml PBS and 0.5 ml FIA, respectively. Starting with the first egg laid, all eggs were collected daily and stored at 4℃ for future extraction of IgY (yolk) and IgA (white) as well as for following ELISA tests until 35 weeks of age when the trial was terminated. All the birds

were bled at 35-week-old before they were sacrificed, and blood samples were centrifuged at 5 000 g for 10 minutes at room temperature. Serum samples were recovered and conserved at -20℃ for ELISA testing.

**Extraction of antibodies from egg white and yolk**

**1 Extraction of egg white antibodies**

A PEG-based Ig isolation method described by Hamal et al. (2006) was modified for antibodies extraction from the egg white. Briefly, the eggshell from the narrowed end was broken, and the egg white was allowed to run into a Falcon tube by gently inverting the egg to facilitate the flow of egg white. Twice the volume of PBS (one tablet dissolved in 200 ml water yields 0.01 M phosphate buffer) (Phosphate Buffered Saline Tablets, Fisher Scientific, Fair Lawn, NJ, USA) was added to the egg white collected, and the contents were mixed thoroughly by shaking. Pulverized Polyethylene glycol 6000 (PEG6000) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added to make a final concentration of 4.7% (wt/vol) and mixed thoroughly until the PEG6000 was completely dissolved. The mixture then centrifuged at 14,000 g for 10 min at room temperature. After centrifugation, the clear supernatant containing the Ig was collected, aliquoted, and stored at -20℃ for the following analysis.

**2 Extraction of egg yolk antibodies**

A chloroform-based method described by Polson (1990) was used for Ig extraction from the egg yolk. After the egg white was taken, the egg yolk was allowed to run into a 50 ml Falcon tube, and the volume was noted. Twice the volume of PBS (0.01M, the same as described above) was added, and the contents were mixed thoroughly by vortex. An equal volume of chloroform (Sigma-Aldrich Corporation, St. Louis, MO, USA) was added, and the contents were mixed vigorously to produce a thick emulsion. After centrifugation (16 300 g, 20 min, room temperature), the mixture separated into 3 distinct layers. The watery phase on the top containing the immunoglobulins was collected, aliquoted, and stored at -20℃ until the following analysis.

**SDS-PAGE and Immunoblotting procedure**

SDS-PAGE followed by immunoblotting were used to prove immunogenicity of these recombinant proteins. Protein samples and molecular weight markers (Amersham High-Range Rainbow Molecular Weight Marker, GE Healthcare Bio-Sciences Corporation, Piscotaway, NJ, USA) were diluted in sample buffer (0.5M Tris, pH 6.8, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 5% mercaptoethanol). Appropriate quantity of each protein (see results) was then loaded into 10% Bis-Acrylamide (Fisher scientific, FairLawn, NJ, USA) separating gels. Gels were run at room temperature in running buffer (25mM Tris, 0.2M glycine, 0.1% SDS) at 100V/10min and then 200V until the dye front reached the bottom of the gel cassette. After SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose membranes (Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON, Canada) using transfer buffer (0.125M Tris-base, 0.1M glycine) by running at 100 V for 1 h. The membranes were blocked

in Tris-buffered saline (TBS) and 2% (wt/vol) skim milk powder (Smucker Foods of Canada Co. Markham, ON, Canada) for 1 h at room temperature and then incubated 2 h with serum in the blocking buffer (TBS containing 2% (wt/vol) skim milk powder). After five washes for 5 minutes each in TBS, the blots were incubated 1 h at room temperature with anti-chicken IgY (IgG) (whole molecule)-peroxidase produced in rabbit (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada). After being washed five times, the blots were developed by incubation in a solution containing $H_2O_2$ (Fisher Scientific, FairLawn, NJ, USA) and 4-chloro-1-naphtol (Sigma-Aldrich Corporation, St-Louis, MO, USA) for 20 min in the dark.

At first, the immunoblot was achieved as follows: mixed antisera against SE from immune layers (naturally infected chickens and vaccinated plus challenged) were used as primary antibody and anti-chicken IgG was used as secondary antibody with sera from naive chickens as the negative control. This protocol did not give satisfactory results even after numerous repetitions. So we decided to produce antibodies directly against each protein from eggs obtained from immunized hens with each recombinant protein. These antibodies were then used as primary antibody with sera from eggs of naive hens as the negative control, and the secondary antibody was the same anti-chicken IgG. Whole-cell proteins of SE, ST and *E.coli*, and OMPs of SE and ST also were used, which had been prepared in our lab as described before (Arockiasamy and Krishnaswamy, 2000; Tran et al., 2010). The various results are described in Results' chapter.

**ELISA tests for specific IgY and IgA levels**

The levels of the anti-GAPDH, anti-Enolase, anti-LpdA, anti-Dps and anti-EF-Tu IgY (in egg yolk and serum) and IgA (in egg white) were determined using Chicken IgG and IgA ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX, USA) respectively, following the manufacturer's instruction. The samples were analyzed in duplicate. IgY and IgA extracted from naive layers were used as the experimental negative control. Each plate had its own set of standards (3.12 to 200 ng/ml for IgY; 15.625 to 1000 ng/ml for IgA). Reagents and buffers were prepared in our laboratory following the specifications of the manufacturer (Bethyl Laboratories, Montgomery, TX, USA). The working dilution of detection antibody used was 1: 5,000 for specific IgY and 1:1 for specific IgA to ensure the effectiveness of the results.

Briefly, flat-bottomed 96-well polystyrene plates (Nunc-Immuno Plates Maxisorp, Inter Med, Denmark) were coated with 20 μg/ml of each recombinant protein for 1 h. Non-specific binding was avoided by incubation with blocking buffer (50 mM Tris, 0.14 M NaCl, 5% skim milk powder, pH8.0) for 30 min. After washing 5 times with washing buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH8.0), 100 μl of each diluted sample was added to each well and incubated 1 h at room temperature. After 5 washes, the plates were incubated with 100 μl of HRP conjugated chicken IgG detection antibodies (Bethyl Laboratories, Montgomery, TX, USA) diluted at 1:75,000 or HRP conjugated chicken IgA detection antibodies (Bethyl Laboratories, Montgomery, TX, USA) diluted at 1:50,000. After washing, samples were incubated with 3,3′,5,5′-Tetramethylbenzidine (TMB) (Bethyl Laboratories, Montgomery, TX, USA) for 15 min, then the reaction was stopped using ELISA stop solution (Bethyl Laboratories, Montgomery, TX, USA) after 15 min. The plates were read at 450 nm using an EL 800 universal microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) and KC

junior software (Bio-Tek Instruments Inc., Winooski, VT, USA). A standard curve describing the relation between the concentration of standards and their absorbance values was generated for each plate, and the antibody concentration of each sample was expressed as micrograms per milliliter or nanogram per milliliter. Additional calculations were carried out to determine the total amount of these antibodies per egg yolk (10-15 ml) and per egg white (20-25 ml).

- Calculation formula for the determination of egg yolk IgY concentration

Each yolk was diluted in twice the PBS volume before IgY extraction, and the work dilution of each extracted sample was 1:5000 for ELISA. So the final contration of IgY in yolk is:

$$\text{Final concentration of yolk IgY (μg/ml)} = \text{Original concentration calculated from ELISA (ng/ml)} \times 5000 \times 3 / 1000$$

- Calculation formula for the determination of egg white IgY concentration

Egg white from each egg was diluted in twice the PBS volume before IgA extraction, and samples after extraction had no more dilution for ELISA. So the final contration of IgA in egg white is:

$$\text{Final concentration of white IgA (ng/ml)} = \text{Original concentration calculated from ELISA (ng/ml)} \times 3$$

# RESULTS

**Production and purity of recombinant proteins**

The N-terminal hexahistidine-tagged proteins were overexpressed in *E. coli* using standard procedures (see Materials and Methods). At first, our purification of produced proteins started using the Qiagen procedure (QIAexpress Kit Type IV, QIAGEN Inc.). The results showed that although overexpression of each protein was obviously present, their purity was not satisfactory due to the presence of numerous contaminant bands as shown with SDS-PAGE (see Figure 1 showing GAPDH and EF-Tu as examples). Since it is crucial to achieve as the highest purity level as possible for each recombinant protein for the next experiments, it was decided to use FPLC. FPLC results showed much higher purity for each protein with much less unwanted bands appearing on the gel (see Figure 2). After purification of the recombinant proteins by FPLC, in order to evaluate their integrity and conservation of the His-tag, an analysis of purified proteins by Anti-His Immunoblotting was done (see Figure 3).

