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Proteomic Analysis of Outer Membrane Proteins in *Salmonella enterica* Enteritidis S

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Salmonella enterica serovar Enteritidis is the predominant agent causing salmonellosis in chickens and other domestic animals. In an attempt to identify antigenic *S*. Enteritidis outer membrane proteins (OMPs) that may be useful for subunit vaccine development, we established a proteomic map and database of antigenic *S*. Enteritidis OMPs. In total, 351 and 301 spots respectively from *S*. Enteritidis strain 270 and strain 350 were detected by two-dimensional gel electrophoresis. Fifty-one antigen-reactive spots were detected by antisera on two-dimensional immunoblots and identified as 12 specific proteins by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. OmpA and DNA starvation/stationary phase protection protein (Dps) were the most abundant proteins among the identified OMPs, comprising 22 and 12 protein species, respectively. Interestingly, we found that the Dps of *S*. Enteritidis is also antigenic. OmpW was also verified to have high antigenicity. These results show that OmpA, Dps, and possibly OmpW are antigenic proteins. This study provides new insights into our understanding of the immunogenic characteristics of *S*. Enteritidis OMPs.

Keywords: Immunoproteome, outer membrane proteins, S*almonella* Enteritidis

Introduction

Foodborne salmonellosis is a continuing major public health concern, despite the implementation of monitoring of environmental contamination. Worldwide, the disease causes 93.8 million cases of illness with 155,000 deaths every year [28]. *Salmonella enterica* serovar Enteritidis (*S*. Enteritidis) is a leading bacterial cause of acute gastroenteritis [32]. Poultry-derived products are considered a major route of human infection with *S*. Enteritidis [3]. The most common routes by which chickens are infected with *S*. Enteritidis are contaminated feed and feces. Infection of chickens with *S*. Enteritidis is initiated by extensive gut colonization. Then, through its interaction with the intestinal epithelium, *S*. Enteritidis invades and spreads to a wide range of tissues [39]. The cell-mediated and humoral responses that form the basis of innate and adaptive protection play important roles in resistance to and clearance of *S*. Enteritidis infection [39].

To prevent salmonellosis caused by *S*. Enteritidis, a commercial vaccine product, Gallimune SE (Merial Animal Health, Lyon, France), has been developed and is used in the poultry industry [44]. However, it contains whole bacteria, which may cause severe side effects in the host. Because of problems with the vaccine, many alternative vaccines to prevent salmonellosis have recently been

investigated, including subunit vaccines, particularly vaccines including outer membrane proteins (OMPs) [19, 35].

In preventing salmonellosis, OMP vaccines are thought to modulate the adaptive immune response to *Salmonella* through the activation of dendritic cells, which act as immune sentinels and play important roles in the regulation of immune responses to antigens [26]. Dendritic cells (DCs), which are plasmacytoid phagocytes, play a key role in the initiation and regulation of an efficient adaptive immune response against pathogens [18, 48]. During infection, these innate cells recognize pathogen-associated molecular patterns from bacteria [45, 46]. Maturing DCs then migrate to secondary lymphoid organs to activate naïve T cells by presenting stimulating antigenic peptides on major histocompatibility complex type I and II receptors [6, 9, 20]. Therefore, DCs constitute the link between innate and adaptive immunity [46]. OmpA is a major protein in the *Salmonella* outer membrane and plays important roles in immune stimulation and the induction of a T helper 1 immune response [26]. OmpA and OmpW have structures that are heat-modifiable by the unfolding of the β-barrel to an α-helix [31]. The biological function of OmpW still remains largely uncharacterized, even though OmpW has been identified in several studies [50].

Biotechnological techniques have been applied to understand the molecular mechanisms of pathogenicity and vaccine-induced immunity. Bacterial OMP databases have been constructed using the techniques of two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Jungblut *et al.* first applied the combination of 2-DE and western blotting to identify bacterial antigens, and OMP databases have been established for several bacteria, including *Helicobacter pylori*, *Bacillus anthrax*, and *Chlamydia trachomatis* [5, 21, 23, 33]. Many previous studies have investigated the OMP profiles of *Salmonella* Typhimurium (*S.* Typhimurium) [11, 15, 40], but no study of *S.* Enteritidis OMPs has been published. Therefore, the present study aimed to investigate the OMPs from *S.* Enteritidis.

In this study, we applied an immunoproteomic approach using 2-DE with immunoblotting to understand the correlation of the discovered proteins with the pathogenicity and immunodominant antigens of *S.* Enteritidis OMPs. In addition, we compared the expression levels of antigens in *S.* Enteritidis isolates from different hosts.

