Chicken Egg Yolk Antibodies Specific for *Salmonella enteritidis* and *S. typhimurium* Against Experimental Salmonellosis in Mice

A Thesis

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by

Lin Fang

In Partial Fulfilment of the

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Chicken Egg Yolk Antibodies Specific for *Salmonella enteritidis* and *S. typhimurium* Against Experimental Salmonellosis in Mice

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ABSTRACT

Lin, Fang. M.Sc., The Faculty of Food and Agricultural Sciences, University of Manitoba. August, 2003. Chicken Egg Yolk Antibodies Specific for *Salmonella enteritidis* and *S. typhimurium* Against Experimental Salmonellosis in Mice. Supervisor: Dr. Gary Crow.

The study describes procedures that were used to isolate and purify three high purity antigens [outer membrane proteins (OMP), lipopolysaccharide (LPS) and fimbriae (FIM)] from *Salmonella typhimurium and S. enteritidis*. Polyclonal antibodies were produced in chickens immunized with the three antigens. The efficacy of purified chicken egg yolk homotypic antibodies specific for OMP, LPS or FIM in controlling experimental salmonellosis in mice was investigated. Mice were challenged orally with 1.5×10^9 colony forming units (c.f. u.) of *Salmonella enteritidis* or 1×10^9 c.f u. of *S. typhimurium* and then orally treated with 0.2 ml of high titer anti-OMP, -LPS or -FIM yolk antibodies 30 min after the challenge and then once each on the following two days. In mice challenged with *S. enteritidis*, antibody treatment resulted in survival rates of 69.2, 46.2 and 40% using OMP, LPS or FIM specific antibodies, respectively, in contrast to only 15.4% in control mice ($p < 0.05$). In the *S. typhimurium* trial, survival rates were 76.9, 58.3 and 36.4% using OMP, LPS or FIM specific antibodies, respectively, in contrast to 0% in the control mice ($p < 0.05$). *In vitro* adhesion of *S. enteritidis* and S. *typhimurium* to HeLa cells was significantly ($p < 0.05$) reduced by each of the anti-OMP, -LPS, and -FIM homotypic antibodies. The results demonstrate

that egg yolk antibodies specific for *Salmonella* OMP, LPS, or FIM will passively protect mice from experimental salmonellosis when administered orally. Of these antibodies, anti-OMP exhibited the highest level of protection *in vivo* and *in vitro*. Other animals and aves may also be protected against salmonellosis by the same antibodies, either singly or combined.

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LIST OF ABBREVIATIONS

BCA ..... bicinchoninic acid

BSA ..... bovine serum albumin

^{o}C ..... degree Celsius

CFA ..... colonization factor agar

cfu ..... colony forming units

DTH ..... delayed Type Hypersensitivity

EDTA ..... ethylenediamine tetraacetic acid

ELISA ..... enzyme-linked immunosorbent assay

ETEC .....*Enterotoxigenic Esherichia coli*

FIM ..... fimbriae

g .....gram

h ..... hour

HEPES ..... 2-ethanesulfonic acid

IFNg ..... interferon-gamma

IgG ..... immunoglobulin G

IgY .....chicken immunoglobulin G

IL-2 ..... Interleukin 2

kDa ..... Kilodalton

LPS ..... lipopolysaccharide

M ..... molarity

MEME ..... Vitacell's Minimum Essential Medium Eagle

mg ..... milligram

min ..... minutes

ml ..... milliliter

mM ..... millimolarity

nm ..... nanometre

OD ..... optic density

OMP ..... outer membrane protein

PBS ..... phosphate buffer saline

PBST ..... phosphate buffer saline with Tween-20

pH ..... acidity

S. ..... *Salmonella*

SDS ..... sodium dodecyl sulfate

SEF ..... *Salmonella enteritidis* fimbriae

STF ..... *Salmonella typhimurium* fimbriae

TCRab ..... T cell antigen receptor

TNFa ..... tumour necrosis factor-a

ug ..... microgram

ul ..... microliter

vol ..... volume

wk ..... week

wt ..... weight

INTRODUCTION

Salmonella enteritidis and *S. typhimurium* are the commonest serotypes which can cause disease in humans and other animals. The global increase in the incidence of *S. enteritidis* and *S. typhimurium* infection is attributed to the consumption of eggs and poultry that harbour the organisms. The incidence of poultry related infections due to *Salmonella* have been steadily increasing in North America in recent years (Müller *et al.*, 1991; Anonymous 1992; Henzler *et al.*, 1994).

In the family, *Enterobacteriaceae,* including the Genus *Salmonella*, pathogenic bacteria have a variety of virulence-associated surface structures such as lipopolysaccharide (LPS), capsular, surface layers, flagella, and fimbriae (or pili). Among them, the outer membrane proteins (OMP) also known as porins, play a role as pathogenicity determinants (Galdiero *et al.*, 1990). OMPs are exposed on the surface of the bacterial cell and they can serve as phage receptors and antigens (Kuusi *et al.*, 1979). Numerous studies have elucidated their biological functions and immunogenic properties (Isibasi *et al.*, 1988). With their physical and biological characteristics, OMPs have been used successfully as vaccine antigens and a number of workers have proven that they are good immunogens, protective in both active and passive immunization studies of mice with sera (Isibasi *et al.*, 1988). LPSs are an integral part of the cell wall of gram negative bacteria. As the name implies, LPSs consist of a lipid core and an outer surface composed of different saccharides, each characteristic of different serovars (O antigens). As a cell surface component, LPSs can interact with the environment (i.e. a host during infection) and exhibit a number of biological activities, including pyrogenicity, lethal toxicity and complement

activation. Fimbriae (FIM) are hair-like structures that emanate from the bacterial cell and allow the bacteria to adhere to solid surfaces. Fimbriae are important virulence determinants because they mediate specific interactions between the bacteria and host cells. Some fimbriae bind specific receptors on the intestinal epithelium and allow intestinal colonization (Clegg *et al.*,1987). The contribution of flagella to the virulence of *Salmonella* has been examined in mouse models (Ciacci-Woolwine *et al*, 1998; Lockman *et al*, 1990, 1992; Weinstein *et al.*, 1984; Carsiotis *et al.*, 1984) and to some extent in chickens (Vercoe *et al.*, 1999; Lee *et al.*,1996; Oyofo *et al.*,1989). Flagella have also been shown to play a key role in pathogenesis by *S. enterica serotype typhimurium*. Flagella help bacteria survive within macrophages (Weinstein *et al.*, 1984), up-regulate tumor necrosis factor alpha (Ciacci-Woolwine *et al.*, 1998) and mediate attachment to epithelial cells. However, there is a paucity of information on the role of flagella in the pathogenesis of *S. enteritidis* in the avian species.

It has been well documented that bacterial virulence can be reduced by passive immunization with antibodies including chicken egg-yolk anti-fimbrial antibodies (Müller *et al.*, 1991; Timo 1980). Anti-K88, -K99, and -987P fimbriae egg- yolk antibodies have been shown to protect piglets from the *E. coli* infection. These results provide further evidence for a role of the surface structure of bacteria as virulence factors and indicated that antibodies against these bacterial surface structures can reduce bacterial pathogenicity. Passive protection by egg-yolk antibodies may serve as a tool for the control intestinal colonization and invasion in *Salmonella* infection. This study was to determine the efficacy of chicken egg yolk antibodies specific for S*almonellae* OMP, LPS, and FIM against experimental salmonellosis in mice *in vivo*

and their ability to inhibit adhesion to intestinal receptors *in vitro* , and thereby possibly extend their efficacy to control *Salmonella* infection in humans and other animals, especially in the poultry industry.

LITERATURE REVIEW

1. Overview of Salmonellosis

Salmonellosis is a common cause of human food-borne illness. It is also an important cause of disease in animals. It has been estimated that 95% of human salmonellosis from foods are from poultry with other food animals being a major reservoir of the disease (Kerr *et al.,* 1992).

The worldwide incidence of *Salmonella* food poisoning has increased dramatically during the last few years. Although the proportion of food-poisoning outbreaks and cases in which the source of infection can be positively identified is small, poultry and poultry products are repeatedly implicated. *Salmonella* from poultry currently enter the human food chain mainly as a result of carcass contamination from infected material or from eggs. *S. typhimurium* and *S. enteritidis*, the strains of *Salmonella* that currently are infecting flocks of table-egg-laying chickens do not generally cause clinical disease in these birds. Rather, it is the colonization of chicken eggs that causes clinical disease in human consumers (Kerr *et al.,* 1992).

S. typhimurium and S. enteritidis have been isolated from the intestinal tract, ovary, oviduct and spleen of naturally infected hens (Poppe *et al.,* 1993). A study analyzing the results of 65 *S. enteritidis* food-borne outbreaks in the United States, found 27 were associated with the consumption of eggs or egg-containing foods (St.Louis *et al.,* 1988). Furthermore, *S. enteritidis* has been isolated from the surface of clean, intact shelled eggs (Humphrey, 1994). Routes of bacterial invasion of the

egg have yet to be defined. Trans-ovarian transmission has been proposed for *S. typhimurium* and *S. enteritidis* (Henzler *et al.*, 1994), although there continues to be controversy. There are two possible routes of *S. enteritidis* contamination of eggs, trans-shell (horizontal) or trans-ovarian (vertical) transmission. The resident site of the pathogen is the yolk, the follicular membrane surrounding the yolk, or the albumen (St. Louis *et al.,* 1988). Translocation of organisms from the peritoneum to the yolk sac or to the oviduct via macro-phages is a possibility suggested by previous studies (Coyle *et al.,* 1988). Egg contamination by penetration of the shell by organisms present in chicken feces deposited on the outside of the egg as it passes through the cloaca is also feasible (St.Louis *et al.,* 1988).

S. enteritidis outbreaks continue to occur in the U.S. and Canada. The CDC (Centers for Disease Control) estimates that 75% of these outbreaks are associated with the consumption of raw or inadequately cooked Grade A whole shelled eggs. However, egg-producing breeder flocks and commercial flocks are also implicated in causing human illnesses.

2. Historical Naming and Classification of *Salmonella*

In 1885, the pioneering American veterinary scientist, Daniel E. Salmon, discovered the first strain of *Salmonella* in pigs when he was attempting to identify the organism that caused swine fever (hog cholera). The organism now is known as *S. choleraesuis.* Due to their importance in pathology, the first *Salmonellae* species were given names (in latinized form) that indicated the disease and animal from which they were isolated, e.g. *S. choleraesuis* (i.e. hog cholera). This form of nomenclature was

abandoned when it was discovered that some *Salmonellae* species were not host specific (i.e. S. *typhimurium* is the causative agent of mouse typhoid and is also a human pathogen). Descriptive naming was replaced by naming newly identified serotypes from the places where they were first isolated, e.g. *S. london, S. havana,* etc. (Old, 1992).

Salmonella are gram-negative facultative anaerobic rods, belonging to the bacterial group, Enterobacteriaceae. There are over 2300 known serovars of *Salmonella* with their classification being controversial (D'Aoust, 1995). The most widely used and best known classification system is the Kauffmann-White scheme (Lim, 1986). In this scheme *Salmonella* are distinguished from each other by antigenic structures such as somatic (O), flagellar (H) and capsular (Vi) antigens. *Salmonella* with common somatic antigens are grouped together and designated into 46 serogroups (Tsang *et al.,* 1991). Serogroups A, B, C, D and E account for 95% of human salmonellosis (Kerr *et al*, 1992). In recent years, concerns have been raised, as particular strains of the bacteria have become resistant to traditional antibiotics, in both animals and humans. *Salmonella* serotype *typhimurium* and *enteritidis* are the most common strains in the North America. *Salmonella* has been known to cause illness for over 100 years.

2.1. The Kauffmann-White scheme

The Kauffmann-White scheme is intended as a diagnostic tool, by which *Salmonellae* species are distinguishable from each other by their particular O (LPS, lipopolysaccharide or somatic), H (flagellar) and Vi (capsular) antigens present on the

cell wall (Old, 1992). Thus, each *Salmonellae* serotype is recognized by its distinct antigenic formula. *Salmonella* serovars sharing the same O-antigens are collected into serogroups (O groups) and arranged alphabetically by H antigens within these groups (Le *et al*, 1974).