**Immunogenicity of recombinant proteins**

First, to prove immunogenicity of these recombinant proteins, mixed antisera against SE from immune chicken (naturally infected chickens and vaccinated plus challenged) were used as primary antibodies and anti-chicken IgG was used as secondary antibodies in a western-blot analysis, with sera from naive chickens as the negative control. Results revealed that this mixed antisera had difficulty to recognize each recombinant protein except for GAPDH (see Figure 4). Because these results were repeatedly obtained, we decided to directly evaluate, in

the chicken, the production of specific antibodies against each protein. For that, we immunized laying hens, and verified if specific antibodies could be produced in the derived eggs.

Five groups of hens were injected with 50 µg of GAPDH, Enolase, LpdA, Dps and EF-Tu respectively and the sixth group was immunized with only PBS as the experimental negative control group. First, eggs were laid when the birds reached 21 weeks of age. Eggs were collected from 21 to 35 weeks of age at which the layers were euthanized. The egg yolk IgY was extracted from eggs using a chloroform-based method (see Material and Method). These IgY were then used in an immunoblotting analysis to test the recognition of interesting recombinant proteins as well as OMPs and whole-cell proteins of SE, ST and *E. coli* (see Table 1). Results showed that there were specific recognitions between antibodies and each recombinant protein and that some corresponding antigens were present in OMPs and whole-cell proteins of different bacterial strains. Non-specific IgY from hens in control group (non-immunized hens) was used as experimental negative control. For this control, recognition between non-specific IgY and each protein was not detectable (see Figures 5 to 9).

**Evaluation of immune response**

To achieve this part, ELISA was used to detect the concentration of IgY in yolk and IgA in the egg white during the 3 injections period. IgY in the sera was also examined prior to euthanasia.

1 Egg yolk specific IgY levels

The levels of specific IgY from egg yolk were examined every week during the laying period for each hen. Generally, the specific IgY level increased and peaked in the 2nd to 3rd week after each injection, then decreased afterward. It tended to stabilize after the 3rd injection. Results also showed that in the stable phase after the 3rd injection, IgY levels against GAPDH, Enolase and Dps (> 1 000 μg/ml) were higher than levels of IgY against LpdA and EF-Tu (< 1 000 μg/ml) (see Figures 10 to14).

2 Sera specific IgY levels

Sera specific IgY levels were evaluated when hens were 35 weeks of age prior to euthanasia. Specific IgY levels were slightly lower in the serum compared to egg yolk lgY levels at the same age in individual bird. Also, IgY levels in the serum against GAPDH, Enolase and Dps (> 1 000 μg/ml) were higher than those against LpdA and EF-Tu (< 1 000 μg/ml) (see Figure 15). This observation was similar to egg yolk specific IgY levels..

3 Egg white Specific IgA level

Specific IgA levels from egg white were examined weekly during the laying period for each hen. For GAPDH, Enolase and Dps groups, the titers of specific IgA were undetectable until the 3rd immunization, and the range of IgA titers was 90-180 ng/ml (see figures 16 to18). For groups of LpdA and EF-Tu, the titers of specific IgA were almost undetectable during the entire laying period following immunizations (data not shown).

**Table 1. Bacterial strains used in assessment of recombinant proteins immunogenicity**

| Bacterial strain | Group specificity | identification | Origin of bacterial strain analysis |
|---|---|---|---|
| *Salmonella* Enteritidis | D | SHY-04-1540 | LEAQ |
| *Salmonella* Typhimurium | B | SHY-2009-03429 | LEAQ |
| *Escherichia coli* | Pathogenic | ECL 14668 | EcL |
| *Escherichia coli* | Non pathogenic | ECL 16142 | EcL |

LEAQ: Laboratoire d'épidémio-surveillance animale du Québec, Canada; EcL: The Reference Laboratory for *Escherichia coli*, Québec, Canada.

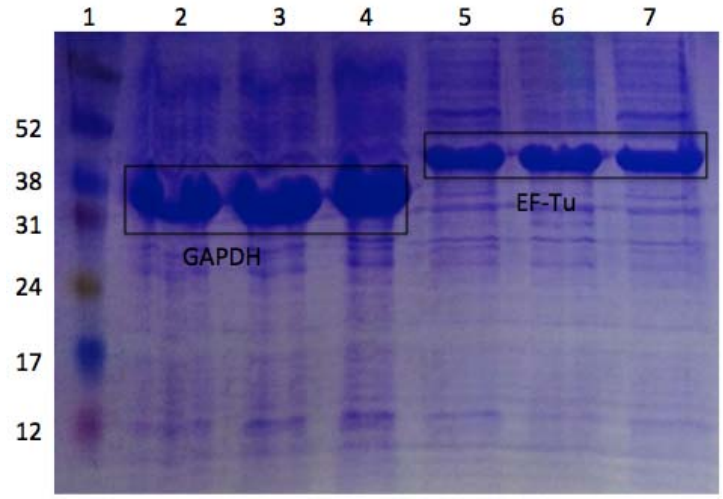



**Figure 1. SDS-PAGE of recombinant GAPDH and EF-Tu purified by Ni-NTA matrix (Qiagen kit).** Lane 1: Molecule weight marker (kDa); Lanes 2-4: three different purification batches of GAPDH (9 μg of protein per well); Lanes 5-7: three different purification batches of EF-Fu (7 μg of protein per well). These batches of GAPDH and EF-Tu were purified with the Ni-NTA matrix from Qiagen Kit.

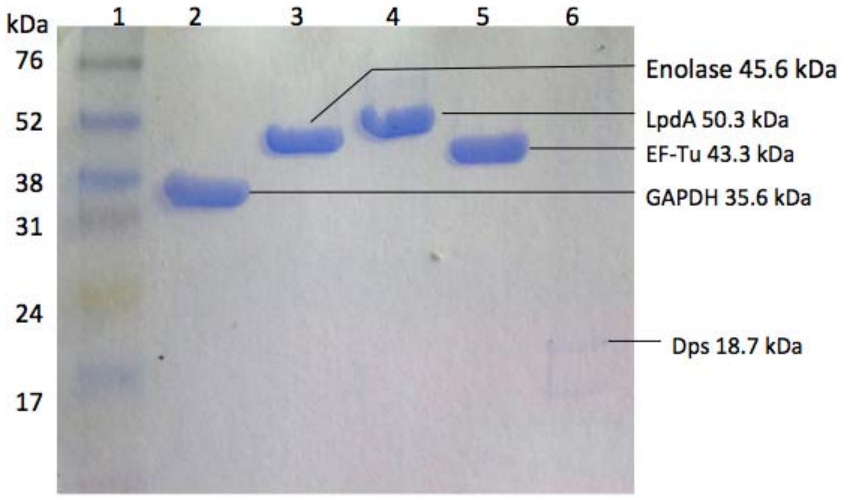



**Figure 2. SDS-PAGE of recombinant proteins purified by FPLC.** Lane 1: MW marker (kDa); Lane 2-6s: GAPDH, Enolase, LpdA, EF-Tu, and Dps, respectively (5 µg of each protein per well).

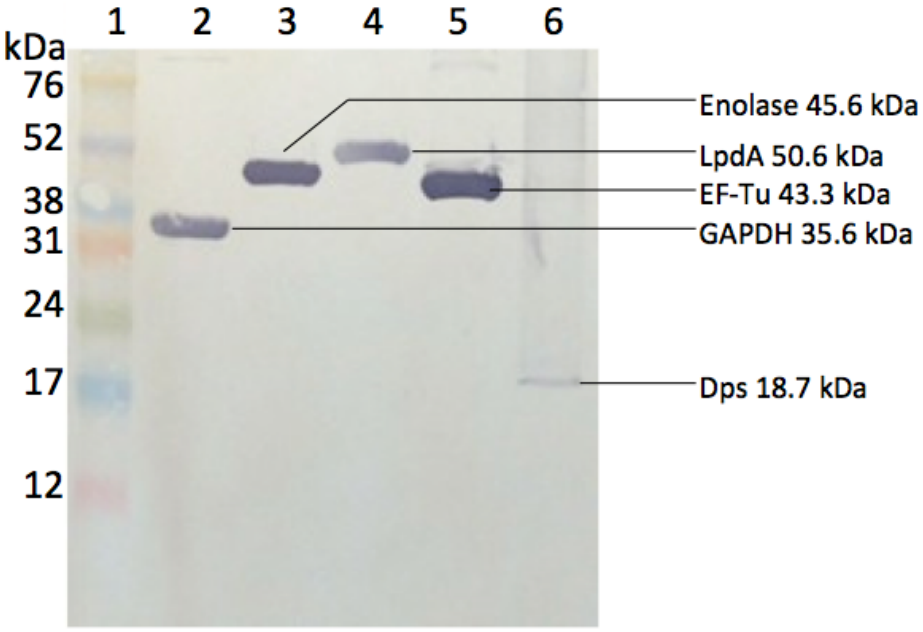



**Figure 3. Anti-His Immunoblotting Analysis of Purified Recombinant Proteins.** Western blot with anti-His as primary antibody shows the His-tag conservation and expected molecular weights of FPLC purified recombinant proteins. Lane 1: MW marker (kDa); Lanes 2-6: GAPDH, Enolase, LpdA, EF-Tu, and Dps, respectively (5 μg of each protein per well).