Materials and Methods

Bacterial Strains and Culture Conditions

The *S.* Enteritidis strains used in this study are listed in Table 1. *S.* Enteritidis strains 270 and 350 were kindly donated by Incheon Veterinary Service Laboratory and Seoul Institute of Health and Environment, respectively. The *S.* Enteritidis strains were cultured in 50 ml of Luria–Bertani (LB) broth overnight at 37°C with aeration. The cultured bacteria were centrifuged at 5,000 ×*g* for 15 min. The pellets were washed twice with ice-cold phosphate-buffered saline (PBS, at pH 7.2) and used for OMP extraction.

Virulence of *S.* Enteritidis Strains from Mice

Female 8-week-old BALB/c mice were used for the virulence study. Six groups of 30 specific pathogen-free mice (control group, 3 mice) were housed in an individually ventilated cage and given sterile food and tap water *ad libitum*. All mice were kept at 22°C, 40%–70% humidity, and under a 12 h light/12 h dark cycle. All animal handling and protocols were reviewed and approved by the Kangwon University Institutional Animal Care and Use Committee (permit no. KW-130829-2). At 8 weeks of age, mice were injected intraperitoneally with 10^5, 10^6, or 10^7 CFU/mouse of bacteria. Mice were observed for 4 dpi, and deaths were recorded daily. The livers and spleens of mice that died and those sacrificed at 4 dpi were separated, weighed, homogenized, and plated on *Salmonella–Shigella* agar (BBL; Becton Dickinson and Company, Sparks, MD, USA). The number of bacteria (CFU) colonizing the organs was counted.

OMP Sample Preparation

OMPs were prepared from whole cells by the method of Barenkamp *et al.* [2]. In brief, bacterial pellets were suspended in 5 ml of 10 mM *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid and were sonicated five times for 20 sec each, separated by 10 sec intervals. The cell debris was then removed by a single centrifugation at 2,500 ×*g* for 20 min. The supernatants were centrifuged at 100,000 ×*g* for 60 min, and the resulting pellets were

Table 1. *Salmonella enterica* Enteritidis strains used in this study.

Strain no.	Origin of isolation	PFGE[a] type	Phage type	Antimicrobial resistance pattern[b]
S. Enteritidis 270[c]	Chicken	A6	PT35	AM-TIC
S. Enteritidis 350	Human	A1	PT21	AM-TIC

[a]PFGE: pulsed-field gel electrophoresis.

[b]AM: ampicillin; TIC: ticarcillin.

[c]*S.* Enteritidis 270 and *S.* Enteritidis 350 were characterized in Kang *et al.* [21].

treated with 1% Sarkosyl solution for 30 min to solubilize the outer membrane. Subsequently, this fraction was pelleted by centrifugation at 100,000 ×*g* for 60 min. OMPs were suspended in buffer solution (7 M urea, 2 M thiourea containing 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate, 1% dithiothreitol, 1% pharmalyte), and the OMP concentration was assayed by the Bradford method [7].

Two-Dimensional Electrophoresis

The method for 2-DE was modified from that of Rabilloud *et al*. [38]. In brief, 200 μg of each protein sample was loaded and separated on an immobilized pH gradient (IPG) strip (4-10 NL IPG, 24 cm; Genomine, Pohang, Korea). The strips were equilibrated and inserted into sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels (20 × 24 cm, 10%–16%) for the second dimension. The 2D gels were silver stained as described by Oakley *et al*. [34]. Quantitative intensity analysis of the digitized images was performed using PDQuest software 8.0 (BioRad, Hercules, CA, USA).

Immunoblot Assay

Antisera against *S*. Enteritidis isolates were prepared in chickens. *S*. Enteritidis was inactivated in 0.01% formaldehyde solution and emulsified in incomplete Freund's adjuvant (InvivoGen, San Diego, CA, USA) according to the manufacturer's protocol. Every 2 weeks, ten 4-week-old chickens were injected subcutaneously with the equivalent of 10^9 CFU of *S*. Enteritidis. Eight weeks after the first injection, the highest-titer sera against *S*. Enteritidis were selected and validated using enzyme-linked immunosorbent assay (Biochek, Foster City, CA, USA).