2.2. Subdividing *Salmonella* species

Each *Salmonella* serovar is considered to be a separate species in the Kauffmann-White scheme of nomenclature (Le, 1987). The concept of "one serovar-one species" is untenable, as over 2300 *Salmonella* serovars have been identified (D'Aoust, 1995; Le, 1987). Several attempts to reduce the number of *Salmonella* species have been reported. Baorman et al in 1944, first propossed that all *Salmonella* isolates should fall within one of three species of *Salmonella.* The three recognized species in the scheme were: *S. choleraesuis, S. typhosa* and S. *kauffinannii.* Kauffmann and Edwards (1952) proposed a similar scheme and identified *Salmonella* on the basis of S. *choleraesuis, S. typhosa* and *S. enterica.* In 1963, Ewing proposed another three species concept based on S. *choleraesuis, S. typhi* and S. *enteritidis.* In the above schemes, all serovars except the first two are included in the last species named. For example, in the Ewing scheme both S. *choleraesuis* and S. *typhi* serotypes are considered *Salmonella* species, with all other *Salmonella* serotypes being grouped in the last category, S. *enteritidis.* Kauffmann (1966) divided the genus *Salmonella* based on biochemical characteristics into four subgenera, S. *kauffinannii* (subgenus I), S. *salamae* (subgenus II), *S. arizonae* (subgenus III) and S. *houtenae* (IV). With the exception of a few species, all serovars in subgenera II to IV are designated by

antigenic formula only, while all those in subgenus I are given names (Old, 1992). This four species system includes *S. arizonae* as a *Salmonella* species. In the approved Lists of Bacterial Names, *Salmonella* is designated as five species, *S. alizonae, S. choleraesuis, S. enteritidis, S. typhi* and S. *typhimurium* (Skerman *et al*, 1980).

Recent DNA homology and numerical taxonomy studies have shown that all *Salmonella* serotypes belong to only two species, *S. enterica* and S. *bongori,* with S. *enterica* subdivided into six subspecies (Le, 1987; D'Aoust, 1995). This two species concept is in use by the World Health Organization (WHO) collaborating centre for reference and research on *Salmonella* (D'Aoust, 1995). This concept uses the antigenic determinants of the Kauffmann-White scheme. Like the Kauffmann-White scheme, all serotypes belonging to subspecies I (enterica) are designated names, while the others are designated by antigenic formula only. In this concept serovars are named as follows, the old Kauffmann-White scheme designation of *S. enteritidis is* now S. *enterica* subspecies *enterica* serovar Enteritidis (D'Aoust, 1995). The two species concept is being challenged by microbiologists and acceptance of this system will be influenced by rabbit (D'Aoust, 1995; Old, 1992). In the following manuscripts, the Kauffmann-White designated names are used.

3. The Biology of *Salmonella* Infection

Neither *S. typhimurium* nor *S. enteriditis* are major clinical pathogens of the chicken unless infection occurs in the very young (<2-day-old) chick. Although *Salmonellae* rapidly become systemic in the 1-2-day-old chick leading to a typhoid-

like disease (i.e. massive systemic infection), infection in the older chick is largely restricted to the intestine. Some *Salmonella* invasion occurs but this is not enough to cause systemic disease (Blankenship *et al.*, 1993). Nonetheless, systemic bacteria may play an important role in bacterial persistence and translocation to the reproductive tract.

Infections of mature hens by *S. enteritidis* are asymptomatic and normal measures of hen health such as weight gain or egg production are unaffected. Furthermore, *S. enteritidis* can specifically colonize the oviduct in the mature hen (Reiber *et al.*, 1995). From this unique niche, the bacteria infect eggs while they form inside the hen, and before the protective shell is in place. Colonization of eggs as they form inside the oviduct is remarkably effective in disseminating *Salmonella*. Chicks that develop from infected eggs eventually become a mature hen, which is infected with *Salmonella* (vertical transmission). Eggs that are destined for the breakfast table may result in the chance contamination of humans. This unusual route of virulence suggests that *S. enteritidis* possesses unique virulence factors that are not found in other *Salmonella* serovars. The importance of understanding this aspect of *Salmonella* infection is twofold (i) human infection arises directly from the consumption of contaminated eggs produced by infected laying hens and (ii) broiler breeders transmit infection to their progeny. In both cases it is likely that a combination of surface contamination with *Salmonella*-infected feces and deposition in the forming egg of the *Salmonella* organisms from either the ovary or oviduct occur (Barrow *et al.*, 1991).

Although a proportion of *S. typhimurium* and *S. enteriditis* are invasive in non-neonatal chickens these numbers are relatively small and the majority of bacteria remain in the gut lumen. Indeed, persistent infection of the caeca with these organisms represents the greatest risk of contamination for food. In the intestine of chicks some damage occurs, particularly in the period just after initial colonization, although this damage rarely results in clinical disease. The precise nature and significance of the intestinal and systemic lesions induced after infection has not been studied in detail.

There is also evidence that the serovar, *S. enteritidis*, is of major public health significance in terms of egg-borne infection as it is able to persist in the reproductive tissues after it has been cleared from the alimentary tract. How this happens is not clear.

4. The main surface antigens of *Salmonella*

The surface components of *Salmonella* provide a protective and yet porous shield against the outside world and are of great significance in the interaction with its habitat when it passes through the acidity of the stomach, the lumen of the gut, the extracellular space of host tissues and the inside of the macrophage (Wary, 1994). The antigens related to their virulence are mainly lipopolysaccharides, fimbriae and outer membrane proteins.

4.1. Lipopolysaccharides of *Salmonella*

LPSs are an integral part of the cell wall of gram negative bacteria (Rietschel *et al.*, 1992). As a cell surface component, LPSs can interact with the environment (i.e. a host during infection) and exhibit a number of biological activities, including pyrogenicity, lethal toxicity and complement activation (Kotina *et al.*, 1990). LPSs are composed of a lipid moiety which is covalently bound to a polysaccharide portion. LPS molecules are anchored to the outer membrane of the cell through their lipid moiety, lipid A. Virtually all biological activities of LPS reside in the lipid A portion. Lipid A is composed of a glucosamine disaccharide and four hydroxy fatty acids (Pearson, 1985). This backbone is ubiquitous and highly conserved among gram negative bacteria.

The polysaccharide part of LPS consists of two regions, the core and the O specific chain. The core is subdivided into the lipid A-proximal inner core and the lipid A-distal outer core. The structural variability of the core within different bacterial species is thought to be limited.

The O-specific chain consists of a repeating oligosaccharide unit which contains up to six sugar residues and may be 70 units long (Peterson *et al.*, 1985). The nature, ring form, type of linkage, and substitutions of the individual monosaccharide residues is characteristic and unique to a bacterial species. Thus, the O-chain is species-specific (Rietschel *et al.*, 1992). In S. *enteritidis,* the oligosaccharide unit consists of a trisaccharide backbone composed of mannose, galactose and rhamnose and a side sugar tyvelose which is attached to the mannose residue. The sugar tyvelose is the unique component of O-antigen factor 9 of Salmonella serogroup D. In contact with the immune system of vertebrate animals, LPS (particularly the O

specific chain) evokes the production of antibacterial antibodies which in turn can be used to identify these bacteria.

4.2. Fimbrial protein

Fimbriae are a family of polymeric proteinaceous surface organelles expressed by many bacteria including several members of the family Enterobacteriaceae (Smyth *et al.*, 1994). Fimbriae are hair-like structures that emanate from the bacterial cell and allow the bacteria to adhere to solid surfaces. Fimbriae of the Enterobacteriaceae are a family broadly divided into two major classes: mannose-sensitive (Type-1) and mannose-resistant, on the basis of the ability of mannose to inhibit the hemagglutinating activity of the fimbriae. Type-1 fimbriae have a channelled appearance due to arrangement of subunits around a hollow core as seen under a transmission electron microscope (Clegg *et al.*, 1987). Fimbriae are important virulence determinants because they mediate interactions between the bacteria and host cells. Some fimbriae bind to the intestinal epithelium and allow intestinal colonization (Lindquist *et al.*, 1987). The expression of fimbriae by certain strains of *Salmonella* was first described nearly 40 years ago (Duguid *et al.*, 1958). However, until recently there was little understanding of the variety of *Salmonella* fimbriae and their functions. In the last 10 years, there has been a renewed interest in the molecular and antigenic characterization and functions of *Salmonella* fimbriae, which has resulted in the identification of their potential as diagnostic and protective antigens (Thorns, 1995). It is now evident that *S. typhimurium* and *S. enteritidis* can express several different types of fimbriae during their life cycle (Paranchych *et al.*, 1988).

The expression of fimbriae is tightly regulated by numerous environmental and cell signals which are still poorly understood. It is assumed, however, that their expression in specific niches in the infected host or in the environment plays an important part in the organism's survival. Current evidence therefore indicates that *Salmonella* can produce numerous fimbriae, which mediate a variety of functions and are important for the maintenance and survival of the organism in the host and its environment (Brubaker, 1985).

Most of *Salmonella* species are able to form type 1 fimbriae, but the physiological functions of the type-1 fimbriae in *Salmonella* are not clear. *S. typhimurium* bearing type-1 fimbriae STF21 were first described by Müller *et al* (1991). The STF21 form pellicles on the surface of static, artobic cultures and can thus reach greater culture densities than do nonpiliated variants (Old *et al.*, 1970). It has been proposed that STF21 mediate the mannose-sensitive adherence of *S. typhimurium* to human intestine (Duguid *et al.*, 1966) and to both human buccal and rat urinary tract epithelial cells (Timo *et al.*, 1980). In orally infected mice, the STF21 increase the virulence of *S. typhimurium*. This was attributed to their role in mediating bacterial adherence to the alimentary tract mucosa (Duguid *et al.*, 1976). *S. enteritidis* has been shown to produce three types of fimbriae, referred to as SEF14, SEF21 and SEF17 (or thin, aggregative pili) that are morphologically, biochemically and immunologically distinct (Müller *et al.*, 1991). The SEF14 fimbriae appear as fine, fibrillar structures, 3-5 nm in diamenter, and are not associated with haemagglutinating activity. Preliminary evidence implied that SEF14 fimbriae might be important virulence determinants, because they induce a protective immune

response in mice. Moreover, the limited distribution of the *sef* operon in *Salmonella* serovars suggested that they might be one the unique virulence factors which contribute to the unusual *S. enteritidis* pathogenesis in chickens (Collinson *et al.*, 1991).

4.3. Outer membrane protein (OMP)

Salmonella has a variety of surface components which are virulence-related. Among them, OMPs also known as porins, play a role as pathogenicity determinants (Galdiero *et al.*, 1990). OMPs are exposed on the surface of the bacterial cell and they can serve as phage receptors and antigens (Kuusi *et al.*, 1979). The OMPs of *S. typhimurium* are present as trimers and form trans outer-membrane water-filled channels, which facilitate the transport of small hydrophilic molecules (Pauln *et al.*, 1998). Under normal growth conditions, *S. typhimurium* express three porins, OmpD (34 kDa), OmpF (35 kDa), and OmpC (36 kDa). The 34k, 35k and 36k proteins, form pores across the outer membrane, through which small hydrophilic compounds can diffuse (Nurmine, 1978), and are therefore called porins. Numerous studies have elucidated their biological functions and immunogenic properties. OMPs, with their physical and biological characteristics, have been used successfully as vaccine antigens. Several research groups have proven that they are good immunogens and are protective in both active and passive immunization studies in mice with sera as the source of protective antibodies (Isibasi *et al.*, 1988).

5. Immunology of *Salmonella* Infection

Although the immune responses of chickens to infection with *S. typhimurium* and *S. enteritidis* have not been functionally dissected, bacteriological studies clearly indicate that immunity to these pathogens is generated. These responses are manifested by the control and the elimination of primary infection and by the development of strong resistance (immunity) to re-challenge with homologous bacterial strains (Barrow *et al.*, 1991; Hassan *et al.*, 1994).

Preliminary studies have indicated that both humoral and cell-mediated immune responses are generated during infection (Hassan *et al.*, 1994). These data clearly indicate that a functional anti-*Salmonella* response develops in the chicken and it is important to dissect this response further. Since the host-*Salmonella* relationship involves both extracellular and intracellular bacteria it is likely that the immune response(s) that are active against infection are equally complex.