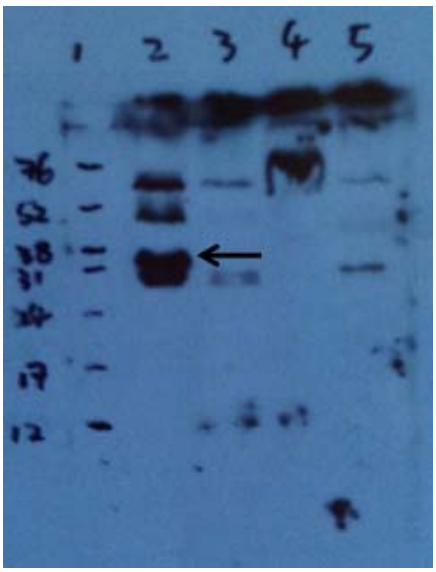



**Figure 4. Fluorescent western blot analysis with antisera against SE from immune layers.** Lane 1: Molecule weight marker (kDa); Lane 2: GAPDH (5µg); Lane 3: Enolase (5µg); Lane 4: LpdA (5µg); Lane 5: EF-Tu (5µg). The arrow points possible band of GAPDH. Primary antibody : antisera against SE from immune layers (1/1 000); secondary antibody : anti-chicken IgY (IgG) (Sigma-Aldrich) (1/10 000).

GAPDH

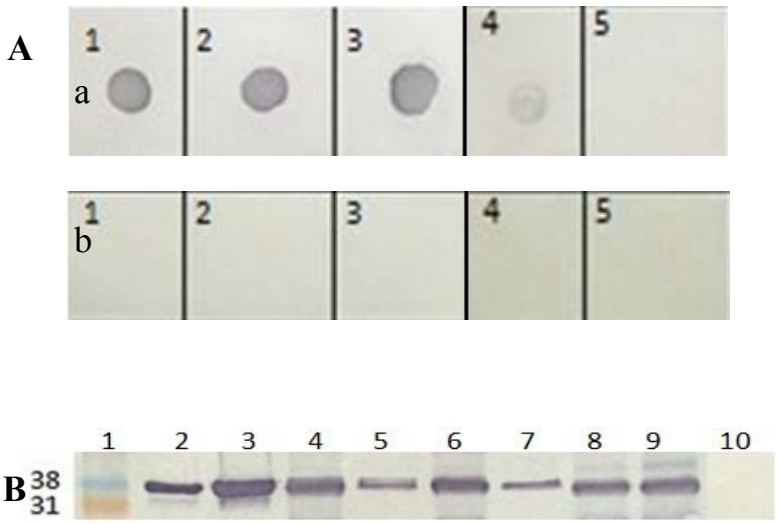



**Figure 5. Immunoblotting analysis with specific IgY against recombinant GAPDH.**

**A** Dot Blot specific IgY against recombinant GAPDH (a) and negative antibody from control group (b). 1-3: recombinant GAPDH: 1μg, 5μg and 10μg, respectively; 4: whole-cell proteins of SE; 5: randomly chosen negative control: EF-Tu (10μg).

**B**.Western Blot with specific IgY against recombinant GAPDH. Lane 1: MW marker (kDa); Lanes 2&3: recombinant GAPDH: 1μg and 5μg; Line 5&7: OMPs of SE and ST, respectively; Lanes 4, 6, 8&9: whole-cell proteins of SE, ST, *E. coli* 14668 and *E. coli* 16142, respectively. Line 10: experimental negative control, using negative antibodies from non-immunized layers and recombinant GAPDH.

Enolase

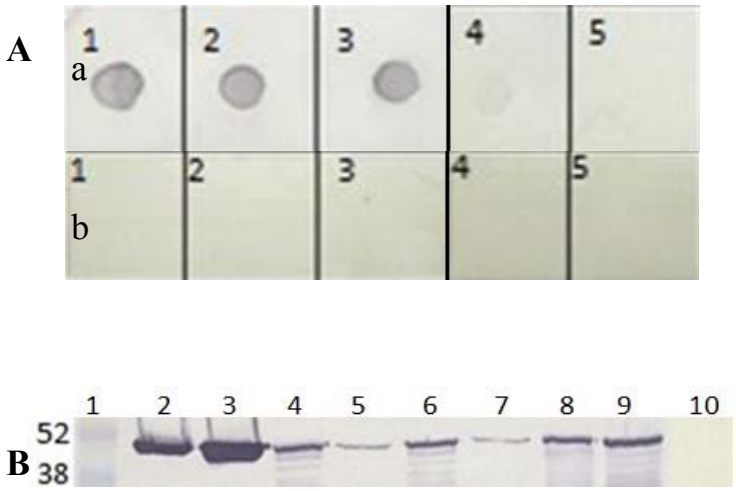



**Figure 6. Immunoblotting analysis with specific IgY against recombinant Enolase**.

**A** Dot Blot with specific IgY against recombinant Enolase (a) and negative antibody from control group (b). 1-3: recombinant Enolase: 1μg, 5μg and 10μg, respectively; 4: whole-cell proteins of SE; 5: randomly chosen negative control: Dps (10μg).

**B**.Western Blot with specific IgY against recombinant Enolase. Lane 1: MW marker (kDa); Lanes 2&3: recombinant Enolase: 1μg and 5μg; Line 5&7: OMPs of SE and ST, respectively; Lanes 4, 6, 8&9: whole-cell proteins of SE, ST, *E. coli* 14668 and *E. coli* 16142, respectively; Line 10: experimental negative control, using negative antibodies from immunized layers and recombinant Enolase.

Dps

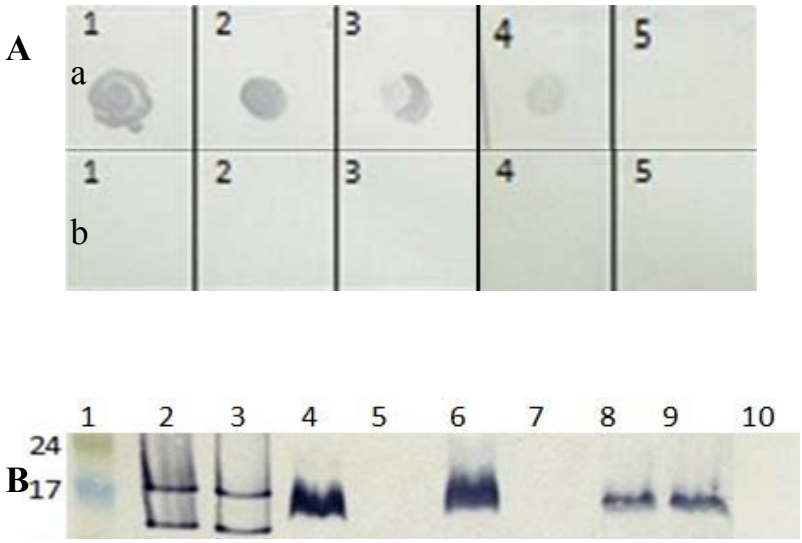



**Figure 7. Immunoblotting analysis with specific IgY against recombinant Dps.**

**A** Dot Blot with specific IgY against recombinant Dps (a) and negative antibody from control group (b). 1-3: recombinant Dps: 1μg, 5μg and 10μg, respectively; 4: whole-cell proteins of SE; 5: randomly chosen negative control: LpdA (10μg).

**B**.Western Blot with specific IgY against recombinant Dps. Lane 1: MW marker (kDa); Lanes 2&3: recombinant Dps: 1μg and 5μg; Line 5&7: OMPs of SE and ST, respectively; Lanes 4, 6, 8&9: whole-cell proteins of SE, ST, *E. coli* 14668 and *E. coli* 16142, respectively; Lane 10: experimental negative control, using negative antibodies from non-immunized layers and recombinant Dps.