The immunoblotting procedure was performed as described by Wu *et al*. [49] with some modifications. The spots on 2-DE gels (*S*. Enteritidis 270) were electroblotted onto PVDF membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and blocked with 5% fetal bovine serum in TBS-T (50 mM Tris-HCl at pH 7.5, 200 mM NaCl, 0.5% Tween 20) at room temperature. Subsequently, membranes were incubated with the prepared antisera at a dilution of 1:100 for 2 h. The membranes were washed three times with TBS-T and incubated with goat anti-chicken IgG-HRP (AbD Serotec, Kidlington, UK; 1:1,000 dilution) for 4 h at room temperature. The membranes were quickly rinsed with TBS-T, followed by development using 3,3′-diaminobenzidine tetrahydrochloride. Finally, the locations of the same spots on the 2-DE gels and the membranes were identified using PDQuest software 8.0 (BioRad).

In-Gel Protein Digestion and MS Protein Identification

For protein identification, matched spots were cut out of the gel and treated with trypsin (Ettan MALDI-TOF Pro; Amersham Biosciences, Piscataway, NJ, USA), as previously described [38]. Spectra were collected from 300 shots per spectrum over an *m/z* range of 600–3,000 and calibrated by two-point internal calibration using trypsin autodigestion peaks (*m/z* 842.5099, 2,211.1046). The data were analyzed using GPS Explorer 3.5 software (Applied Biosystems, Foster City, CA, USA). Identification of the proteins was based on searches of the *Salmonella* database in the National Center for Biotechnology Information databases (2013.01). A score greater than 66 from Mascot server ver. 2.3 (Matrix Science, London, UK) was accepted as significant ($p < 0.05$). The search parameters were (i) trypsin, as the cleaving enzyme; (ii) to allow for missed cleavage; (iii) carbamidomethyl (C), as a fixed modification; (iv) oxidation (M), as a variable modification; and (v) 0.1–0.2 Da, as a mass tolerance for the peptide ions (*m/z*).

Results and Discussion

Virulence Comparisons of *S*. Enteritidis Isolates in Mice

The level of bacterial colonization of the organs of infected mice was assessed to compare the virulence of *S*. Enteritidis isolates from different hosts by a model of dose-dependent infection of mice *in vivo* (Table 2). The mortality rates of mice infected with *S*. Enteritidis 270 at all the doses were slightly higher than those infected with *S*. Enteritidis 350 with the exception of 10^6 CFU dose. However, no significant difference was observed. All mice died by 4 days post infection (dpi). We also found that the levels of colonization by *S*. Enteritidis 270 were slightly higher than those by *S*. Enteritidis 350 at the same time. In particular, *S*. Enteritidis 270 in the liver showed 0.12–0.4 log higher levels of colonization than *S*. Enteritidis 350, and significant differences ($p < 0.05$) between the two strains in

Table 2. Virulence of *S*. Enteritidis in mice.

Strain no.	Recovery of *S*. Enteritidis (log CFU/g)						
	Liver				Spleen		
	Dose[#] (CFU)	10^5	10^6	10^7	10^5	10^6	10^7
S. Enteritidis 270		8.77[a]	8.80[a]	8.96[a]	9.46[a]	9.67[a]	9.80[a]
S. Enteritidis 350		8.37[b]	8.55[b]	8.84[a]	9.31[a]	9.31[a]	9.63[a]

[#]Frozen stock of SE was transferred in LB broth to activate *S*. Enteritidis, and three doses of *S*. Enteritidis were intraperitoneally injected into 8-week-old mice.

[ab]Significant differences ($p < 0.05$) between serovars were determined by two-way ANOVA and are denoted by the letters.

bacterial colony forming units (CFU) recovered from the liver were shown at all doses except 10^7 CFU. *S*. Enteritidis 270 in the spleen showed 0.15–0.36 log higher levels of colonization than *S*. Enteritidis 350. Based on these results, we could conclude that *S*. Enteritidis 270 was more virulent than *S*. Enteritidis 350.

2-DE Profiles and Immunoblot Assay from *S*. Enteritidis Isolates

Protein spots on 2-DE were visualized within the molecular weight (MW) range of 10–200 kDa and isoelectric points (p*I*) of 4–10 (Fig. 1). In total, 351 and 301 spots were counted on the maps of strains 270 and 350, respectively, by the PDQuest software. The 2-DE gel of *S*. Enteritidis 270 was electroblotted onto a polyvinylidene difluoride membrane, and OMPs on the membrane were immunodetected with *S*. Enteritidis-specific chicken antisera (Figs. 1A and 1C). Fifty-one spots were detected, all of which were also found on the 2-DE gel of *S*. Enteritidis 350 (Fig. 1B, Supplementary Data 1). Subsequently, the 51 spots detected by the antisera were identified as 12 different proteins, and their abundance levels in the two gels were compared (Table 3). The detailed Mascot search results, mass lists, and MS spectra are provided as supporting information. MWs of OMPs were distributed in the range of 22.59–38.38 kDa, and the results corresponded with theoretical MWs from previously observed OMPs in *Salmonella* species located at 18, 23, 36, 37, 38, 39, 42, 43, and 45 kDa [11, 14, 15, 40]. All the spots for OMPs were distributed in the range of p*I* 4.27–5.82 and were similar to those reported for other bacterial OMPs [1, 51].