In terms of functional studies of the immune system-*Salmonella* interaction, these have largely been restricted to mammalian hosts (principally the mouse). Immunity in the murine host to *S. typhimurium* is dependent upon TCRab T cells, B cells, IFNg and TNFa (Hormaeche *et al.*, 1994). Nonetheless, the host-pathogen relationships between *Salmonella* and the murine or avian host are distinct and the infection is likely to be controlled by very different immune effector mechanisms. For example, there are many immune cell populations unique to the mucosal immune system such

as the intra-epithelial, lamina propria and Peyer's patch lymphocytes that are functionally distinct from systemic cell populations. It is also clear that the requirements for induction and regulation of immune responses at intestinal sites can be quite different to the requirements for systemic responses (Roberts *et al.*, 1996). Moreover, *S. typhimurium* and *S. enteritidis* are found mostly in the gut lumen of chickens. IgA-dependent effector mechanisms may play a more important role in chickens than in the murine experimental models of typhoidal (systemic) disease. Indeed, there are many differences between the immune system of chickens and those of mouse and human, such as the ontogeny of B cells and the diversification of antibodies, the T cell control of the IgA response, the levels of systemic TCRgd T cells and the differential responses to infectious pathogens dependent upon the MHC (Davison *et al*, 2000). Thus, for a number of reasons it is important to determine the immunological mechanisms that operate against mucosal infection of chickens with *S. typhimurium* and *S. enteriditis* and this may be a focus of future research.

6. Epidemiology of *Salmonella* Infections

Salmonella infection is one of the most important problems in commercial poultry. There has been considerable variation in the occurrence of the most common *Salmonella* serovars in poultry in different countries and at different times. *S. typhimurium* has been among the most common serovars isolated from poultry in many countries, especially during the period from about 1950 until the late 1970s, it was the commonest *Salmonella* serovar isolated from poultry and other birds in the UK. During the period 1968 to 1973, it accounted for 41.1% of all isolates and was followed by *S. enteritidis* at 6.2%. However, during the last 10 to 15 years, *S.*

enteritidis has replaced *S. typhimurium* as the commonest serovar in poultry in many countries worldwide.

At the same, human salmonellosis caused by *S. enteritidis* infections has also increased dramatically worldwide in the past decade (Rodrigue *et al.,* 1990). In many countries, *S. enteritidis* has surpassed *S. typhimurium* as the most commonly isolated serovar (Poppe *et al.,* 1994). In the United Kingdom (UK), a 250 % increase in human isolations of *S. enteritidis* has been reported for the years 1982 to 1992 (Roberts *et al.*, 1996). In the early 1970s, *S. enteritidis* isolates from human in the United States (US) accounted only for 5% of the total (Tauxe, 1991). This number, however, increased to 20% by the year 1989, particularly in the northeastern states. In recent years, S. *enteritidis* outbreaks have occurred in most states (Madden, 1990). In Canada, the increase in the number *S. enteritidis* infections has not been as dramatic as in other countries (Poppe, 1994). However, *S. enteritidis* infections have increased to 16% ranking this pathogen second to *S. typhimurium* as the most commonly isolated serovar (Khakhria *et al.,* 1994).

In a study of domesticated farm animals, 3.5 times more *Salmonella* serotypes were identified from birds as compared to cattle, pigs and sheep (Haigh *et al.*, 1991). Poultry and poultry products, including foods made with shell eggs are an important vehicle of *S. enteritidis* infections in humans (Humphrey *et al,* 1988; St Louis *et al,* 1988). Contaminated feed can introduce *Salmonella* serotypes to the farm and animals can become infected either by eating contaminated feeds or from the environment that has been contaminated (Bryan, 1981). Large scale farming confines large number of animals in crowded areas, often sharing the same foods and water,

and *Salmonella* can spread by cross-infection. The presence of *S. enteritidis* in Canadian commercial layer flocks has been reported (Poppe *et al.*, 1993). *S. enteritidis* was isolated from environmental samples (dust/fluff, eggbelt samples and feces) in eight of 295 randomly selected flocks (2.7%). Naturally occurring infections of *S. enteritidis* in layer flocks are usually mild or subclinical with little effect on egg production (Poppe *et al.,* 1994). In the past, the main source of *Salmonellae* in poultry was the caecal contents of infected birds (Rampling *et al.,* 1989). However, this pathogen has been isolated from the ovaries, oviducts, liver, heart and spleen of condemned broilers and necropsied layer hens (Rampling *et al.,* 1989; Poppe *et al.,* 1994). *S. enteritidis* shares the same surface antigens with avian adapted *S. gallinanon* and *S. pullorum*. These similarities may account for the adaptation of certain *S. enteritidis* phage types to poultry (Rampling *et al.*, 1989). Shell eggs and foods containing eggs are a major vehicle of *S. enteritidis* infection in humans (Coyle *et al.,* 1988; St. Louis *et al.,* 1988). Implicated foods include ice cream, egg sandwiches, scrambled eggs, hollandaise sauce, eggnog, homemade pasta and bread coatings. Epidemiological studies of egg-associated *S. enteritidis* outbreaks show that improper food handling is the cause of these outbreaks (Morris, 1990). Pooling of eggs, exposing eggs to time and temperature abuse, eating raw or undercooked eggs, mixing egg shells with egg contents and poor blending sanitation have been identified as factors increasing the risk of infections (Morris, 1990). A recent outbreak in Canada of *S. enteritidis* occurred in an accredited care facility (Anonymous, 1992). This outbreak involved 95 confirmed cases of *S. enteritidis* PT 13. The most likely vehicle of infection was ready-to-eat foods contaminated by raw foods processed in

the same vertical mixer. Once identified as the source of contamination, the mixer was examined and dried egg was found under the mixer blade. An environmental swab of the egg revealed *S. enteritidis* PT 13.

Shell eggs are equipped with physical and chemical defence systems against bacteria (Parkinson, 1966). Physical barriers include the egg shell and the shell membranes. The pH of the albumen, which may reach 9 to 10, impedes microbial growth and is one the chemical defences of eggs. Antimicrobial agents such as lysozyme, which degrades the cell wall of gram-positive bacteria; conalbumin, which chelates iron; avidin, which binds biotin and apoprotein, which ties up riboflavin are also present in eggs (Parkinson, 1966). Once the egg is broken, the physical defences are no longer a barrier and homogenization of the egg contents can neutralize chemical agents.

There are two possible routes for *Salmonella* to enter shell eggs, trans-shell infection (horizontal) and trans-ovarian infection (vertical). In trans-shell infections, *Salmonella* present on the shell can pass through the pores of the egg when water and temperature differences occur between the egg and the surrounding environment (Lock *et al.,* 1992). A study by Haigh and Beets (1991) showed that immersing eggs into a broth containing *Salmonella* resulted in the contamination of the egg contents with the organisms. Egg pores are large enough to allow the entry of bacteria (Haigh *et al.*, 1991). Gast and Beard (1990) report a positive correlation between egg shell contamination and the presence of *S. enteritidis* PT 13 in fecal samples in hens artificially infected.

For *S. enteritidis,* there is evidence to suggest that the most probable route of entry into the egg is via trans-ovarian infection. *S. enteritidis* has been isolated from the contents of clean, intact shell eggs (Humphrey, 1994). Furthermore, *S. enteritidis* has been isolated from reproductive tissue of naturally and artificially infected hens in the absence of intestinal carriage (Gast *et al.*, 1990; Humphrey, 1994; Poppe *et al.,* 1993). Humphrey *et al* (1988) found that 72 % of naturally contaminated eggs had less than 20 cells of *S. enteritidis.* In a study of artificially infected hens, all yolk contents were *Salmonella*-negative, even though *S. enteritidis* was isolated from the albumen (Gast *et al.*, 1990). If *S. enteritidis* were infecting the yolk, larger number of bacteria should have been isolated from the egg contents (Humphrey *et al.*, 1994). These studies suggest that the most probable site of contamination within the egg is either the albumen or the vitelline membrane and not the yolk (Humphrey *et al.*, 1994).

7. Current Precautionary Measures for *Salmonella* Infection in Poultry

Salmonellae are among the major worldwide bacterial pathogens in chickens (Bryan, 1981). Prevention of *Salmonella* infection is important for poultry health and for the food industry. Currently, no efficient methods are available. Nevertheless, regulatory authorities in many countries have adopted regulations in an effort to prevent and control the occurrence and spread of *Salmonella* in the poultry industry. In general, they specify measures to avoid the introduction of *Salmonella* on the farm and to control *Salmonella* in flocks of layers as outlined below:

7.1. Clean the production chain from the top: The major strategy to control *Salmonella* in poultry should be directed forwards the cleaning of the production

chain from the top to prevent *Salmonella* introduction through breeder flocks. Control measures to prevent the introduction and spread of *Salmonella* infection in breeder flocks should concentrate on high standards of animal management accompanied with bacteriological and serological monitoring of the flocks (Wray *et al.*, 1994). *Salmonella* control programs toward *S. typhimurium* and *S. enteritidis* in chicken breeder flocks were adopted in the European Union by the Zoonoses Directive (92/117/EEC). These measures must be coupled with meticulous attention at all stages of hatching egg production and sanitation (Hafez *et al.*, 1995). Hatching egg sanitation should not be used to replace cleanliness but to complement and support it. All equipment used in hatcheries should be thoroughly cleaned and disinfected before eggs are placed on them. In Canada, eggs from layer flocks infected or environmentally contaminated with *S. enteritidis* have been sent to egg breaking stations for pasteurization. When human infection was traced to a layer flock, the flock has been destroyed and compensation has been paid.

7.2. Feed hygiene: *Salmonella* contaminated feed has been recognized as the most common source of infection in poultry flocks. The level of contamination frequently varies between the time of feed processing and its delivery. Different approaches have been used to reduce the contamination of feed ingredients as well as finished feed (Häggblom, 1993). They include: effective decontamination of feed; strict separation between clean and unclean parts in feed mills (the clean part starts directly after treatment of the feed); reduction of dust contamination within feed mills; hygienic storage conditions for all feed ingredients; efficient microbiological control measures throughout feed mill including finished feed; and finally, regular cleaning and

disinfection of the mill. Cleaning and inspection programs for feed transportation vehicles must be carried out to maintain the hygienic quality of finished feed. Only vehicles that are proven to be clean and free from *Salmonella* contamination are allowed to transport treated feed.

In conclusion, practices to control *Salmonella* have been promoted and implimented by the poultry industry in several countries. However, the complete elimination of *Salmonella* has been difficult to achieve (Davies *et al.*, 1996).

8. *Salmonella* Vaccines Issues

S. enteritidis and *S. typhimurium* are the most commonly isolated infectious enteric bacterial pathogen of animals. The observation that calves exposed to low doses of virulent *Salmonella* are protected against subsequent high dose virulent challenge suggests prevention of salmonellosis via vaccination is possible (Smith *et al.*, 1980). Most of the *Salmonella* vaccines licensed for commercial use in the United States are formalin inactivated, aluminum hydroxide adjuvanted products. In experimental studies, the reported efficacy of *Salmonella* bacterins ranges from good to ineffective (Steinbach *et al.*, 1994). The overall consensus of these reports is that vaccination of cattle with *Salmonella* bacterins provides partial protection against a *Salmonella* challenge. The absence of controls limits the interpretation of empirical reports describing the application of these vaccines in commercial production systems (Hunter, 1977).

The antigens responsible for inducing protective immunity to *Salmonella* are unknown. Application of *Salmonella* outer membrane proteins, in particular porins, as

vaccines has demonstrated the capacity of these proteins to induce protective immunity against an experimental *Salmonella* challenge (Kuusi,1979). Porin vaccines are capable of inducing protective immunity to *Salmonella* infection in mice, chickens, turkeys and rabbits (Charles, 1993).

Salmonella outer membrane proteins induce a cell mediated immune response in immunized mice characterized by Delayed Type Hypersensitivity (DTH) reactions and Interleukin 2 (IL2) and Interferon-gamma (IFN-g) production in *in vitro* assays (Galdiero *et al.*, 1993). *Salmonella* porin vaccines have not been evaluated but it has been noted that animals infected with S*almonella* mount a strong humoral and cellular immune response to these antigens. *Salmonella* infections in animals also elicit DTH and *in vitro* blastogenesis responses against porins from heterologous *Salmonella* serotypes (Robertsson, 1982).