LpdA

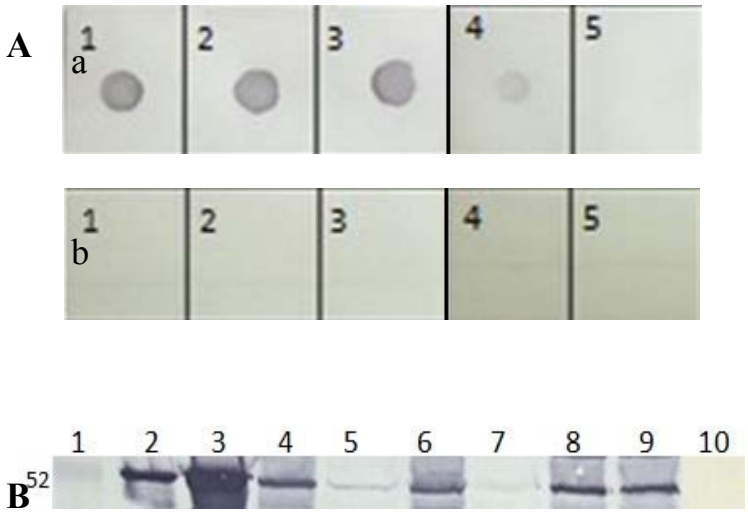



**Figure 8. Immunoblotting analysis with specific IgY against recombinant LpdA**.

**A** Dot Blot with specific IgY against recombinant LpdA (a) and negative antibody from control group (b). 1-3: recombinant LpdA: 1μg, 5μg and 10μg, respectively; 4: whole-cell proteins of SE; 5: randomly chosen negative control: Enolase (10μg).

**B**.Western Blot with specific IgY against recombinant LpdA. Lane 1: MW marker (kDa); Lanes 2&3: recombinant LpdA: 1μg and 5μg; Line 5&7: OMPs of SE and ST, respectively; Lanes 4, 6, 8&9: whole-cell proteins of SE, ST, *E. coli* 14668 and *E. coli* 16142, respectively; Lane 10: experimental negative control, using negative antibodies from non-immunized layers and recombinant LpdA.

EF-Tu

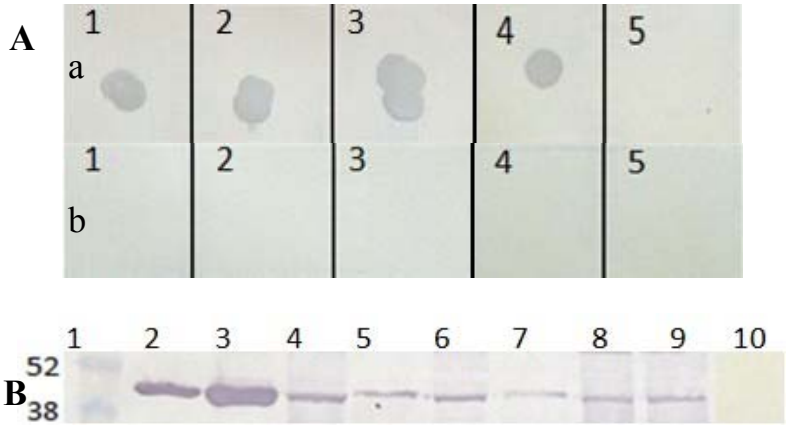



**Figure 9. Immunoblotting analysis with specific IgY against recombinant EF-Tu**.

**A** Dot Blot with specific IgY against recombinant EF-Tu (a) and negative antibody from control group (b). 1-3: recombinant EF-Tu: 1μg, 5μg and 10μg, respectively; 4: whole-cell proteins of SE; 5: randomly chosen negative control: GAPDH (10μg).

**B**.Western Blot with specific IgY against recombinant EF-Tu. Lane 1: MW marker (kDa); Lanes 2&3: recombinant EF-Tu: 1μg and 5μg; Line 5&7: OMPs of SE and ST, respectively; Lanes 4, 6, 8&9: whole-cell proteins of SE, ST, *E. coli* 14668 and *E. coli* 16142, respectively. Line 10: experimental negative control, using negative antibodies from non-immunized layers and recombinant EF-Tu.

GAPDH

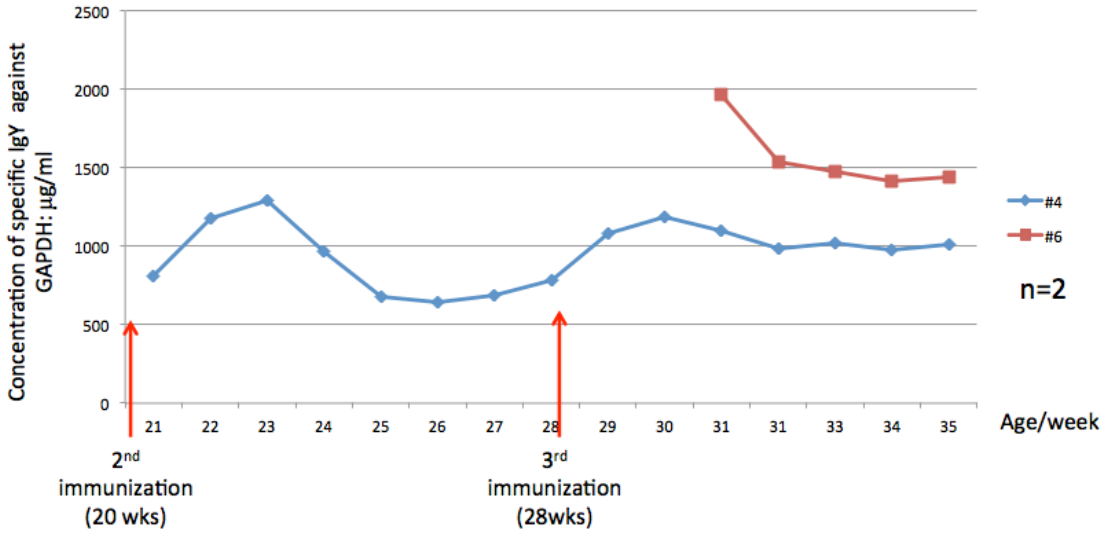



**Figure 10. Quantification of specific IgY against recombinant GAPDH in egg yolk of laying hens during post-immunization period analyzed using chicken IgG ELISA quantitation set**. Data shows the concentration of yolk specific IgY from laying hens, tagged as #4(♦) and #6(■), immunized 3 times with recombinant GAPDH. Two arrows stand for the time of 2nd and 3rd immunization, respectively. #5 was a poor layer and was not included in the results.

Enolase

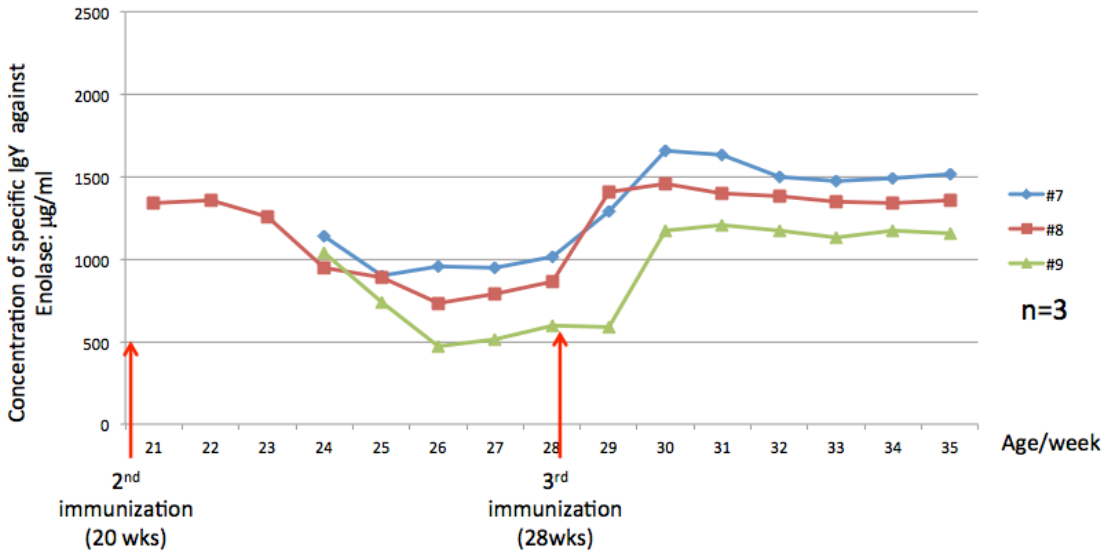



**Figure 11. Quantification of specific IgY against recombinant Enolase in egg yolk of laying hens during post-immunization period analyzed using chicken IgG ELISA quantitation set.** Data shows the concentration of yolk specific IgY from laying hens, tagged as #7(♦), #8(■) and #9(▲), immunized 3 times with recombinant Enolase. Two arrows stand for the time of 2nd and 3rd immunization, respectively.