Analysis and Identification of Antigenic Proteins from *S*. Enteritidis Isolates

Among the 51 antigenic spots listed in Table 3, OmpA was the most abundant protein of the isolated OMPs, and 22 protein species of OmpA were distributed in the range of 28.03–38.38 kDa and p*I* 4.27–5.82. OmpA has been studied extensively in *Escherichia coli* and is essential for bacterial survival and pathogenesis [4, 42]. OmpA is also believed to stimulate a strong antibody response [37]. Previous studies demonstrated that OmpA from gram-negative bacteria activated macrophages and dendritic cells to produce cytokines [43, 47], which implies that OmpA is immunogenic and is a possible candidate for a subunit vaccine [12, 13, 24, 27]. Twelve spots identified as a DNA starvation/stationary phase protection protein (Dps) had the second highest abundance level. Although the majority of these antigenic spots were distributed in the range of 18.42–19.72 kDa (p*I* 4.40–6.39), two spots were

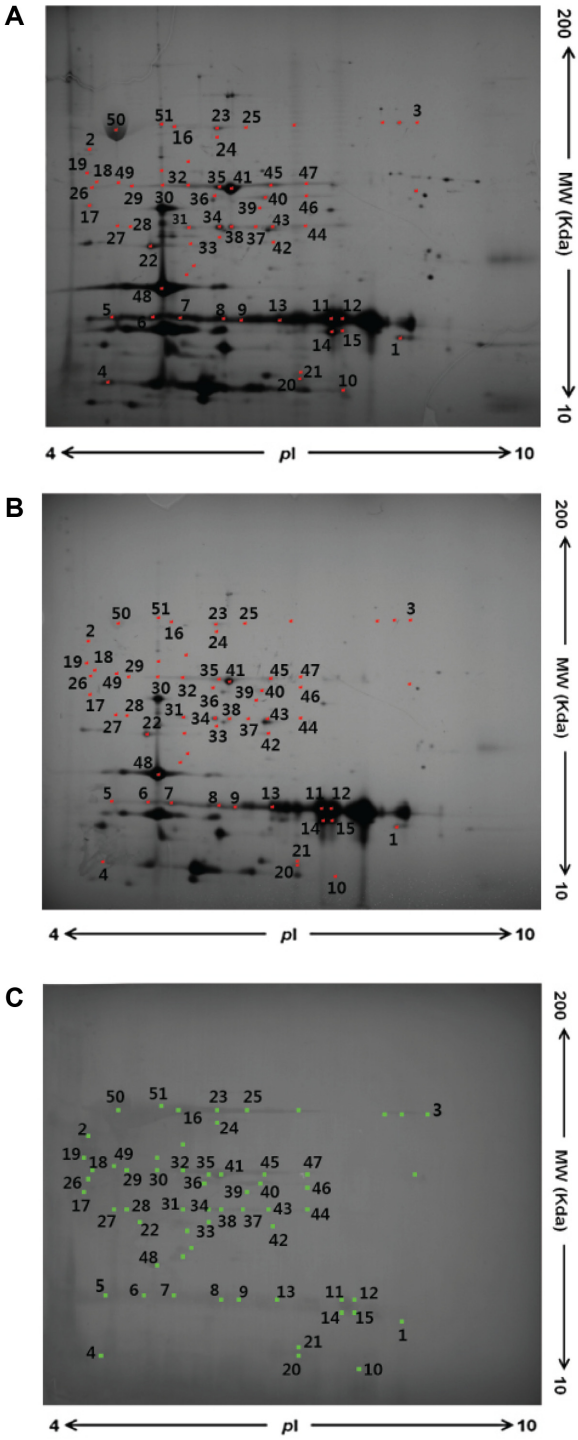

Fig. 1. Matched silver-stained 2-DE maps of OMPs isolated from (**A**) *S*. Enteritidis 270 and (**B**) *S*. Enteritidis 350, and (**C**) immunoblotted membrane of *S*. Enteritidis 270 OMPs.
(**A** and **B**) First-dimension analysis, IPG 4–10; separation distance, 24 cm. Second-dimension analysis, SDS–PAGE (10%–16%, 20 × 24 cm). Image analysis and the quantification of protein spots were performed with PDQuest software 8.0. (**C**) A total of 51 spots corresponded with the immunoblotted membrane. Spots without numbers were not identified.