Currently, different live and inactivated vaccines have been developed and used in an attempt to reduce *Salmonellae* shedding and colonization especially for *S. typhimurium* and *S. enteritidis* infections. However, only a few vaccines are currently available commercially (Vielitz *et al.*, 1992; Springer *et al.*, 1996). One of the vaccines did not eliminate the infection in birds previously infected with a field S*almonella* strain, but it may have reduced the number of carriers (Mayer *et al.*, 1998). In contrast, *E. coli* ETEC fimbrial vaccines, such as F4 (K88), F5 (K99), F6 (987P) and F41, have been successfully used and are available commercially. The rationale for their development was the prevention of gut colonization by inhibiting adhesion mediated by fimbriae. Whether a similar approach will be successful for *Salmonella* vaccines has yet to be tested, but evidence is becoming available that the

many fimbriae of *Salmonella* may be virulence determinants that are possibly associated with tissue-specific adherence. Fimbriae may also prove to be good vaccine candidates for *Salmonella*. Others studies with egg-yolk antibodies against *S. typhimurium and S. enteritidis* fimbriae, OMP and LPS in a mice model demonstrated solid protection from oral challenge and a significant reduction in the extent of invasion, persistence and shedding of the challenge strain. The contribution to protection in chicken made by the fimbriae, OMP or LPS remain unknown but worthy of further investigation.

9. The Advantage of Egg yolk Antibodies Used for Prevention Intestinal Diseases in Animals

The benefits of avian antibodies have been recognized for several decades and their use offers many advantages compared to mammalian antibodies. The only avian species from which antibodies are highly defined and easily accessible is the chicken. The major serum antibody in chicken is immunoglobulin G (IgG or IgY) which is transported into the egg in a manner similar to the placental transfer of IgG in mammals (Coleman, 2000). The protection against pathogens that the relatively immunocompetent newly hatched chick has is through transmission of antibodies from the mother via the egg (Wang *et al.*, 2000). The yolk of immunized chickens is a rich and inexpensive source as polyclonal antibodies. Chicken IgG is found mainly in the egg yolk, whereas its concentration in egg white is very low. A hen will produce 5 to 6 eggs in one week each having a yolk volume of approximately 15 ml. The antibody concentration in the yolk may be higher than that in the serum (Li *et al.*,

1998). Therefore, in 1 wk a hen produces egg antibodies equivalent to 90 to 100 ml of serum or 180 to 200 ml of whole blood. This could be compared to an immunized rabbit, which yields approximately 20 ml whole blood/wk when repeatedly bled, and thus a comparatively small amount of antibody can be produced by each rabbit (Li *et al.*, 1998). Only large mammals such as cows or horses can produce more antibodies than a laying hen. However, the blood collection procedure is time consuming and painful for animals. Furthermore, the cost of feeding and handling is considerably lower for a hen than for a rabbit and larger animals.

Crude egg yolk may also be used as an antibody source; of particular interest is the oral administration of specific antibodies to prevent bacterial infection. Chicken egg-yolk antibodies when administered orally have been used for passive immunization against infectious diarrheal diseases in animals (Marquardt, 2000). Therefore, these antibodies offer practical means of controlling certain intestinal diseases (scours) caused by microorganisms such as enterotoxigenic *Escherichia coli* (ETEC) in animals such as the early weaned pig. Studies have demonstrated that egg-yolk antibodies obtained from hens immunized with a strain of ETEC, K88, were highly effective in protecting 3 to 14 day-old piglets against the pathogenic effects of this organism. Piglets fed egg-yolk with anti-ETEC antibodies only had transient diarrhea, nearly all survived and all of the survivors gained weight. In contrast, control piglets that were treated with egg-yolk powder that did not contain the specific antibodies had severe diarrhea, were dehydrated, lost weight and several died within 48 h (Marquardt, 2000). Specific bovine Ig has been used to prevent rotavirus infections in infants (Ebina, 1985; Brussow *et al.,* 1987) and travellers' diarrhea

(Tacket *et al.,* 1988). Lately, oral administration of egg yolk antibodies has also been successfully used for the prevention of bacterial infections in other animal models. It has been used to prevent rotavirus infections in mice (Ebina *et al.,* 1990), *Escherichia coli* infections in rabbits (O'Farrelly *et al.*, 1992) and piglets (Wiedemann *et al.,* 1990), and caries in rats (Hamada *et al.,* 1991).

It has been shown in a previous study (Yokoyama *at al.*, 1992) that antibodies against various outer surface antigens were partially effective in the control of salmonellosis in mice. This is the only study that has been reported in the literature using this new approach for the control of *Salmonella* infection.

The objectives of this study described in this thesis were to confirm the validity of results obtained by Yokoyama *at al.* (1992) and to determine if three surface antigens, as identified in the Yokoyama study, could be used to not only control salmonellosis in a mouse model but also to inhibit the binding of *Salmonella in vitro* to an epithelium cell line (Hella cells). The antibodies were to be obtained from the yolk of hens that were immunized against the OMP, FIM (type I) or LPS from two of the most common serovars of *Salmonella: S. enteritidis* and *S. typhimurium.* The six antigens were to be isolated in as pure a form as possible using modifications of previously published procedures so as to maximize antibody titer and to ensure specificity of effect. The antibodies elicited by the antigens were to be purified from the yolk of eggs, and the titres (relative concentration of each specific antibody) were to be assayed using an Enzyme-linked Immunosorbent Assay (ELISA) developed in our laboratory. *In vitro* studies with Hela cells and *in vivo* studies with mice were carried out to determine if each antibody could block the binding of *Salmonella in*

vitro or passively (oral administration) to prevent death in mice challenged with the organism.

In all of these studies, bacterial virulence was reduced by passive oral immunization with chicken egg yolk antibodies. The beneficial effects observed with the use of chicken egg yolk antibodies against intestinal infection should encourage further studies on their prophylactic value in the control of salmonellosis in animals and humans.

MATERIALS AND METHODS

1. Reagents and Materials

All the organic and inorganic chemicals were analytical grade quality.

1.1. Chemicals and reagents. Prestained protein molecular weight markers, Coomassie brilliant blue R-250, Tween 20, alkaline phosphatase substrate, bicinchoninic acid (BCA, protein assay reagent), Bradford reagent, alkaline phosphatase-conjugated rabbit anti-chicken immunoglobulin G (IgG), a standard molecular weight reference (Sigma marker), Freund's complete and incomplete adjuvants, sodium dodecyl sulfate (SDS), diethanolamine, HEPES (N-2-hydroxyethylpiperazinc-N-2 ethane suifonic acid), bovine pancreas ribonuclease and dexyribonuclease, Sepharose 4B, Triton-X 100, sucrose, poly-L-lysine, and bovine serum albumin (BSA) , silver stain kit were from Sigma, St. Louis, Mo. The 96-well ELISA plates (Falcon 3911) were from VWR Canlab, Winnipeg, Manitoba, Canada; the Lab-Tek Chamber Slide System was from Nalgene Nunc International, Naperville, IL, USA; instant skim milk powder (Carnation) was from Nestle, Don Mills, Ontario, Canada; 0.2-mm-pore-size nitrocellulose membrane was from Bio-Rad Laboratories, Richmond, Calif.; 0.45-mm-pore-size membrane filter (MSI) was from Fisher Scientific, Nepean, Ontario, Luria broth was from Becton Dickinson, Sparks, MD, USA; casamino acid, yeast extract, agar powder were from Difco, Detroit, MI, USA; All other chemicals and reagents were of analytical grade.

1.2. Buffers and Media. The following buffers or solutions were used: LPS buffer, 50 mM sodium phosphate buffer (pH 7.0) containing 5 mM EDTA and 0.05% sodium azide; 0.01 M HEPES (N-2-hydroxyethylpiperazinc-N-2 ethanesulfonic acid) buffer; 20 mM Tris hydrochloride buffer (pH 7.5) containing 0.2% SDS; 10 mM Tris buffer, pH 7.5, containing 0.05% NaN3; 1 mM HCl; 1 M NaCl; 0.1 M bicarbonate, pH 9.0; 0.15 M ethanolamine, pH 10.5; phosphate buffered saline (PBS), 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2; 20 mM $MgCl_2$ containing 0.1 ug /ml of bovine pancreas ribonuclease and dexyribonuclease; PBST, 0.01 M sodium phosphate, 0.15 M NaCl, 0.05% Tween 20; transfer buffer, 48 mM Tris base, 39 mM glycine, 20% methanol, 0.037% SDS; and alkaline phosphatase substrate, 1 mg/ml in 10 mM diethanolamine and 0.5 mM $MgCl_2$, pH 9.5; bacteria preservation media, 0.8% of Bacto Nutrient Broth (Difco 0003); 20.0% glycerol; Vitacell's Minimum Essential Medium Eagle (MEME) culture media.

1.3. Animals

1.3.1. Chickens: A total of 60 five-month-old white Leghorn laying hens were from the University of Manitoba and were used for the production of egg yolk antibodies. The hens were divided into 6 groups, each group containing 10 chickens. They were injected with six different antigens

1.3.2. Mice: A total of 110 BALB/c 5-week-old mice were randomly distributed into two groups, each group containing 4 subgroups of from 10 to 13 mice. The mice were obtained from the University of Manitoba Animal Colony.

The mice and chickens used in this study were managed according to the Canadian Council on Animal Care (CCAC) standards (CCAC, 1993).

1.4. Bacteria Strains and Hella cells

1.4.1. *Salmonella* strains: *S. enteritidis* ATCC-13076 and *S. thyphimurium* ATCC-13311 were obtained from Dr. Blank, Food Science, University of Manitoba,Winnipeg, Manitoba, Canada. They were stored at -70 °C in bacteria preservation media.

1.4.2. Human epithelium Hela cell line: The Hela cell line CCL-2 was obtained from ATCC, 10801 University Blvd. Manassas, VA, USA. They were stored in liquid nitrogen following the procedure described in ATCC protocol.

2. Preparation of Antigens

2.1. OMP (outer membrane protein) preparations from *S. enteritidis* and *S. typhimurium* were prepared using the method of Nurminen (1978) and Schnaitman (1971) with modifications. Bacteria were suspended in 0.01 M HEPES buffer and homogenized. After centrifugation at 12,000 x g at 4 °C. The bacterial cells were resuspended in 0.01 M HEPES and sonicated. The centrifugation was repeated and the supernatant was collected and subjected to ultracentrifugation at 200,000 x g for 45 min at 4 °C. The bacterial pellet was treated with 2% Triton-X 100 in 0.01 M HEPES and ultracentrifuged. The procedure was repeated with 2% Triton-X 100 and 5 mM EDTA in 0.01 M HEPES, and ultracentrifuged. The sediment which contained the OMP was resuspended in PBS and dialysed against the same buffer. The purity of

each OMP preparation was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12 % acrylamide gels.

2.2. LPS (lipopolysaccharide) preparation from *S. enteritidis* and *S. typhimurium*: Bacterial LPS extracts were prepared using the modified method of Johnson and Perry (1976). A total of 1 g of freeze-dried *Salmonella* cells was resuspended in 20 ml of LPS buffer and sonicated three times for 30 s at 300 W at 4 °C using the sonicator (SONICS model CV26, Fisher). The suspension was diluted to 100 ml with 20 mM $MgCl_2$ containing 0.1 ug /ml of bovine pancreas ribonuclease and dexyribonuclease, and incubated for 10 min at 37 °C and for an additional 10 min at 60 °C. Then, the suspension was mixed with an equal volume of pre-heated 90% phenol and incubated for 15 min at 70 °C. The final suspension was centrifuged at 1500 x g for 1 h, and the aqueous layer was collected and dialyzed for 1 wk at 4 °C against distilled water which was changed daily. The LPS extracts were lyophilized and stored at -20 °C until used.

2.3. SEF 14 (fimbriae) preparation from *S. enteritidis*

The procedures for the purification of SEF 14 followed those described previously with modifications (Feutrier *et al.*, 1986). Briefly, after 48 h of growth in static CFA broth, cells were harvested by centrifugation (4,000 x g, 10 min, 4°C), and suspended in cold 0.15 M ethanolamine buffer (pH 10.5), and fimbriae were separated from the cells by homogenization in a omogenizer (Heidolph Diax900, Germany) for 2 min. The cells were pelleted by centrifugation and suspended in

ethanolamine buffer, and the blending and centrifugation procedures were repeated. The supernatants were combined, ammonium sulfate was added to 10% saturation, and the mixture was stirred overnight at 4 °C and centrifuged (20,000 x g, 10 min, 4 °C). The supernatant was then treated with 3 volumes of -20 °C acetone, allowed to stand on ice for 10 min, and centrifuged (15,000 x g, 10 min, 0.5 °C). The acetone was decanted, the pellet was suspended in ethanolamine buffer, and the acetone precipitation and centrifugation steps were repeated. A total of 1 ml of ice-cold ethyl ether was added to the pellet, the top of the pellet was carefully rinsed to avoid disturbing the pellet, the ether was poured off, and the precipitate was suspended in ethanolamine buffer. Crystalline ammonium sulfate was added to 40% saturation and stirred overnight at 4 °C, and the precipitate was collected by centrifugation as described above. After suspension in ethanolamine buffer and centrifugation at 120,000 x g for 60 min at 4 °C, the supernatant was dialyzed extensively against 20 mM Tris hydrochloride buffer (pH 7.5) containing 0.2% SDS and centrifuged for 3 h at 120,000 x g at 20 °C. The pellet was then suspended in ethanolamine buffer and dialyzed against sterile distilled water, and lyophilized.