Dps

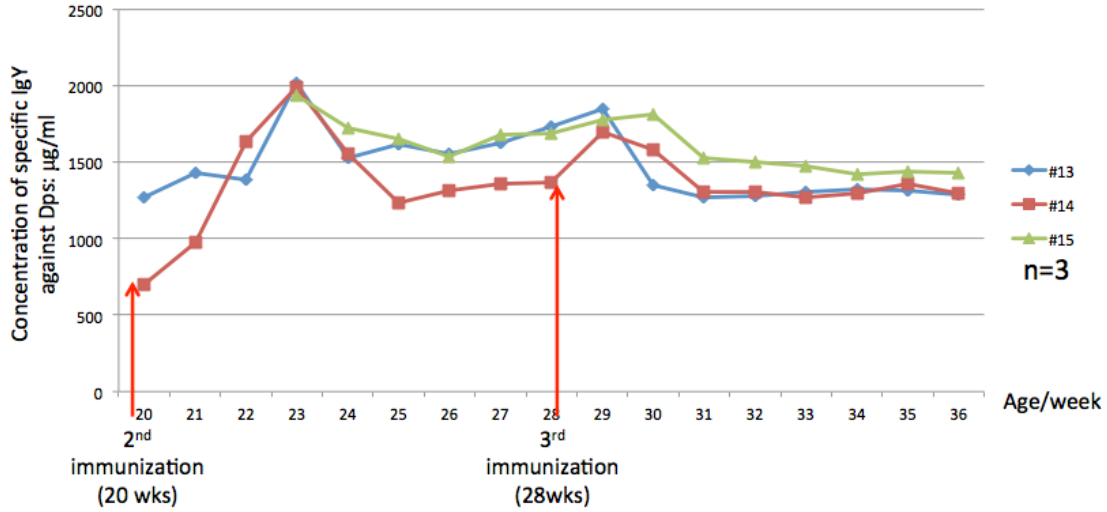



**Figure 12. Quantification of specific IgY against recombinant Dps in egg yolk of laying hens during post-immunization period analyzed using chicken IgG ELISA quantitation set.** Data shows the concentration of yolk specific IgY from laying hens, tagged as #13(♦), #14(■) and #15(▲), immunized 3 times with recombinant Dps. Two arrows stand for the time of 2nd and 3rd immunization, respectively.

LpdA

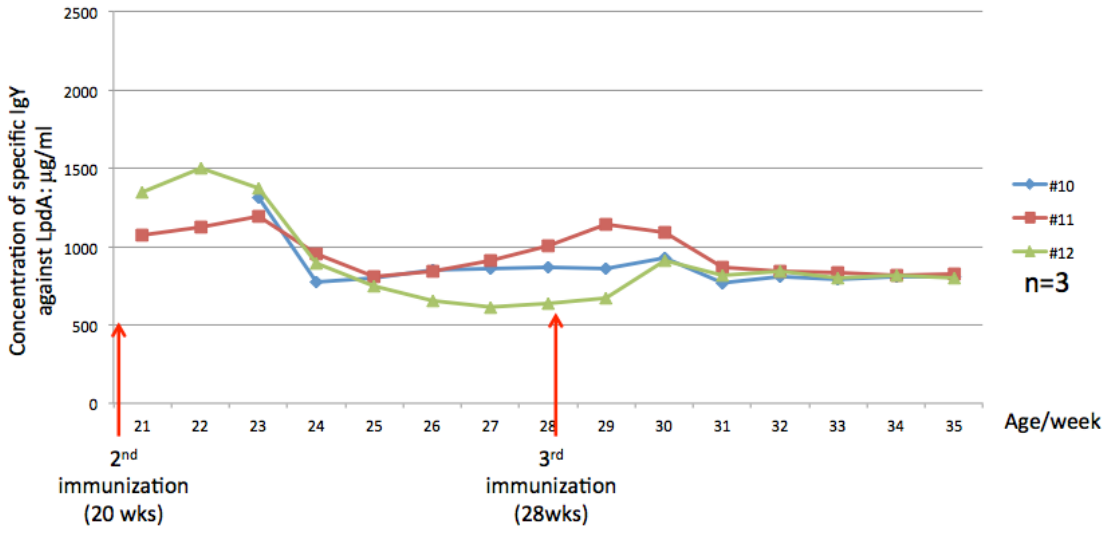



**Figure 13. Quantification of specific IgY against recombinant LpdA in egg yolk of laying hens during post-immunization period analyzed using chicken IgG ELISA quantitation set.** Data shows the concentration of yolk specific IgY from laying hens, tagged as #10(♦), #11(■) and #12(▲), immunized 3 times with recombinant LpdA. Two arrows stand for the time of 2nd and 3rd immunization, respectively.

EF-Tu

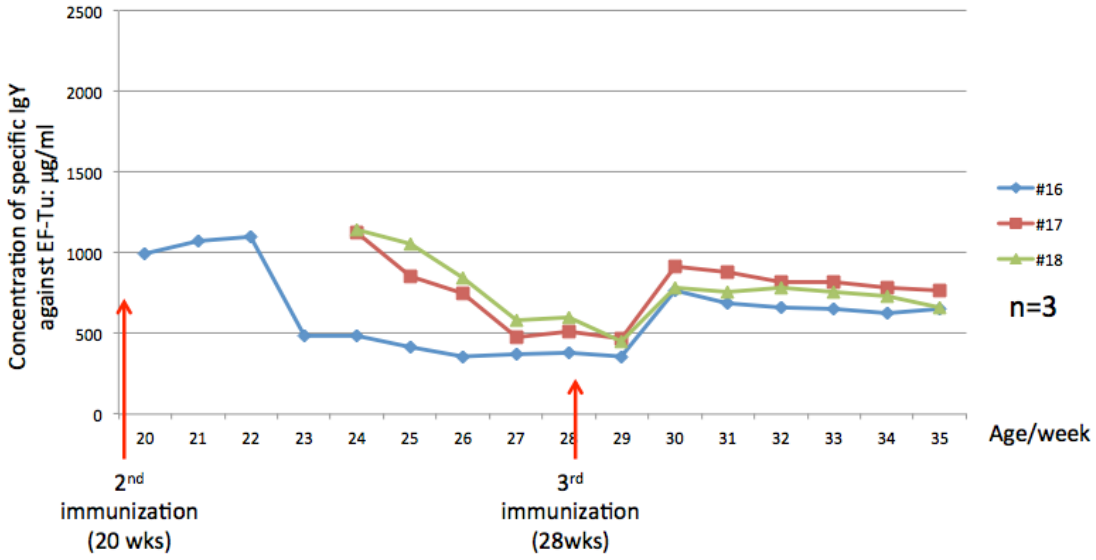



**Figure 14. Quantification of specific IgY against recombinant EF-Tu in egg yolk of laying hens during post-immunization period analyzed using chicken IgG ELISA quantitation set.** Data shows the concentration of yolk specific IgY from laying hens, tagged as #16(♦), #17(■) and #18(▲), immunized 3 times with recombinant EF-Tu. Two arrows stand for the time of 2nd and 3rd immunization, respectively.

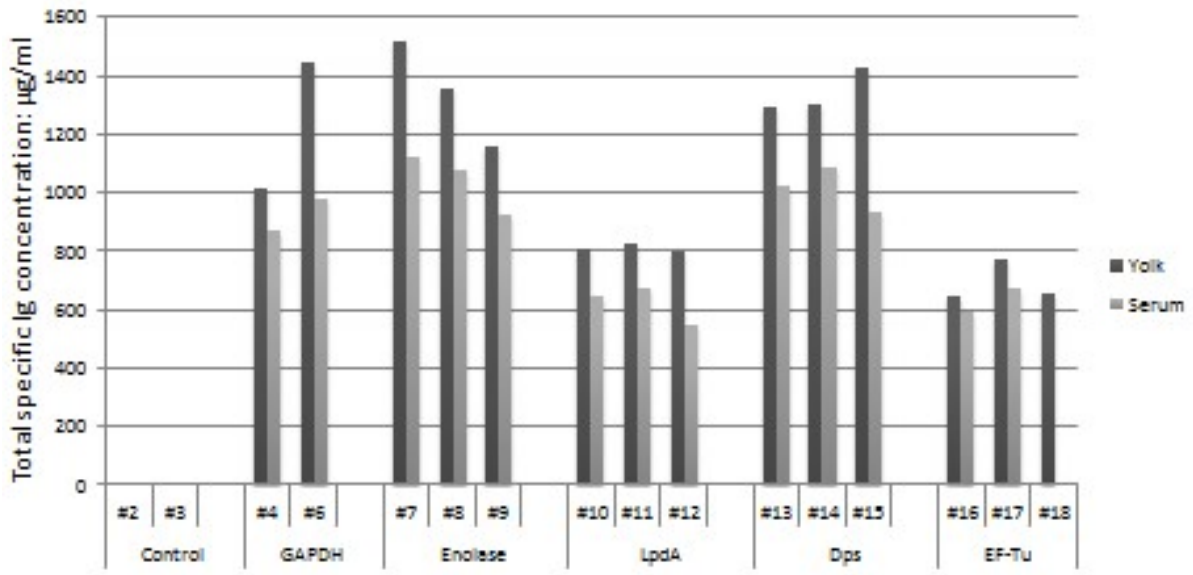



**Figure 15. Comparision of specific IgY in egg yolk and serum**. Data shows that the concentration of specific IgY in egg yolk and serum at 36 week of age prior to euthanasia. The dark column and the light column stand for IgY from yolk and serum, respectively. Each number stands for one hen, and three hens per group. #1 and #5 were poor layers so they were not included in the results. For the control group, mixed 5 recombinant proteins (2 μg for each protein) were used, and no recognition signal between IgY and proteins was detected.