Table 3. Immunoreactive proteins identified and a comparison of their abundance in different strains.

Spots	Identified proteins	Gene symbols	Functional categories	Average sequence coverage (%)	Average abundance level[a]		
					Gel		Membrane
					S. Enteritidis 270	*S*. Enteritidis 350	*S*. Enteritidis 270
1	Dihydrolipoamide dehydrogenase	*dld*	Energy production and conversion	29	1,477.05	381.98	47.55
12	DNA starvation/ stationary phase protection protein	*dps*	Inorganic ion transport and metabolism	81	3,745.53	871.14	10.89
1	Flagellar capping protein	*fliD*	Flagellar apparatus	65	638.41	212.90	20.99
1	Flagellar hook-associated protein	*flgL*	Cell motility	47	465.36	ND	27.21
2	Flagellar hook protein	*flgE*	Cell motility	41	31.85	ND[b]	8.42
1	Maltoporin	*lamB*	Carbohydrate transport and metabolism	51	652.48	ND	27.31
1	MltA-interacting protein	*mipA*	Cell wall/membrane biogenesis	90	6,478.10	8,312.64	1.00
3	Outer membrane channel protein	*tolC*	Efflux system	58	802.92	258.56	44.44
22	Outer membrane protein A	*ompA*	Cell envelope	51	2,915.11	1,557.99	30.48
5	Phase 1 flagellin	*fliC*	Cell motility	38	24,729.68	155.51	6.44
1	Outer membrane protein W	*ompW*	Cell envelope	72	3.62	20.42	65.45
1	Antimicrobial peptide resistance/ lipid A acylation protein	*pagP*	Transfer of palmitate	49	3,985.91	2,025.45	29.01

[a]The abundance level was calculated by dividing the sum of spot intensities by the number of spots.

[b]ND: not detected.

located at 14.00 and 14.54 kDa (p*I* 4.38 and 6.39), which is a close match with their theoretical values. This protein is encoded by the *dps* gene and plays a role in defense against hydrogen peroxide [8]. Recently, it was demonstrated that Dps in *Salmonella* Gallinarum (*S*. Gallinarum) is antigenic [10]. *S*. Gallinarum was grown and harvested under the same conditions as the *S*. Enteritidis in this study, and, interestingly, this is the first study to report the antigenicity of Dps in *S*. Enteritidis. The third major group was flagella-related proteins, including a flagellar capping protein, a flagellar hook-associated protein, a flagellar hook protein, and a phase 1 flagellin. Other identified proteins were a dihydrolipoamide dehydrogenase, a maltoporin, a MltA-interacting protein, and an antimicrobial peptide resistance/lipid A acylation protein. Outer membrane-related proteins, including an outer membrane channel protein and OmpW, were also detected. Whereas 22 OmpA spots were detected, only one antigenic spot for OmpW was identified, at 22.59 kDa (p*I* 4.73), as listed in Table 3. These results are consistent with those of previous studies showing that OmpA is a well-conserved and major OMP in *S*. Typhimurium and *E. coli*, of which there are 100,000 copies per cell in *E. coli* [29, 35, 41]. However, OmpW was reported as a minor protein in *E. coli* [36]. Singh *et al*. [40] also listed the immunogenic OMPs of *S*. Typhimurium. The major protein was OmpA, with minor proteins including OmpW, OmpD, OmpX, and OmpS1. Other identified proteins were peptidoglycan-associated lipoprotein precursor, nucleoside-specific channel-forming protein, and VacJ lipoprotein. In this study, the sequences of the most abundant OmpA and the two identified OmpW of *S*. Typhimurium were highly matched with those of *S*. Enteritidis; however, other identified proteins were not matched with each other. This may be because of the different methods used for OMP extraction and gel electrophoresis.

To date, very little information has been available regarding the function of OmpW [29]. Recently, Gil *et al*. [16, 17] reported that OmpW plays a role in the response to oxidative damage and that it functions as a porin. Previous studies demonstrated that the OmpW isolated from *S*. Typhimurium showed immunogenic characteristics in *Salmonella*-induced reactive arthritis and that OmpW from *Vibrio cholerae* was immunogenic during infection [25, 30, 40].