2.4. STF 21 (fimbriae) preparation from *S. typhimurium*

The STF 21 frimbriae were purified by using deoxycholate and concentrated urea according to Korhonen (9) with modifications. After growth for 48 h in static Luria broth, cells were harvested and suspended in cold 10 mM Tris buffer, pH 7.5, containing 0.05% NaN3.

The pili were removed from bacterial cells by three short successive treatments in a Heidolph Diax900 homogenizator. The mixture was then centrifuged at 12,000 x g for 30 min, the cells were discarded and the suspended pili were precipitated by 50% saturation with ammonium sulfate. After dialysis, deoxycholate was added to the suspension to a final concentration of 0.5% and the suspension was dialyzed against deoxycholate-containing buffer for 48 h at 4 °C. The deoxycholate-insoluble material was removed in a centrifuge (10,000 g, 10 min) as deoxycholate solubilized the pili completely, whereas, most of the outer membrane proteins remained in the deoxycholate-insoluble fraction.

The supernatant was concentrated and layered on a 10-60% (w/w) sucrose gradient prepared in the deoxycholate-containing buffer. The gradients were run for 20 h at 200,000 xg in a SW27 rotor in an ultracentrifuge. The pili banded at a density of 1.10-1,15 gcm^{-3} and were clearly separated from the deoxycholate micelles and from the cell wall material. The pili containing fraction was collected and deoxycholate and sucrose were removed by dialysis.

After dialysis, the pili solution was concentrated and crystalline urea was added to a final concentration of 6 M. The solution was kept at room temperature for 2 h and eluted through a Sepharose 4B column with 6 M urea buffer as the eluant. Fractions in the void volume of the Sepharose 4B column were collected and urea was removed by dialysis.

The purified pili gave only one band in SDS-PAGE and had a molecular mass of 21 kDa.

3. Antigen Purity Assays by SDS-PAGE Electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970) with modification. About 20 ul (0.5 mg/ml) of sample was used per lane. Isolated samples were boiled for 10 min in SDS-PAGE sample buffer. The samples were subjected to electrophoresis on 12% gels poured with a 4% stacking gel. The gels were stained with 0.5% Coomassie blue R25 at room temperature for 30 min. The protein bands were visualized by destaining with 20% methanol and 5% acetic acid. The molecular masses were determined by using a standard molecular mass reference (Sigma marker).

LPS gels were silver stained using the method of Kittelberger and Hilbink (1993) with modifications. Details of the procedure were provided with the kit (Sigma). Briefly, after removal from the electrophoresis chamber, the gel was placed in 300 ml of ethanol-acetic acid Fixing Solution. The solution was changed three times every 20 min followed by rinsing the gels three times, each with 300 ml of deionized water for 10 min. The gels were placed in 300 ml of Silver Equilibration Solution and allowed to equilibrate with gentle agitation for 30 min. They were then rinsed for 20 sec with 300 ml of distilled water and the water was poured off when finished. The Developer Solution (150 ml) was poured over the gel. After 5-8 min the Developer Solution was removed from the gels and a second 150 ml aliquot of the Developer Solution was added to the gel. The Developer Solution was poured off when the desired band appeared. It was then rapidly replaced with Stop Solution for 5 min. The gels were rinsed three times, each time for 10 min with 300 ml distilled water.

4. Determination of Antigen Protein Concentration

The Bicinchoninic Acid method was used for the determination of antigen protein concentration following the manufacturer's recommendations (Sigma). Briefly, 1 part of Reagent B was added to 50 parts Reagent A and mixed well. Each standard and protein sample was pipeted (0.1 ml) into test tubes along with 0.1 ml of diluent for blanks. The Working Reagent containing Bicinchoninic acid was added to each tube and mixed well. All tubes were incubated at 37 °C for 30 min, and then cooled to room temperature. The absorbance of each tube against a water reference was measured at 562 nm. The absorbance of the blank was subtracted from the values found. The concentration of protein was determined from the standard curve.

5. Egg-yolk Antibodies Production

Five-month-old white Leghorn chickens were immunized with antigens for the development of egg-yolk specific antibodies. Antigen preparation containing 0.5 mg of each antigen in 0.5 ml of PBS were emulsified in Freund's complete adjuvant (1:1) were injected i.m. into the breast muscles of chickens. The antigens injected into each of six hens were OMP, LPS or fimbriae (SEF 14 or ST 21) from *S. enteritidis* or *S. typhimurium*. Three weeks after the first injection, chickens received booster injections of the same dosage by the same route of administration and this was repeated 2 wk later. Freund's incomplete adjuvant was used in place of the complete adjuvant for the second and third injections. The eggs were collected 1 wk after the final injection and were stored at 2-4 °C until processed. Eggs were also collected prior to immunization for the assay of antibody titer.

6. Ig Y Isolation from Chicken Egg Yolk

Yolk antibody (IgY) was purified from the eggs by ammonium sulfate precipitation (Daniel *et al.*, 2001). Briefly, approximately 500 ml of yolk was diluted with 6 volumes of water at 4°C. Precipitated material was allowed to settle for 16 h. The supernatant (3.5 liters) was removed, clarified by the addition of 1M sodium carbonate (pH 9) to 10 mM, and concentrated to 500 ml by diafiltration (Pellicon 50-kDa-cutoff filters, 0.1 mm^2; Millipore Corp.). The antibody was precipitated with 20% (wt/vol) ammonium sulfate and resuspended in 100 ml of phosphate-buffered saline (PBS). Residual salts were removed by buffer exchange with PBS, and the antibody was lyophilized. The final mass was 2.4 g, containing approximately 1.5 g of IgY (based on optical density at 280 nm and enzyme-linked immunosorbent assay [ELISA] comparison to affinity-purified IgY). Pre-immunization antibody was similarly isolated from the yolks of nonimmunized hens.

7. Antibodies Titre Assayed by ELISAs (enzyme-linked immunosorbent assay)

7.1. Titration of fimbriae and OMP antibodies

IgY extracts were assayed for the titre of specific antibodies (IgY) by using an ELISA with alkaline phosphatase as the substrate. The procedure was similar to that used by Metcalfe *et al.* (1991). Purified fimbriae or OMP (1ug/well) were immobilized on polystyrene microtiter plates for 2 h at 37°C, washed three times with PBST, blocked with 5% skim milk powder for 2 h at 37°C, and washed with PBST. Twofold serial dilutions of each IgY extract preparation were then added to each well

(100 ul), and the plates were incubated for 2 h at 37°C. After washing with PBST, alkaline phosphatase-labeled goat anti-swine IgG (100 ul; 1:3,000) was added, and the plates were incubated for 1 h at 37°C. All plates were then washed with PBST, substrate (100 ul) was added, and the mixture was incubated for 30 min at 37°C. Absorbency was determined at 405 nm in an ELISA plate reader (model 450; Bio-Rad, Hercules, CA).

7.2. Titration of LPS antibodies

The technique described by Engvall and Perlmann (1972) was used with modifications. A solution (100 ul) of polylysine in 0.01 M phosphate-buffered saline at pH 7.2 (PBS) was placed in disposable polystyrene microplates. The solution was incubated overnight at room temperature. Aliquots (100 ul) of 1 ug of LPS suspended in PBS were then placed in poly-L-lysine-precoated plates, and incubated for 1 h at 37 °C. The plates were washed three times with PBS containing 0.05% Tween 20 (PBST). Twofold serial dilutions of each IgY extract preparation were then added to each well (100 ul), and the plates were incubated for 2 h at 37°C. After washing with PBST, alkaline phosphatase-labeled rabbit anti-chicken IgG (100 ul; 1:3,000) was added, and the plates were incubated for 1 h at 37°C. All plates were then washed with PBST, substrate (100 ul) was added, and the mixture was incubated for 30 min at 37°C. Absorbency was determined at 405 nm in an ELISA plate reader.

8. Determination of the Concentration of Bacteria

Bacteria were grown in 300 ml of Luria broth at 37 °C for 18 h followed by centrifugation at 1000 x g at 4 °C. The cells were suspended in 1 ml of sterile saline. Serial dilutions of this initial bacteria solution was carried out by pipeting 1 ml of the suspension into 9 ml of sterile saline (1:10), and then 1 ml of this new solution into another 9 ml of sterile saline (1:100) followed by $1:10^3$, $1 : 10^4$, $1: 10^5$, $1 : 10^6$, $1 : 10^7$ and $1:10^8$ dilutions. The $1 : 10^6$, $1 : 10^7$ and $1:10^8$ dilutions of the initial sample were pipeted (0.1 ml) onto appropriately labeled Petri dishes coated with plate count agar. The plate count agar contained casein peptone, 10 g; tryptone, 5 g; yeast extract, 5 g; glucose, 10 g; agar, 15 g; and distilled water, 1000 ml. It was autoclaved for sterilization and poured into sterile Petri dishes. The 0.1 ml of suspension was spread over the entire surface of the agar using a sterile glass spreader. The plates were incubated in an inverted position in the incubator for 24 to 48 h at 37°C. The colonies on plates exhibiting 50-150 colonies were counted. The number of CFU/ml in the original 1 ml of bacteria solution were calculated. (CFU: colony forming units). The initial bacteria solution was then diluted with sterile saline to the desired concentration.

9. *In vitro* Adherence Assay

This overall procedure was modified to Khoramian *et al.*, (1990).

9.1. Tissue Culture Method

Vitacell's Minimum Essential Medium Eagle (MEME) culture media (ATCC, 10801 University Blvd. Manassas, VA, USA.) was warm up to 37 °C in an incubator for 30

min. Five milliliters of the media was transferred to each Lab-Tek Chamber Slide System.

The vial containing the Hela cells was taken from the liquid nitrogen and thawed by gentle agitation for 2 min in a 37 °C water bath. The vial was removed from the water bath and decontaminated by dipping in 70 % ethanol. The Hela cells (0.2 ml) were transferred to Lab-Tek Chamber Slide System and incubated at 37 °C in an air atmosphere containing 5% of CO_2. Prewarmed media was used to feed the cells 3 times per week. The cells were visually inspected frequently (twice a day) under an invert microscope. The growing culture was kept until a mono-lay of cells formed.

9.2. Bacteria binding

Bacteria were grown in Luria broth at 37 °C for 18 h. Cells were harvested by centrifugation (1,000 x g, 5 min, 4°C) and suspended at a concentration of approximately 1 x 10^7 c.f.u. /ml in MEME. The bacteria (1 ml) were mixed with 1 ml of four-fold serial diluted egg yolk antibody solution (in MEME 1:10 dilution of original purified IgY sample) at 37 °C for 15 min. After a period of incubation, the medium was aspirated from each chamber and 1ml suspension of bacteria, or bacteria plus antibody was added onto each slide chamber with Hela cells as described above. This was followed by incubation at 37 °C for 30 min.

9.3. Tissue culture staining and bacteria numbering

The slide chambers were washed three times with warm PBS, fixed with cold acetone, and stained with Giemsa solution. The number of bacteria attached to 10

HeLa cells of each slide was determined under a microscope (400x) and the average number of bacterial cells attached to each HeLa cell was calculated.

10. Preparation of Bacteria for Challenge

S. entritidis and *S. typhimurium* were grown on Luria agar for 24 hr at 37 °C. Bacteria were collected in 2 ml of sterile saline from each plate. The bacterial concentration was determined and diluted with sterile saline to a final concentration of *S. enteritidis* suspension of 1.5 x 10^9 c.f.u./ ml and S. *typhimurium* of 1 x 10^9 c.f.u. /ml. The mice were challenged with the bacterial preparations within 30 min.