GAPDH

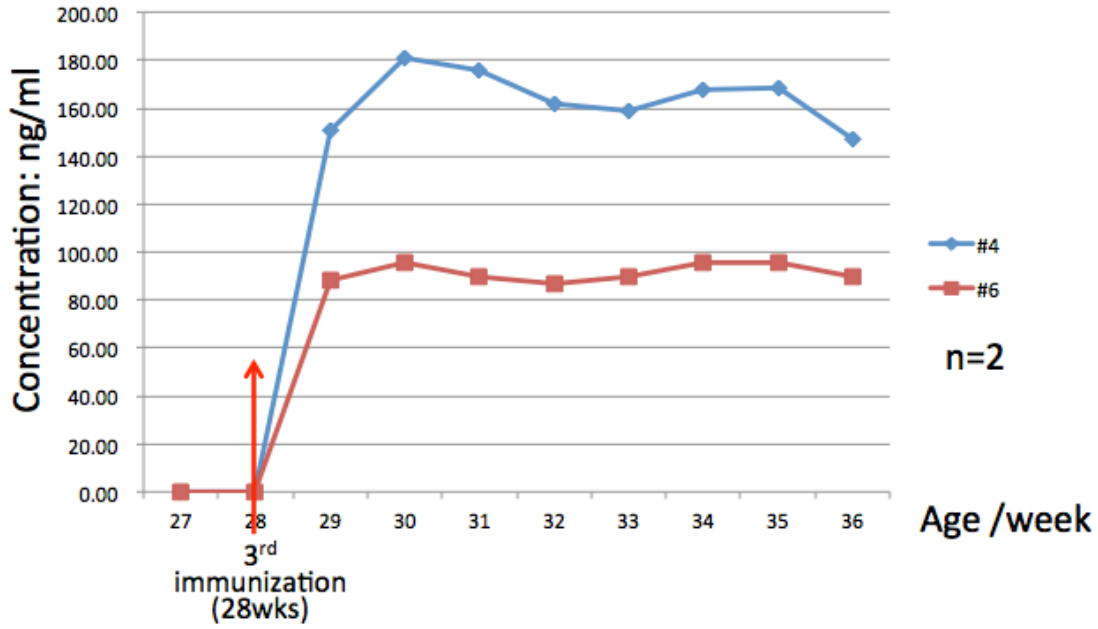



**Figure 16. Quantification of specific IgA against recombinant GAPDH in egg white of laying hens during post-immunization period analyzed using chicken IgA ELISA quantitation set.** Data shows the concentration of specific IgA in white of each egg from laying hens, tagged as #4(♦) and #6(■), immunized 3 times with recombinant GAPDH. The arrow stands for the time of 3rd immunization. The specific IgA in the egg white was not detectable before the 3rd immunization. #5 was a poor layer and was not included in the results.

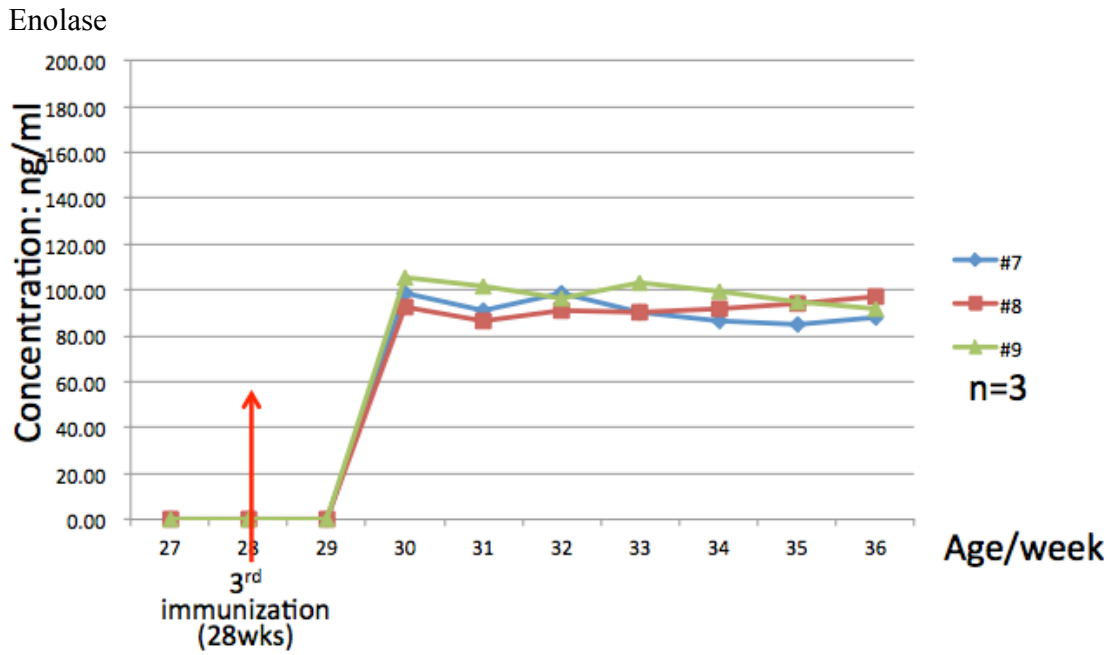



**Figure 17. Quantification of specific IgA against recombinant Enolase in egg white of laying hens during post-immunization period analyzed using chicken IgA ELISA quantitation set.** Data shows the concentration of specific IgA in white of each egg from laying hens, tagged as #7(♦), #8(■) and #9(▲), immunized 3 times with recombinant Enolase. The arrow stands for the time of 3rd immunization. The specific IgA in the egg white was not detectable before the 3rd immunization.

Dps

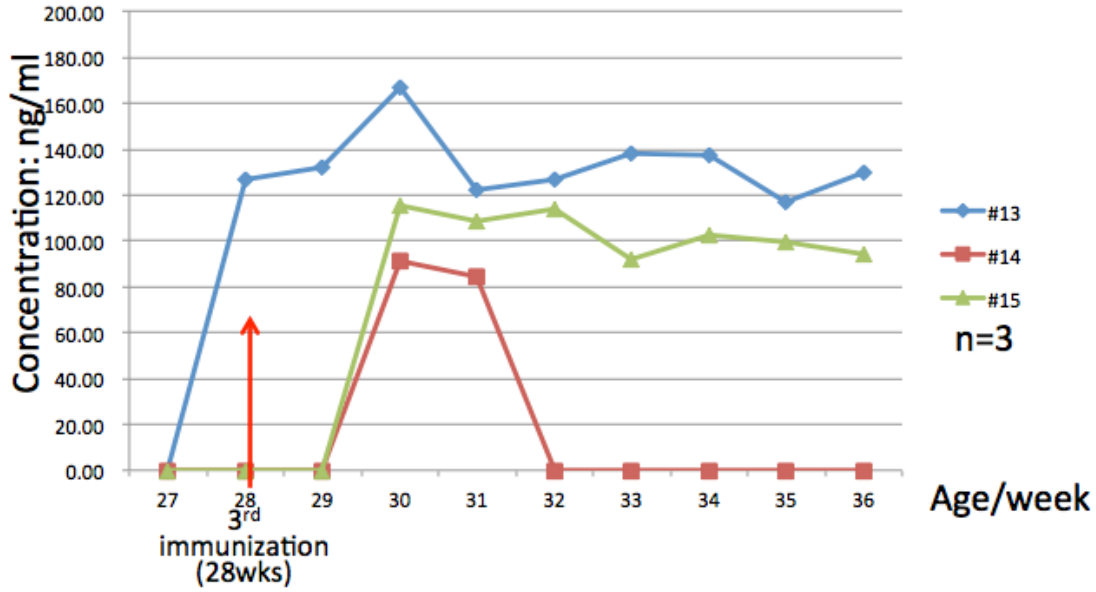



**Figure 18. Quantification of specific IgA against recombinant Dps in egg white of laying hens during post-immunization period analyzed using chicken IgA ELISA quantitation set.** Data shows the concentration of specific IgA in white of each eggfrom laying hens, tagged as #13(♦), #14(■) and #15(▲), immunized 3 times with recombinant Dps. The arrow stands for the time of 3rd immunization. The specific IgA in the egg white was not detectable before the 3rd immunization

# GENERAL DISCUSSION

**Choice of candidate proteins**

According to results of a previous study in our lab, five candidate proteins have been chosen and were identified as: 1- Lipoamide dehydrogenase (LpdA) (EC 1.8.1.4), 2-Enolase (EC 4.2.1.11), 3 - Elongation factor-Tu (EF-Tu), 4 - Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12), 5- DNA-binding protein from starved cells (Dps).