As shown in Table 3, all the proteins isolated from *S*. Enteritidis 270 showed higher abundances than those from *S*. Enteritidis 350, with the exception of the MltA-interacting protein and OmpW. However, only one spot was detected each for the MltA-interacting protein and OmpW. This result is consistent with the relative virulence

of the two *S*. Enteritidis isolates in mice (Table 2). The average antigenicity of the 12 proteins isolated from *S*. Enteritidis 270 and *S*. Enteritidis 350 did not correlate with their average abundance. Interestingly, OmpW showed the highest antigenicity of the 12 identified proteins, and double the average level of OmpA (Table 3). This result indicates that OmpW may be a promising subunit vaccine if produced by genetic amplification techniques. We also believe that OmpW needs further study to identify its immunogenic characteristics, and to determine whether it is protective.

This study was focused on identifying an effective host immune response to antigenic *S*. Enteritidis OMPs, and it demonstrated *via* immunoproteomic techniques that OmpA, Dps, and possibly OmpW are proteins with high abundance and immunogenicity. The results in this study can be used to identify candidate antigen proteins that can elicit host immune responses to invading *S*. Enteritidis bacteria. Further studies are necessary to investigate the efficacy of these antigenic proteins in protecting against *S*. Enteritidis infection in an animal model.

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References

1. Alam SI, Bansod S, Kumar RB, Sengupta N, Singh L. 2009. Differential proteomic analysis of *Clostridium perfringens* ATCC13124; identification of dominant, surface and structure associated proteins. *BMC Microbiol.* **9:** 162.
2. Barenkamp SJ, Munson RS Jr, Granoff DM. 1981. Subtyping isolates of *Haemophilus influenzae* type b by outer-membrane protein profiles. *J. Infect. Dis.* **143:** 668-676.
3. Baumler AJ, Hargis BM, Tsolis RM. 2000. Tracing the origins of *Salmonella* outbreaks. *Science* **287:** 50-52.
4. Belaaouaj A, Kim KS, Shapiro SD. 2000. Degradation of outer membrane protein A in *Escherichia coli* killing by neutrophil elastase. *Science* **289:** 1185-1188.
5. Bini L, Sanchez-Campillo M, Santucci A, Magi B, Marzocchi B, Comanducci M, *et al*. 1996. Mapping of *Chlamydia trachomatis* proteins by immobiline-polyacrylamide two-dimensional electrophoresis: spot identification by N-terminal sequencing and immunoblotting. *Electrophoresis* **17:** 185-190.
6. Bousso P, Robey E. 2003. Dynamics of CD8+ T cell priming by dendritic cells in intact lymph nodes. *Nat. Immunol.* **4:** 579-585.
7. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
8. Calhoun LN, Liyanage R, Lay JO Jr, Kwon YM. 2010. Proteomic analysis of *Salmonella enterica* serovar Enteritidis following propionate adaptation. *BMC Microbiol.* **10:** 249.
9. Chen H, Schifferli DM. 2003. Construction, characterization, and immunogenicity of an attenuated *Salmonella enterica* serovar Typhimurium *pgtE* vaccine expressing fimbriae with integrated viral epitopes from the *spiC* promoter. *Infect. Immun.* **71:** 4664-4673.
10. Cho Y, Sun J, Han JH, Jang JH, Kang ZW, Hahn TW. 2014. An immunoproteomic approach for characterization of the outer membrane proteins of *Salmonella* Gallinarum. *Electrophoresis* **35:** 888-894.
11. Chooneea D, Karlsson R, Encheva V, Arnold C, Appleton H, Shah H. 2010. Elucidation of the outer membrane proteome of *Salmonella enterica* serovar Typhimurium utilising a lipid-based protein immobilization technique. *BMC Microbiol.* **10:** 44.
12. Crocquet-Valdes PA, Diaz-Montero CM, Feng HM, Li H, Barrett AD, Walker DH. 2001. Immunization with a portion of rickettsial outer membrane protein A stimulates protective immunity against spotted fever rickettsiosis. *Vaccine* **20:** 979-988.
13. Dumetz F, Lapatra SE, Duchaud E, Claverol S, Le Henaff M. 2007. The *Flavobacterium psychrophilum* OmpA, an outer membrane glycoprotein, induces a humoral response in rainbow trout. *J. Appl. Microbiol.* **103:** 1461-1470.
14. Encheva V, Wait R, Begum S, Gharbia SE, Shah HN. 2007. Protein expression diversity amongst serovars of *Salmonella enterica*. *Microbiology* **153:** 4183-4193.
15. Encheva V, Wait R, Gharbia SE, Begum S, Shah HN. 2005. Proteome analysis of serovars Typhimurium and Pullorum of *Salmonella enterica* subspecies I. *BMC Microbiol.* **5:** 42.
16. Gil F, Hernandez-Lucas I, Polanco R, Pacheco N, Collao B, Villarreal JM, *et al*. 2009. SoxS regulates the expression of the *Salmonella enterica* serovar Typhimurium *ompW* gene. *Microbiology* **155:** 2490-2497.
17. Gil F, Ipinza F, Fuentes J, Fumeron R, Villarreal JM, Aspee A, *et al*. 2007. The *ompW* (porin) gene mediates methyl viologen (paraquat) efflux in *Salmonella enterica* serovar Typhimurium. *Res. Microbiol.* **158:** 529-536.
18. Granucci F, Ricciardi-Castagnoli P. 2003. Interactions of bacterial pathogens with dendritic cells during invasion of mucosal surfaces. *Curr. Opin. Microbiol.* **6:** 72-76.
19. Hamid N, Jain SK. 2008. Characterization of an outer membrane protein of *Salmonella enterica* serovar Typhimurium that confers protection against typhoid. *Clin. Vaccine Immunol.*