11. Animal Challenge and Antibody Administration

A total of 110, 5 wk-old mice weighting 20 to 25 g were randomly distributed into two groups. Each group contained 4 subgroups. The procedure for the challenge exposure of mice with the bacteria was performed as described by Yokoyama *et al*, (1992) with modifications. Group I (*S. enteritidis*): four subgroups with 15 mice per group were inoculated orally with 0.2 ml of *S. enteritidis* suspension (1.5 x 10^9 c.f.u./ ml). Group II (*S. typhimurium*): four subgroups with ten mice per group were inoculated orally with 0.2 ml of *S. typhimurium* (1 x 10^9 c.f.u. /ml). All mice in each trial were given 0.2 ml of the challenge infectious dose as specified above and about 30 min later, 0.2 ml antibody solution consisting of anti- OMP, anti- LPS, anti- FIM or control antibody from sham immunized chickens were given to subgroups 1, 2, 3, and 4, respectively. The antibody solution was given to each mouse three times a day at 8 h intervals for three consecutive days. The bacterial inoculum and antibodies were administered by gastric incubation using a sterilized blunt needle attached to a 1

ml tuberculin syringe. Mice were observed daily for two wk for clinical signs of infection and the survival (%) of the mice was calculated.

12. Statistical Analysis

The designs in both challenge experiments and *in vitro* binding studies were Completely Randomized Designs (CRD). In challenge experiments, 10 to 13 mice replicates per treatment were used. In binding studies, each treatment has 10 replicates. The statistical significance of differences ($P<0.05$) in survival rates between the treated and control groups was assessed by using the Chi-square test, and the mean differences ($P<0.05$) in bacteria adhesion *in vitro* between the control and antibody treated groups was assessed by analysis of variance and Tukey's test.

RESULTS

Purification of OMPs, LPSs and FIMs

1. Purification of OMP from *S. typhimurium* and *S. enteritidis*

The procedure for OMP isolation and purification from *S. typhimurium* and *S. enteritidis* was as described in Materials and Methods. The proteins obtained from both strains of *Salmonella* by this method ranged in size from 33 to 36 kDa (Fig. 1a and 1b). The OMP from *S. typhimuriun* exhibited three main bands having a molecular size of 34, 35, and 36 kDa (Fig.1a). These values are similar to those previously described by Marjatta (1978). The OMP from *S. enteritidis* only showed two main bands of 34 and 35 kDa. SDS-PAGE analysis of these preparations indicated that of the collective purity of the OMP's were greater than 90 %.

Fig.1. SDS-PAGE analysis of purified OMP from *S. typhimurium* (Fig.1a) and S. *enteritidis*(Fig.1b) showing: lane 1, molecular weight marker; 2, purified OMP with three main protein bands of 34, 35 and 36 kDa or 3, purified OMP with two main protein bands of 34 and 35 kDa. The numbers to the left of each Figure are the molecular masses of the each marker protein in kDa.

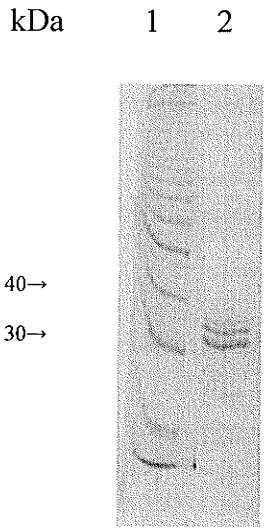


Fig. 1a

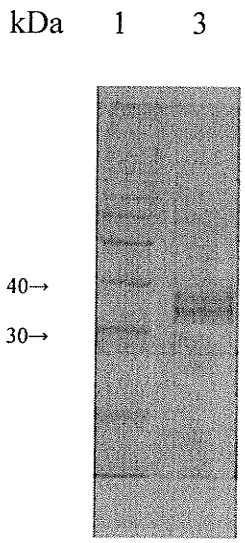


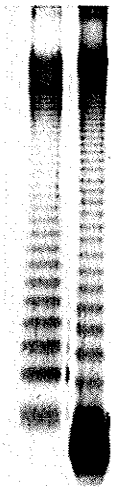
Fig. 1b

2. Purification of LPSs from *S. typhimurium* and *S. enteritidis*

The LPSs extracted from *S. typhimurium* and *S. enteritidis* are shown in Fig. 2. (lanes A and B). Silver staining of SDS-PAGE gels revealed that both of LPSs are heterogeneous, with bands in a form of ladder-like patterns typical for smooth, gram-negative bacteria (Hitchcock *et al*, 1983). These bands represent the LPS molecules containing increasing lengths of O antigen chains (oligosaccharide unit), and are also typical of those reported for *S. typhimurium* (Peterson *et al.*, 1985) and *S. enteritidis* (Valtonen *et al.*, 1975).

Fig. 2. Silver stained SDS-PAGE of LPSs extracted from *S. typhimurium* (lane A) and *S. enteritidis* (lane B) showing ladder banding from the presence of different lengths of O side-chain.

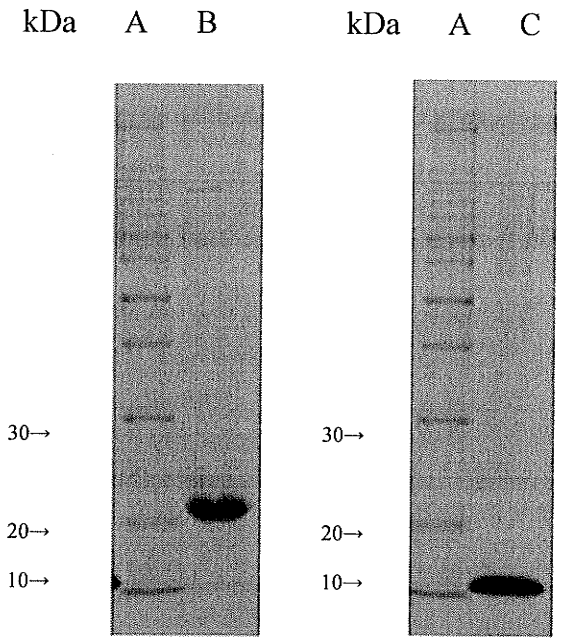
A B



3. Purification of FIMs from *S .typhimurium* and *S. enteritidis*

Two different purification procedures were followed for SEF14 and STF 21 as described as described in Materials and Methods. Further purification was performed by gradient ultracentrifugation and gel filteration through a Sepharose 4B column. STF 21 from *S .typhimurium* was obtained in high purity and had a molecular weight of 21 kDa (Fig. 3B). The 14-kDa fimbrial antigen from *S. enteritidis* obtained by precipitate in 0.2 % SDS also yielded a preparation of high purity (Fig 3C).

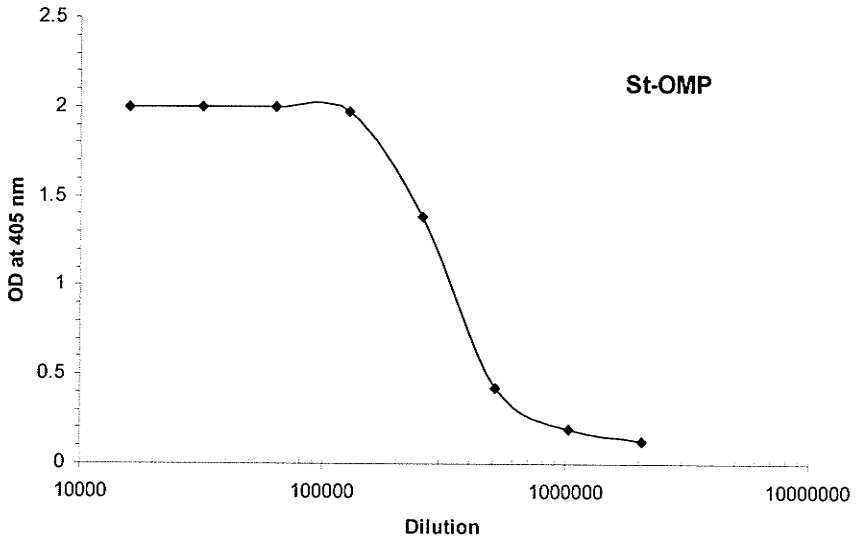
Fig.3. SDS-PAGE analysis of purified *S. typhimurium* STF 21 and *S. enteritidis* SEF14 showing: lane A, mol.-wt markers; B, purified *S. typhimurium* STF21; C, purified *S. enteritidis* SEF14. The numbers to the left of each Figure are the molecular masses of each protein in kDa.


kDa
A
B
30→
20→
10→
kDa
A
C
30→
20→
10→

Antibody titres of purified IgY to *Salmonella* OMP, LPS and FIM

In this study, the purified chicken egg yolk IgYs were used for oral administration in the challenge trials with mice and the in vitro adhesion studies with Hela cells. The amount (titer) of egg yolk IgY antibody was assessed by ELISA. The titers of the OMP, LPS and FIM specific antibodies were 3 $x10^5$, 1 x 10^5, and 5 x 10^5, respectively, for *S. typhimurium* (Fig. 4, 5, 6, st-OMP). Those for the OMP, LPS, and FIM for *S. enteritidis* were 2.5 $x10^5$, 1 x 10^5, and 5.5 x 10^5, respectively (Fig. 4, 5, 6, se-OMP). The titers of egg yolk IgY of the non-immunized hens were < 10^2 against the 3 different antigens. Cross-reactivity studies with the different antibodies from the same strain of *Salmonella* demonstrated that there was essentially no-cross reactivity of the antibodies with the two antigens that were not the immunogens.

Fig.4. Typical ELISA curves for the quantitation of egg yolk IgY titre against *S. typhimurium* (upper Fig) and *S. enteritidis* (lower Fig) OMP. Titre was the dilution of antibody required to give an OD that was 50% of the maximum OD. Each value is the average of triplicate analysis. The SE for all value was less than 5 % of the mean and, therefore, are not shown


St-OMP
OD at 405 nm
2.5
2
1.5
1
0.5
0
10000
100000
1000000
10000000
Dilution

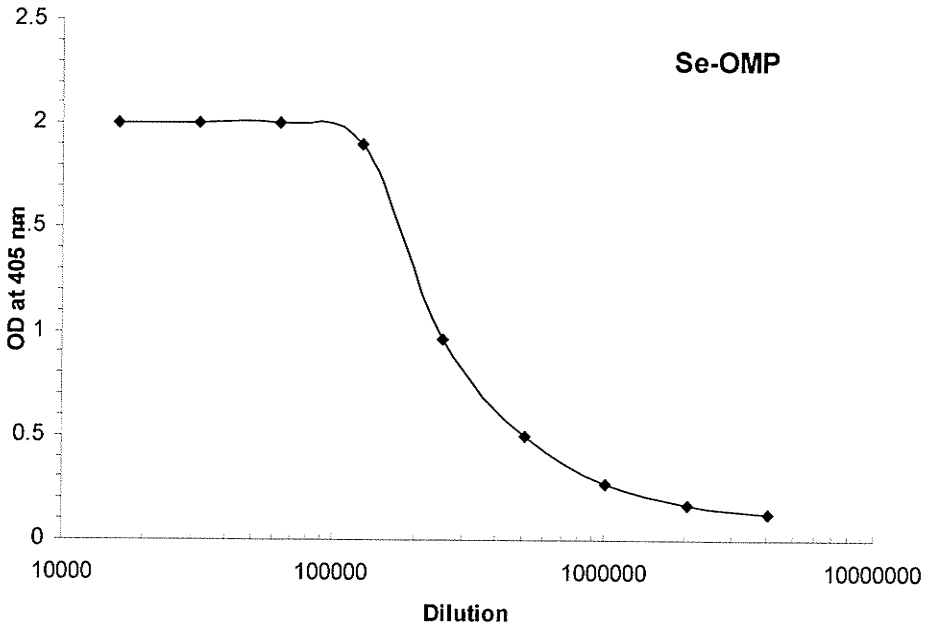
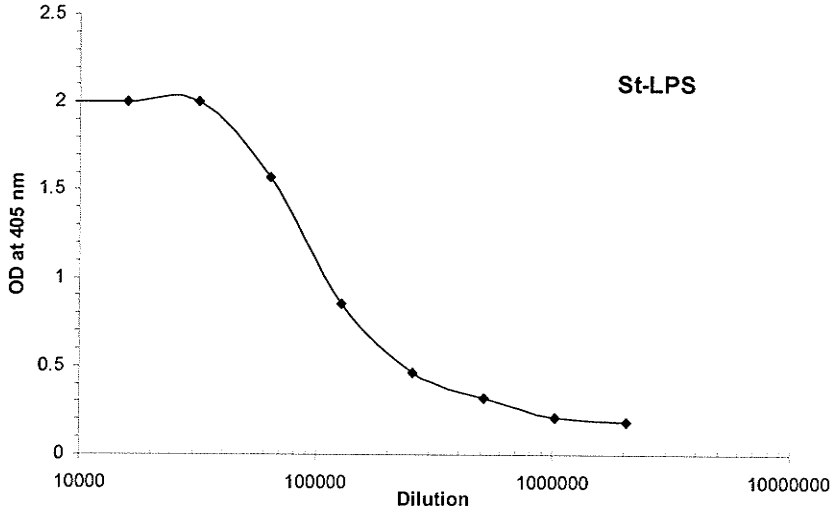

Se-OMP
OD at 405 nm
2.5
2
1.5
1
0.5
0
10000
100000
1000000
10000000
Dilution

Fig.5. Typical ELISA curves for the quantitation of egg yolk IgY titre against *S. typhimurium* (upper Fig) and *S. enteritidis* (lower Fig) LPS. Titre was the dilution of antibody required to give an OD that was 50% of the maximum OD. Each value is the average of triplicate analysis. The SE for all value was less than 5 % of the mean and, therefore, are not shown.