The selection of candidate proteins was based on their biological and immunological characters. The immunological functions of these proteins have already been reported in different species including bacterial, and fungi (as described in the Literature Review). These proteins are well expressed and conserved at the genetic level in both SE and ST (the gene encoding each of these proteins is found with high identity (>99%) in SE and ST based on Genebank, NCBI). In our previous research, antibodies against these proteins were detected in layers that were naturally infected with SE, as well as in vaccinated and challenged layers. The surface expression of all these proteins has been reported (as described in the Literature Review). These proteins are functionally involved in DNA and RNA activities, or in center metabolism and energy production. In addition, they have been reported as virulent factors associated with bacterial adhesion and invasion to the host cell during the infection. Interestingly, Dps is able to protect the bacterial DNA in certain starving environments.

**Production and purification of selected recombinant proteins**

Affinity tags are highly efficient tools when they are used with recombinant DNA techniques, which allow for a minor modification of proteins of interest leading to efficient detection, characterization and purification. The most widely used affinity tags for recombinant proteins purification is the polyhistidine tag. It is frequently used because of its low immunogenicity and small size. In addition, many proteins function with the polyhistidine tag positioned at either the N- or C-terminus, and purification methods can be carried out under both native and denaturing conditions (Young et al., 2012). The genes of selected recombinant proteins of this project were cloned in pQE30 vectors, which allowed a 6xhis-tag was installed at the N-terminal of recombinant proteins. There are various advantages of placing a tag at the N-terminal end, such as an efficient translation from initiation sites of pQE30 and this tag also can be removed easily (Sachdev and Chirgwin, 1998).After overexpressions by induction with IPTG, affinity-purification of these recombinant proteins was achieved with the use of Ni-NTA, which exhibited high affinity for adjacent histidine residues.

For this protein purification, we used a batch procedure described by QIAGEN at first. This procedure entails binding the protein to a Ni-NTA resin in solution and then packing the protein-resin complex into a column for the washing and elution steps. Since imidazole competes with his-tag to bind Ni-NTA, the washing step was achieved by twice volume of 20 mM imidazole to the bacterial lysate. Single concentration of imidazole in small volume of washing buffer could not wash out all the unwanted proteins because these nonspecific binding proteins with various numbers of consecutive histidine residues have different binding strength with Ni-NTA matrix and various concentrations of imidazole are necessary to remove them. From our previous SDS-PAGE result (see Figure 1), there were a lot of non-targeted

proteins (additional bands) appearing with recombinant proteins. Recombinant proteins with poor purity are not optimal to be used, because the contaminants might interfere with specificity of all further detection and evaluation, both *in vitro* and *in vivo*. This is why we finally decided to use FPLC to perform the purification. Therefore, different gradients of imidazole were used to wash out as many as possible of unwanted proteins slowly and efficiently before the elution of target proteins. Because imidazole would compete with his-tag to bind Ni-NTA, different gradients of imidazole were used to remove as many unspecific binding proteins as possible and elute targets proteins with high purity at the end of the process (Kuo and Chase, 2011). Purified proteins used in our later experiments still contain a 6xhis-tag, which was believed to be too small to interfere with the structure and function of the recombinant proteins as discussed before.

Dps showed two bands after purification, a finding similar to that of Hanna *et al.* in 2008. According to results of SDS-PAGE and immunoblot with anti-His, it implies that the upper band seemed to be recombinant Dps with his-tag (see Figures 2 and 3) and the other one might be degradations resulting from the various steps of expression and fractionation (Hanna *et al*., 2008). The relatively high salt concentration (300 mM NaCl) in buffers used during purification procedure might be the crucial reason for these degradations according to Stephani et al. (2003), who reported the degradation of Dps *in vitro* after its isolation from bacteria. Surprisingly, for the other proteins, no degradation bands after FPLC purification were detectable. This could imply that they might be resistant to the experimental reagents used in the procedure in order to maintain their integrities. Indeed, there is little information regarding their degradation during purification previously reported.

**Immunogenicity of selected recombinant proteins**

GAPDH, Enolase, LpdA and EF-Tu were reported previously as immunogenic in other species including bacteria, fungi and parasite while there was little information about immunogenicity of Dps as discussed before. In the current study, their immunogenicities in SE and ST are of the higher interest.

The evaluation of immunogenicity of selected proteins was achieved by immunoblotting. First, we assumed that if these proteins conserved in both SE and ST were immunogenic, they would be recognized by the antibody generated against these serovars. So the antisera from layers immunized with SE were used as primary antibody to detect their immuno-reactions with selected proteins. Unfortunately, specific recognition between antibody and selected proteins could not be efficiently displayed (data not shown). To resolve this problem, we decided to boost the sensitivity of the immunoblot, and then a high sensitive fluorescent western-blot with signal exposed on X-ray film was performed (Faoro et al., 2011). Except GAPDH, the other recombinant proteins were undetectable as well as many non-specific bands were visible (see Figure 4.). There are many possible reasons for this. One could be the low purity of proteins. These proteins were isolated from *E. coli*, which has numerous proteins in common with *Salmonella* Enteritidis. When these common proteins were not removed effectively, they tended to interfere with the result by reacting with antibodies. Another reason could be the various quantities of specific antibodies against each selected protein contained in antisera. The antibodies contained in the antisera included not only antibodies against selected

proteins, but also antibodies against many other SE whole cell proteins. So the quantities of antibodies against each selected protein might not be enough to have clear and convincing results to demonstrate their immunogenicity. The result also implied that quantities of specific antibodies for each protein might be various in this mix antisera and that only antibodies against GAPDH were present in sufficient quantities for specific recognition. Moreover, other facts might be potentially involved as well, such as GAPDH could be more immunogenic than other proteins so GAPDH might be able to generate more antibodies than other proteins, or avidities of different specific antibodies were various. After numerous repetitions with all the modifications of condition parameters without any better result, we decided to change the method and tried to produce antibodies *in vivo* against selected proteins directly. This is why laying hens were immunized intramuscularly (IM) with selected proteins respectively, then egg yolk IgY was extracted and specificities of these antibodies were later analyzed with western-blot. We assumed that if our selected proteins were immunogenic, they should be able to stimulate immune responses, and to induce antibodies against each of them. To our satisfaction, our new western-blot results supported this hypothesis. It showed that these yolk IgYs were able to recognize each recombinant protein we produced (Figures 2 to 6).

The inoculum (1 ml) was administered to the hens intramusculary at 2 sites on the pectoral muscle. The dose of antigen (50 μg) was chosen on the basis of various similar procedures devoted to production of specific egg yolk antibodies against antigenic proteins. With the same conditions (protein, IFA as adjuvant, and IM injection), it appears that a range of 10 μg – 100 μg of protein could produce sufficient amounts of specific IgY antibodies to be identified (Khan et al, 2003; Erhard et al, 2000).

We also found that these antibodies could recognize each protein conserved in whole-cell proteins and OMPs (outer membrane proteins) of SE and ST, the only exception being Dps that was not detected in OMPs (see Figure 7). This result was in accordance with a previous report on GAPDH, Enolase, LpdA and EF-Tu as bacterial surface-expressed proteins; this later study also reported that these proteins could be expressed on surface of SE and ST. This finding is important because the induced antibodies might find and bind to these surface-expressed proteins in order to eliminate infection and neutralize bacterial virulence. In previous researches, all these five proteins, including Dps, were reported to be able to be expressed on cytoplasmic membrane. Dps is a DNA-binding protein, and when bacteria are suffering from some severe adverse environment conditions and stress, Dps is expressed in large quantity to protect the bacteria from the oxidative stress and limited nutrients (Martinez and Kolter, 1997). We assumed that Dps might appear in OMPs in large quantity produced when cells are stressed based on study of Brown et al in 2012, which implied that *Salmonella* outer membrane might be enriched with Dps in a phagosome-mimicking condition. Since our OMPs were extracted from bacterial cell growing under suitable conditions, Dps might not be expressed on the outer membrane or in a quantity not sufficient to be detected.