15: 1461-1471.
20. Itano AA, Jenkins MK. 2003. Antigen presentation to naive CD4 T cells in the lymph node. *Nat. Immunol.* **4:** 733-739.
21. Jungblut PR, Grabher G, Stoffler G. 1999. Comprehensive detection of immunorelevant *Borrelia garinii* antigens by two-dimensional electrophoresis. *Electrophoresis* **20:** 3611-3622.
22. Kang ZW, Won HK, Kim EH, Noh YH, Choi HW, Hahn TW. 2011. Protective effects and immunogenicity of *Salmonella* Enteritidis killed vaccine strains selected from virulent *Salmonella* Enteritidis isolates. *Korean J. Vet. Res.* **51:** 21-28.
23. Kimmel B, Bosserhoff A, Frank R, Gross R, Goebel W, Beier D. 2000. Identification of immunodominant antigens from *Helicobacter pylori* and evaluation of their reactivities with sera from patients with different gastroduodenal pathologies. *Infect. Immun.* **68:** 915-920.
24. Koizumi N, Watanabe H. 2003. Molecular cloning and characterization of a novel leptospiral lipoprotein with OmpA domain. *FEMS Microbiol. Lett.* **226:** 215-219.
25. Kurupati P, Teh BK, Kumarasinghe G, Poh CL. 2006. Identification of vaccine candidate antigens of an ESBL producing *Klebsiella pneumoniae* clinical strain by immunoproteome analysis. *Proteomics* **6:** 836-844.
26. Lee JS, Jung ID, Lee CM, Park JW, Chun SH, Jeong SK, *et al*. 2010. Outer membrane protein a of *Salmonella enterica* serovar Typhimurium activates dendritic cells and enhances Th1 polarization. *BMC Microbiol.* **10:** 263.
27. Li H, Xiong XP, Peng B, Xu CX, Ye MZ, Yang TC, *et al*. 2009. Identification of broad cross-protective immunogens using heterogeneous antiserum-based immunoproteomic approach. *J. Proteome Res.* **8:** 4342-4349.
28. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, *et al*. 2010. The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clin. Infect. Dis.* **50:** 882-889.
29. McClean S. 2012. Eight stranded beta-barrel and related outer membrane proteins: role in bacterial pathogenesis. *Protein Pept. Lett.* **19:** 1013-1025.
30. Nandi B, Nandy RK, Mukhopadhyay S, Nair GB, Shimada T, Ghose AC. 2000. Rapid method for species-specific identification of *Vibrio cholerae* using primers targeted to the gene of outer membrane protein OmpW. *J. Clin. Microbiol.* **38:** 4145-4151.
31. Nandi B, Nandy RK, Sarkar A, Ghose AC. 2005. Structural features, properties and regulation of the outer-membrane protein W (OmpW) of *Vibrio cholerae*. *Microbiology* **151:** 2975-2986.
32. Nandre R, Matsuda K, Lee JH. 2014. Efficacy for a new live attenuated *Salmonella* Enteritidis vaccine candidate to reduce internal egg contamination. *Zoonoses Public Health* **61:** 55-63.
33. Nilsson I, Utt M, Nilsson HO, Ljungh A, Wadstrom T. 2000. Two-dimensional electrophoretic and immunoblot analysis of cell surface proteins of spiral-shaped and coccoid forms of *Helicobacter pylori*. *Electrophoresis* **21:** 2670-2677.
34. Oakley BR, Kirsch DR, Morris NR. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* **105:** 361-363.
35. Okamura M, Ueda M, Noda Y, Kuno Y, Kashimoto T, Takehara K, Nakamura M. 2012. Immunization with outer membrane protein A from *Salmonella enterica* serovar Enteritidis induces humoral immune response but no protection against homologous challenge in chickens. *Poult. Sci.* **91:** 2444-2449.
36. Pilsl H, Smajs D, Braun V. 1999. Characterization of colicin S4 and its receptor, OmpW, a minor protein of the *Escherichia coli* outer membrane. *J. Bacteriol.* **181:** 3578-3581.
37. Puohiniemi R, Karvonen M, Vuopio-Varkila J, Muotiala A, Helander IM, Sarvas M. 1990. A strong antibody response to the periplasmic C-terminal domain of the OmpA protein of *Escherichia coli* is produced by immunization with purified OmpA or with whole *E. coli* or *Salmonella* Typhimurium bacteria. *Infect. Immun.* **58:** 1691-1696.
38. Rabilloud T, Kieffer S, Procaccio V, Louwagie M, Courchesne PL, Patterson SD, *et al*. 1998. Two-dimensional electrophoresis of human placental mitochondria and protein identification by mass spectrometry: toward a human mitochondrial proteome. *Electrophoresis* **19:** 1006-1014.
39. Sheela RR, Babu U, Mu J, Elankumaran S, Bautista DA, Raybourne RB, *et al*. 2003. Immune responses against *Salmonella enterica* serovar Enteritidis infection in virally immunosuppressed chickens. *Clin. Diagn. Lab. Immunol.* **10:** 670-679.
40. Singh R, Shasany AK, Aggarwal A, Sinha S, Sisodia BS, Khanuja SP, Misra R. 2007. Low molecular weight proteins of outer membrane of *Salmonella* Typhimurium are immunogenic in *Salmonella* induced reactive arthritis revealed by proteomics. *Clin. Exp. Immunol.* **148:** 486-493.
41. Singh SP, Williams YU, Miller S, Nikaido H. 2003. The C-terminal domain of *Salmonella enterica* serovar Typhimurium OmpA is an immunodominant antigen in mice but appears to be only partially exposed on the bacterial cell surface. *Infect. Immun.* **71:** 3937-3946.
42. Sonntag I, Schwarz H, Hirota Y, Henning U. 1978. Cell envelope and shape of *Escherichia coli*: multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. *J. Bacteriol.* **136:** 280-285.
43. Soulas C, Baussant T, Aubry JP, Delneste Y, Barillat N, Caron G, *et al*. 2000. Outer membrane protein A (OmpA) binds to and activates human macrophages. *J. Immunol.* **165:** 2335-2340.
44. Springer S, Lindner T, Ahrens M, Woitow G, Prandini F, Selbitz HJ. 2011. Duration of immunity induced in chickens by an attenuated live *Salmonella* Enteritidis vaccine and an inactivated *Salmonella* Enteritidis/Typhimurium vaccine. *Berl. Munch. Tierarztl. Wochenschr.* **124:** 89-93.
45. Steinman RM, Hawiger D, Nussenzweig MC. 2003. Tolerogenic dendritic cells. *Annu. Rev. Immunol.* **21:** 685-711.