St-LPS
OD at 405 nm
2.5
2
1.5
1
0.5
0
10000
100000
1000000
10000000
Dilution

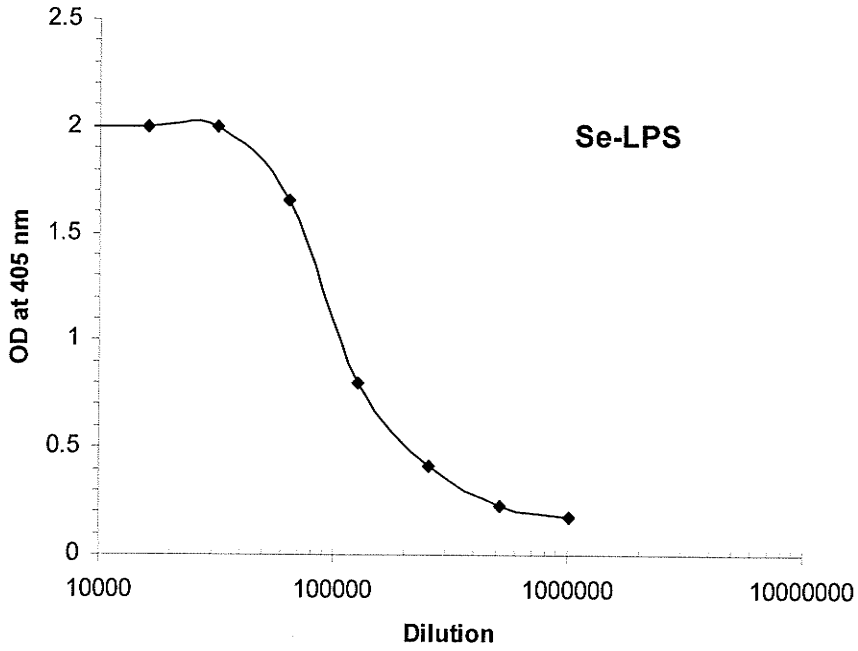
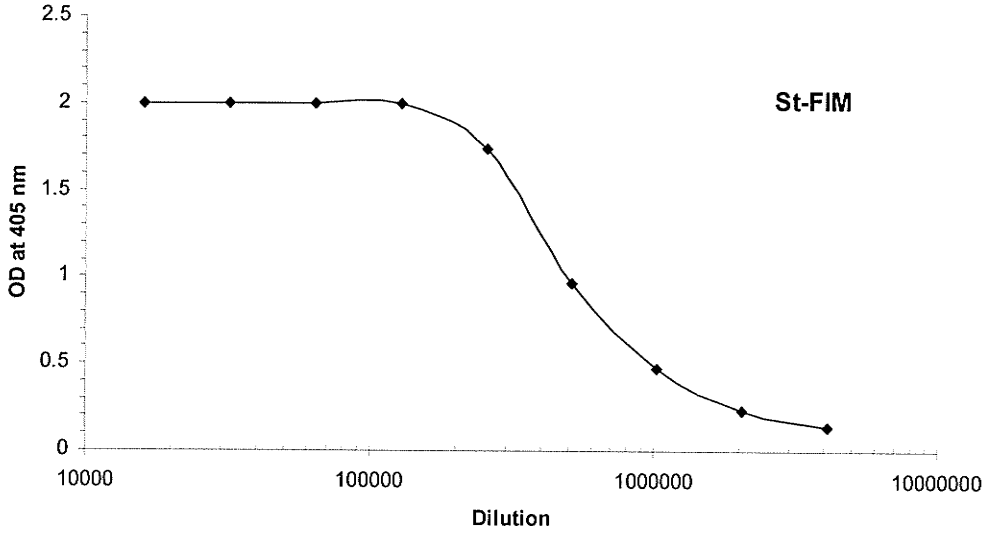
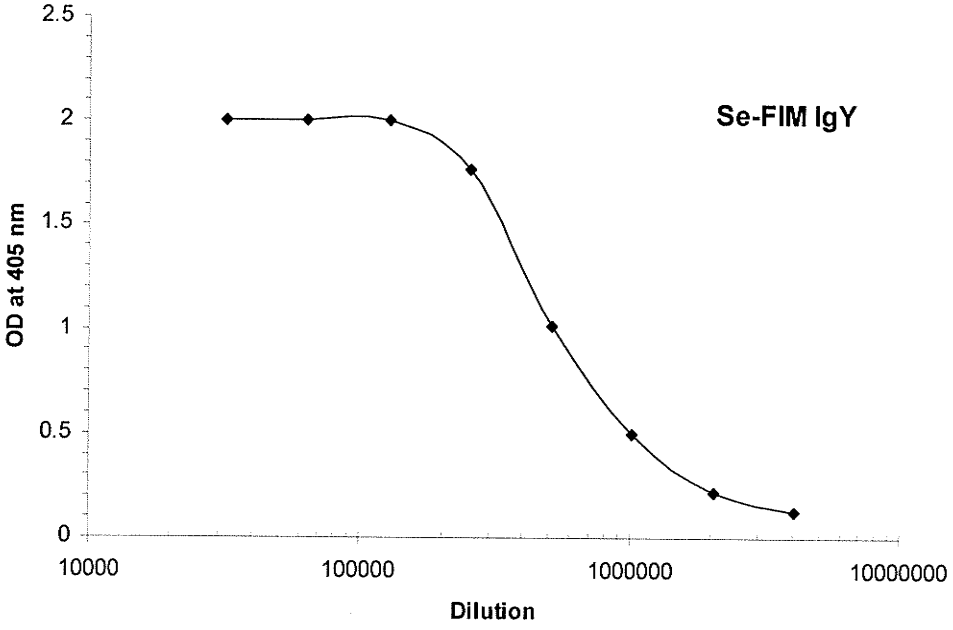

Se-LPS
OD at 405 nm
2.5
2
1.5
1
0.5
0
10000
100000
1000000
10000000
Dilution

Fig.6. Typical ELISA curves for the quantitation of egg yolk IgY titre against *S. typhimurium* (upper Fig) and *S. enteritidis* (lower Fig) FIM. Titre was the dilution of antibody required to give an OD that was 50% of the maximum OD. Each value is the average of triplicate analysis. The SE for all value was less than 5 % of the mean and, therefore, are not shown.


St-FIM
OD at 405 nm
2.5
2
1.5
1
0.5
0
10000
100000
1000000
10000000
Dilution


Se-FIM IgY
OD at 405 nm
2.5
2
1.5
1
0.5
0
10000
100000
1000000
10000000
Dilution

3. Clinical evaluation of mice after a challenge exposure with *Salmonella* and passive immunization with one of the three anti *Salmonella* antibodies

The clinic responses of mice after a *Salmonella* challenge and subsequent treatment with antibodies (IgY) are shown in Fig.7 and 8.

a. Mice in Group A. The clinical responses of mice after a S. *typhimurium* challenge and subsequent treatment with antibodies are shown in Fig. 7. Mice in subgroup 1 (OMP antibody) had the highest survival rate, with a 76.9% recovery rate from *S. typhimurium* infection. Mice in subgroup 2 (LPS antibody) and subgroup 3 (STF 21 antibody) had survival rates of 58.3 and 36.4%, respectively, whereas control mice in subgroup 4 administered non-immune egg yolk antibodies all died. The difference in the survival rates between the OMP antibody subgroup and the control subgroup was statistically different ($p < 0.05$). The susceptible mice manifested the following clinical signs after infection: lethargy, anorexia, and death. No overt diarrheal disease was observed.

b. Mice in Group B. The clinical responses of mice after the *S. enteritidis* challenge and subsequent treatment with antibodies are shown in Figure 8. Mice in subgroups 1 (OMP antibody), 2 (LPS antibody) and 3 (SEF 14 antibody) had a survival rates of 69.2, 46.2 and 40%, respectively, whereas control mice in subgroup 4 administered non-immune egg yolk antibodies only had a survival rate of 15.4%. The difference in the survival rates between the OMP antibody group and the control group was

statistically significant ($p<0.05$). The susceptible mice manifested the following clinical signs after infection: lethargy, anorexia, and death. Overt diarrheal disease was not observed in the control group.

Fig.7. Protection of mice initially challenged with *S. typhimurium* and orally administered with three different egg-yolk antibodies to *S. typhimurium* over a 14 day period. The controls were treated with pre-immune antibodies collected from hens prior to being immunized. See Materials and Methods for further details. The initial number of mice in each treatment group ranged from 10 to 13.

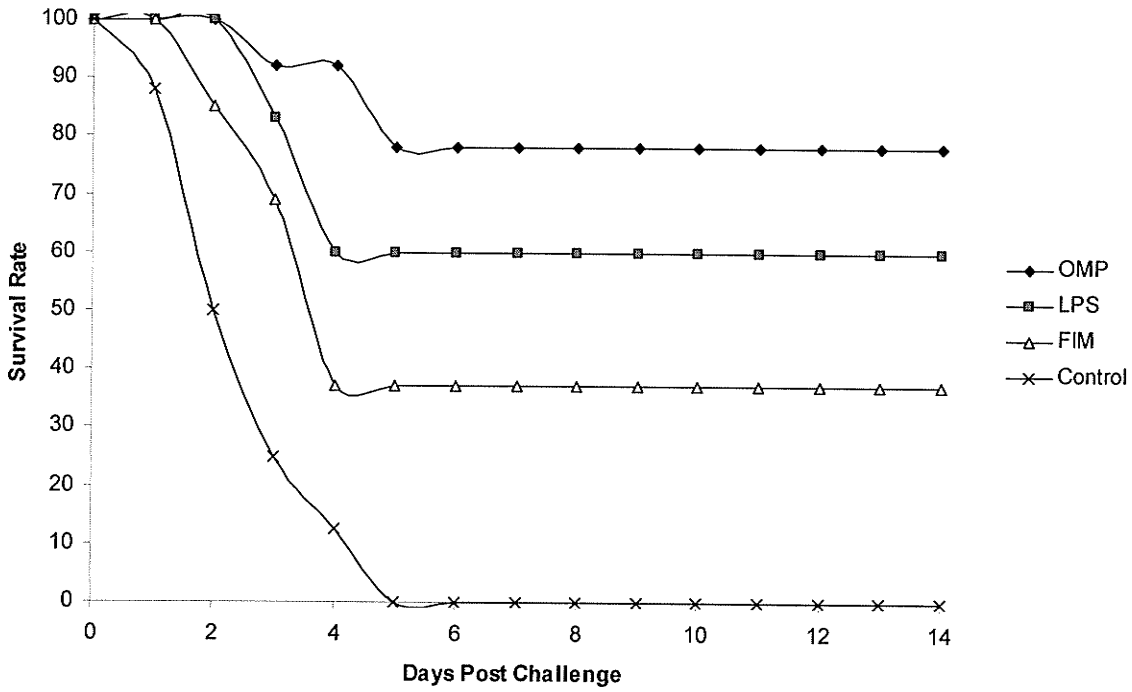
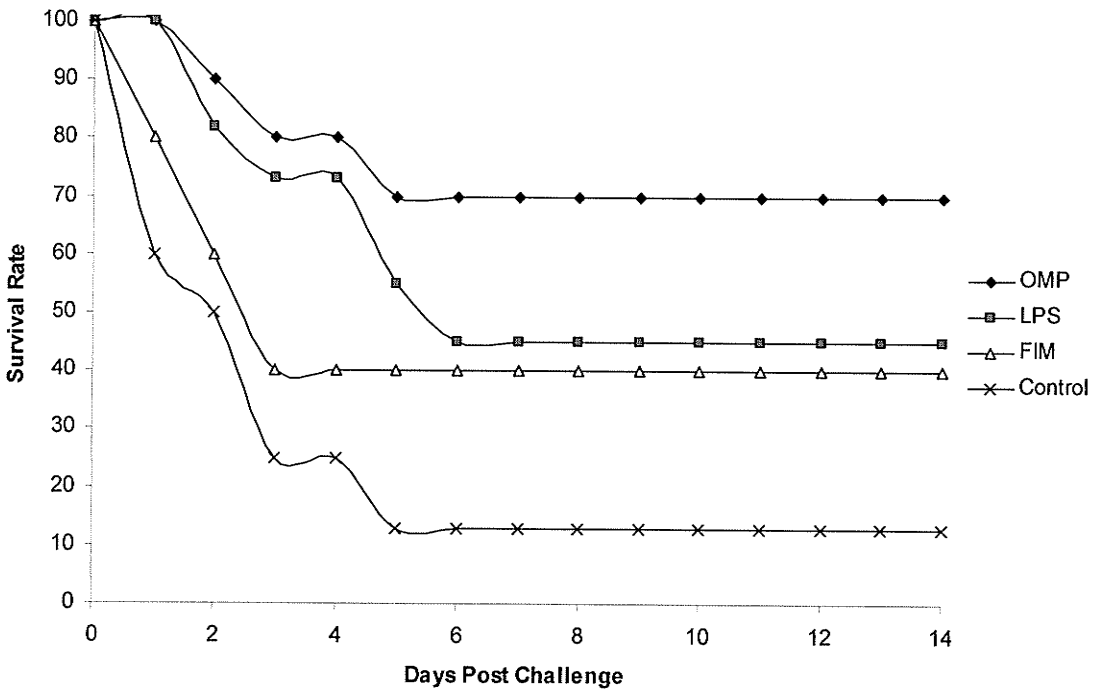