According to data of Genebank, all these proteins are highly identical in SE, ST and *E. coli*, so all the selected proteins of SE and ST should be recognized in whole-cell proteins of *E. coli*. This finding of cross reactivity suggested that antibodies induced by proteins of SE and ST might be able to recognize and eliminated *E. coli* infection, which is also a common pathogen in chicken.

Hens and egg production

In this experiment, 18 Special-pathogen-free (*Salmonella* free) White Leghorn hens arrived at 14 weeks of age, and were separated by cages individually marked with the numbers #1 to #18. Feces were collected and detected weekly to ensure that hens remained *Salmonella* free. For almost hens, the first eggs were laid at around 21 weeks of age, and the production for each normal bird was around 5 to 7 eggs per week. Among these birds, #1, #5 and #6 were exceptions for different behaviors. Bird #1 had difficulty to get feed (no matter the feed texture presented) so it developed more slowly than other birds and gained less weight. It laid no egg during the experiment, which was possibly because of malnutrition. For bird #5, its antibody levels in both yolk and serum were significantly low (at least less than half of other birds). This humoral immune deficiency sign could be caused by various factors such as inherent deficiencies or bacteria or virus infections. For bird #6, its laying was delayed for about 10 weeks compared to the others, and started at 31 weeks of age; otherwise it had normal physical development and antibody levels. Because of these behaviors, Birds #1 and #5 were not included in the results while bird #6 was included due to its normal antibody levels obtained.

Egg antibody production

The commercially available chicken IgY ELISA quantitation set and chicken IgA ELISA quantitation set were used to assess antibody (IgY and IgA) produced in eggs and sera. The

highlight of this method was that it is a quantitative protocol so that it can provide an idea about antibody quantities. In addition, these sets are widely used in recent researches for antibody quantifications (Natsuda et al., 2010; Rocha et al., 2011; Nandre et al., 2012; Nandre et al., 2013). Because of all these advantages, this was the optimal protocol to be used. The working dilutions of our sample antibodies (1:5 000 for IgY and 1:1 for IgA) were decided by the effectiveness of result readings and calculating according to the manufacturer's instructions (Bethyl Laboratories). The concentration of IgY in egg yolk was much higher than that of IgA in egg white, and the optimal dilution of samples was necessary to select to ensure the OD of final ELISA plates reading was comparable to standard values (as described in Materials and Methods) and detectable. This is why we used working dilutions of 1:5 000 and 1:1 for IgY and IgA respectively.

**Specific egg yolk IgY levels**

IgY is recognized as an important maternal antibody that is selectively secreted from the circulation of the hen into the egg yolk and then absorbed across the yolk sac membrane into the embryonic circulation. It can protect newborn chicks against pathogens before development and maturity of their own immune system one to two weeks after hatch. It has been reported that the amount of total IgY in yolk ranges from 60 to 150 mg/yolk of which 3-15 mg/yolk are antigen-specific (Kovacs-Nolan and Mine, 2012). Our results are in accordance with these data, with quantities of specific yolk IgY ranging from 4 to 17 mg/yolk. Yolk specific IgY levels induced by recombinant proteins showed a similar trend during the laying period (Figures 7 to 11). IgY levels increased after each injection, and reached a peak

2-3 weeks later, then decreased afterward. Levels were also more stable after the 3rd injection than those after the 2nd injection. Also we found that the amount of IgY in the yolk was correlated with the IgY concentrations in serum, as reported in previous work (Figure 12) (Kramer and Cho, 1970; Loeken and Roth, 1983; Hamal *et al*., 2006).

**Specific egg white IgA levels**

During egg formation, IgA induced by local immunity is deposited into the egg white in the oviduct as maternal antibodies. Its major function in the newly hatched chick, as a protective Ig in the alimentary tract, is to provide protection to young chicks until their own immune response become fully effective (Hamal, 2006; Kovacs-Nolan and Mine, 2012). During the whole experimental period, specific IgA induced by GAPDH, Enolase and Dps were not detectable until 1 to 2 weeks after the 3rd injection, and the levels ranged from 90 to 180 $\mu$ g/ml (Figures 13-15), whereas, the specific IgA against LpdA and EF-Tu were almost undetectable during the whole trial time (data not shown). When compared together, the results showed specific antibody levels in yolk, serum and white appeared positively correlated among each other, and GAPDH, Enolase and Dps might have induced higher antibody levels that LpdA and EF-Tu.

Because IgA is a major mucosal immunoglobulin and because its presence in the egg is due to mucosal secretions deposited into the egg white, the finding of specific IgA against GAPDH, Enolase and EF-Tu in the egg white after immunization would suggest that these three proteins might be able to stimulate mucosal immune response. This is a very valuable finding

for the development of an oral vaccine because if specific IgA against these proteins could be produced even by intramuscular administration, then it is expected that these proteins could induce a strong local mucosal immune response if oral administration is used, which is the optimal route to stimulate local mucosa immune response.

Although the quantities of specific IgA were very low, this was the first time that specific IgA against bacterial proteins in egg white are observed,. In the past, the research about specific IgA from egg white has been very scarce, and there is hardly any study about egg white specific IgA against protein antigen. For virus antigen, Hamal et al. in 2006 tried to test specific IgA against Newcastle disease virus (NDV) and infectious bronchitis virus (IBV) in egg white but no satisfactory result was obtained.

*Salmonella* Enteritidis and *Salmonella* Typhimurium being enteric pathogens, mucosal immunity (secretory IgA) plays a fundamental role in the protection against *Salmonella* infection (Hackett 1993). In this study, because our objective was to assess the immunogenicity of selected recombinant proteins, as the birds were inoculated with the selected proteins intramuscularly, we did not determine the mucosal immune response directly. We assumed that immunization of layers with the selected recombinant proteins by routes that induce a strong mucosal immune response such as oral administration might potentially increase the production of mucosal antibody (secretory IgA). Although the present study indicated that these selected candidate proteins were immunogenic and can stimulate the production of specific antibodies in eggs, both IgY and IgA, the results should be considered preliminary because of the administration route and the lack of a large study size. Therefore,

future experiments should involve a larger group of birds and replicates, as well as direct assessment of mucosal immune response such as secretory IgA evaluation, especially after determining the suitable route for administering the selected proteins in layers.

# CONCLUSION

In conclusion, we found the satisfactory techniques to produce and purify recombinant proteins, and also demonstrated immunogenicity of these selected recombinant proteins and their potentials to be a new subunit vaccine against SE and ST in chickens in this research.

First, pQE30 vector is useful tool to install a 6xhis tag to the N-terminal of each protein, then after overexpression in *E.coli* with affection of IPTG, these recombinant proteins would be purified by FPLC using the specific affinity between his-tag and Ni-NTA resin column. These gained proteins were with high purity and well conserved the His-tag, which suggest that techniques we used to produce and purify recombinant proteins are ideal to achieve a satisfying result.

Because antibodies collected from hens injected with recombinant proteins could recognize each of them with immunoblot tests, we demonstrated that these proteins could induced a specific antibody response in hens which demonstrates the immunogenicity of these proteins. Moreover, these specific antibodies induced by recombinant proteins could also recognize specific antigens in whole-cell proteins and OMPs of SE and ST, which implied a possible recognition between these antibodies and bacterial cells. The results also suggested that antibodies induced by proteins of SE and ST might be able to recognize and eliminated *E. coli* infection, which is also a common pathogen in chicken, due to the existence of the cross reaction between antibodies against recombinant proteins and *E.coli* whole-cell proteins.

In addition, we evaluated the titer of specific antibodies induced by proteins in blood (IgY), egg yolk (IgY) and egg white (IgA) using ELISA. We found that GAPDH, Enolase and Dps

induced and maintained higher level of specific antibodies than LpdA and EF-Tu in blood, egg yolk and white. Also, specific IgA against LpdA and EF-Tu were not detectable during our study. IgA is a very important antibody produced by local mucosal immune response which is also a really significant aim about the oral vaccine against SE infection; therefore it is very interesting to find that GAPDH, Enolase and Dps could even induce IgA production by intramuscular injection, which is not the optimal administration route for mucosal antibodies production. Based on these findings, we demonstrated that these five selected recombinant proteins, which are GAPDH, Enolase, Dps, LpdA and EF-Tu, were immunogenic since they were capable to stimulate the production of specific antibodies *in vivo* (blood, egg yolk and egg white) after immunization of layers. However, their abilities of stimulating local mucosal responses and the production of local sIgA via oral administration are not proved directly and need to be further studied.

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