46. Tobar JA, Carreno LJ, Bueno SM, Gonzalez PA, Mora JE, Quezada SA, Kalergis AM. 2006. Virulent *Salmonella enterica* serovar Typhimurium evades adaptive immunity by preventing dendritic cells from activating T cells. *Infect. Immun.* **74:** 6438-6448.
47. Torres AG, Li Y, Tutt CB, Xin L, Eaves-Pyles T, Soong L. 2006. Outer membrane protein A of *Escherichia coli* O157:H7 stimulates dendritic cell activation. *Infect. Immun.* **74:** 2676-2685.
48. Wick MJ. 2003. The role of dendritic cells in the immune response to *Salmonella*. *Immunol. Lett.* **85:** 99-102.
49. Wu M, Stockley PG, Martin WJ 2nd. 2002. An improved western blotting technique effectively reduces background. *Electrophoresis* **23:** 2373-2376.
50. Wu XB, Tian LH, Zou HJ, Wang CY, Yu ZQ, Tang CH, *et al*. 2013. Outer membrane protein OmpW of *Escherichia coli* is required for resistance to phagocytosis. *Res. Microbiol.* **164:** 848-855.
51. Ying T, Wang H, Li M, Wang J, Wang J, Shi Z, *et al*. 2005. Immunoproteomics of outer membrane proteins and extracellular proteins of *Shigella flexneri* 2a 2457T. *Proteomics* **5:** 4777-4793.