Survival Rate
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
Days Post Challenge
OMP
LPS
FIM
Control

Fig. 8. Protection of mice initially challenged with *S. enteritidis* and orally administered with three different egg-yolk antibodies to *Senteritidis* over a 14 day period. The controls were treated with pre-immunization antibodies. See Materials and Methods for further details. The initial number of mice in each treatment group ranged from 10 to 13.


100
90
80
70
60
50
40
30
20
10
0
Survival Rate
0
2
4
6
8
10
12
14
Days Post Challenge
OMP
LPS
FIM
Control

4. Effect of antibodies on *Salmonella*-mediated bacterial adhesion to Hela cells *in vitro*

The antibodies against OMP, LPS and FIM of *Salmonella* inhibited the binding of *S. typhimurium* and *S. enteritidis* to human epithelium cell line Hela in a concentration-dependent manner (Tables 1 and 2). OMP, LPS and FIM antibodies at the high concentration were effective at inhibiting the binding of *Salmonella* ($p < 0.05$).

Table 1. Effect of egg yolk antibody on *in vitro* adhesion of *S. typhimurium* to HeLa cells

	Antibody Titer[1]	Mean ± standard deviation of adherent *Salmonella* cells per 10 Hela cells at different antibody dilutions		
		x 10	x 40	x 160
OMP	1×10^5	12 ± 7^a	21 ± 8^a	35 ± 14
LPS	1×10^5	21 ± 11^a	31 ± 7	44 ± 9
FIM	1×10^5	19 ± 9^a	32 ± 12	47 ± 12
Control	$< 1 \times 10^2$	61 ± 8^b	56 ± 5^b	62 ± 10

[1] Original titer was adjusted to titers given below with PBS.

[a, b] means in the same column with different superscripts differ significantly ($p < 0.05$) based on a Tukey's test.

Table 2. Effect of egg yolk antibody on *in vitro* adhesion of *S. enteritidis* to HeLa cells

	Antibody titer[1]	Mean ± standard deviation of adherent *Salmonella* cells per10Hela cells at different antibody dilution		
		x 10	x 40	x160
OMP	1×10^5	9 ± 6^a	19 ± 8^a	34 ± 12
LPS	1×10^5	17 ± 7^a	31 ± 9	46 ± 11
FIM	1×10^5	21 ± 8^a	33 ± 13	41 ± 8
Control	$< 1 \times 10^{2b}$	57 ± 14^b	55 ± 7^b	63 ± 8

[1] Original titer was adjusted to titers given below with PBS.

[a, b] means in the same column with different superscripts differ significantly ($p < 0.05$) based on a Tukey's test.

DISCUSSION

The objectives of this study were to determine if antibodies against three outer surface antigens from *S. typhimurium* and *S. enteritidis* would provide passive protection *in vitro* and *in vivo* against the corresponding pathogens. Each antigen was purified to more than 95% purity prior to injection into laying hens so as to ensure that the antibody response was specific. In order to accurately measure antibody titer and reduce interference from other components, the antibodies were partially purified so as to remove the lipid and non-IgY components. This greatly facilitated the development of the six different ELISAs required to quantitate antibody titer and the use of the antibodies in the *in vitro* Hela cell studies. Presumably, it would not have been necessary to purify the antibodies for the *in vivo* challenge studies since previous studies with other antibodies, especially *E. coli* strain K88 (F4), have demonstrated that the antibodies were highly effective when administrated in the presence of other egg constituents (Marquardt, 2000). Cross-reactivity studies among the different antigens confirmed that the purified antibodies only reacted with their corresponding antigens and not the other antigens from the same *Salmonella* serovar. Also the fimbrial antigens were strain specific which provided a basis for the specific determination of either strain of *Salmonella* using the immunobased ELISA. These assays would be of great value to regulatory personnel and the industry, because very few specific assays are available commercially.

In mice challenged with *S. enteritidis*, antibody treatment resulted in survival rates of 69.2, 46.2 and 40% using OMP, LPS or FIM specific antibodies, respectively,

in contrast to only 15.4% in control mice. In the *S. typhimurium* trial, the survival rates were 76.9, 58.3 and 36.4% using OMP LPS or FIM specific antibodies, respectively, in contrast to 0 % in control mice. These data demonstrate that all three antibodies from each *Salmonella* strain were effective in the partial control of the pathogens. This study provided similar data and confirmed the studies of Yokoyama *et al* (1994) on the use of chicken egg yolk antibodies raised against the OMP, LPS, and FIM of *S. typhimurium* and *S. enteritidis* for the control of experimental salmonellosis in mice.

The *in vitro* studies with Hela cells demonstrated that the antibodies were also highly effective at inhibiting the binding of each *Salmonella* serovar to specific receptors on the surface of Hela cells, although they were not able to completely block the binding. The results, nevertheless, strongly suggest that each of the three antigens are virulence factors involved in the colonization of the intestinal mucosa. The data, therefore, suggest that the antibodies can serve as a valuable tool to elucidate the importance and role of OMP, LPS and FIM on the surface of *Salmonella*, particularly the role of OMP and its constituent porins, as pathogenicity determinants in mice or other animals. In this study each of the antibodies for either organism was tested alone and the efficacy of combinations were not investigationed. It is, hypothesized, that the use of two or more of the antibodies together would have further increased their efficacy as presumably more then one surface antigen is involved in the binding of *Salmonella* to the mucosal surface, a presequisite for pathogenicity (Isaacson, 1994).

This proposal is further supported by the work of Vander Velden *et al.*, (1998) where they constructed several mutants of *S. typhimurium* that had defective adherence antigens. They reported that *Salmonella* defective in any one of four different outer surface antigens had a reduced affinity for intestinal receptors, and that the binding affinity was synergistically reduced when two or more of the adhesion factors were defective with essentially no binding of the organism being observed when four of the adhesions were defective. Similarly, it is hypothesized that the neutralization of two or more of the binding factors such as OMP, FIM or LPS by the homologous antibodies would be more effective than that achieved by any one antibody alone. This possibility, however, has not be been tested but many provide a basis for the complete inhibition of the binding of *Salmonella* to intestinal receptors of mice and other animals and, therefore, provide a means of greatly reducing its pathogenicity. Both the *in vitro* and the *in vivo* studies suggest that anti-OMP antibodies were more efficacious in the inhibition of both serovars of *Salmonella* than the other two antibodies. Further dose response studies are required to confirm these observations, especially, as suggested above when combinations of the antibodies are used. Also the role of each of the three porins that constitate the OMP need to be further evaluated. Preliminary studies by other researchers (Pai *et al.*, 1992) have shown that they have a similar sequence of amino acids since the anti-OMP protein from one form of porin cross-reacts with each of the others to varying degrees.

In conclusion, these studies demonstrate that each of the three different antigens are involved in the binding of *Salmonella* to intestinal receptors and that the inhibition of the interaction of the organism with intestinal receptors considerably

reduces the pathogenicity of *Salmonella* with anti-OMP antibodies appearing to be more effective then the anti-FIM or anti-LPS antibodies. The approach is promising and further protection may be achieved by using combinations of two or more of the anti- adherence antibodies. Although the current *in vivo* studies were with mice, similar results may also be achieved with other animal species such as the pigs and with poultry. Also, antibody treatment, in a manner similar to that achieved with other intestinal pathogens such as *E. coli* K88 (F4) (Marquardt, 2000) may facilitate clearance of the organism from the intestinal tract, one of the most common routes for transmission of this pathogen into foods. The use of specific egg yolk antibodies to control salmonellosis in animals and the shedding of the organism into foods would be of considerable benefit as it is estimated that the economic losses caused by *Salmonella* in North America annually is 0.6 to 3.5 billion dollars in addition to the loss of human lives (Buzby *et al.*, 1996). This problem is becoming more serious as the use of other control agents such as antibiotics are being banned, or are not effective because of antibiotic resistance. There is a need for further research in order to fully explain the ability of *Salmonella* antibodies to control food-borne diseases and to further elucidate the mechanism by which the different adhesion factors contribute to the pathogenicity of *Salmonella.*

SUMMARY

The objective of the study was to determine if antibodies against specific outer-membrane antigens from two serotypes of *Salmonella* (*S. typhimurium* and *S. enteritidis*) would inhibit the binding of the organisms onto the surface of Hela cells and would passively protect mice that had been previously challenged with either strain of *Salmonella enteritidis* or *S. typhimurium*. Three purified antigens [outer membrane protein (OMP)], lipopolysaccharide (LPS) and fimbriae (FIM)) were isolated in relatively pure form and antibodies against each of the antigens was produced in the yolk of eggs from laying hens that had been hyperimmunizaed with the different antigens. Mice that had been challenged orally with 1.5×10^9 cfu of *Salmonella enteritidis* or 1×10^9 cfu of *S. typhimurium* were orally treated with 0.2 ml of the homologous anti-OMP, -LPS or -FIM yolk antibody three times a day for three consecutive days. Antibody treatment of the mice challenged with *S. enteritidis* resulted in survival rates of 69.2, 46.2 and 40% using OMP, LPS or FIM specific antibodies, respectively, in contrast to only 15.4% for the control mice. In the *S. typhimurium* trial, the survival rate was 76.9, 58.3 and 36.4% using OMP, LPS or FIM specific antibodies, respectively, in contrast to 0% in control mice. In vitro adhesions of *S. enteritidis* and *S. typhimurium* to HeLa cells were greatly reduced by antibody treatment. For example, in the studies with *S. typhimurium* the adhesion per 10 Hela cells in the absence of specific antibodies was 61 ±8 (= SE) whereas in the presence of the highest concentration of anti-OMP, -LPS and –FIM only 12 ± 7 ($p<0.05$), 21 ± 11 ($p<0.05$) and 19 ± 9 ($p<0.05$) CFU of the organisms were bound,

respectively. Similar results were obtained when anti-*S. enteritidis* antibodies were used. The results demonstrate that egg yolk antibodies specific for *Salmonella* OMP, LPS, and FIM reduce the binding of *Salmonella* to Hela cells and provide considerable passive protection to mice from experimental salmonellosis when the antibodies were administered orally. Of these antibodies, anti-OMP exhibited the highest level of protection in both the *in vivo* and *in vitro* studies.

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