Bangl. J. Vet. Med. (2008). 6 (2): 145–151

IMMUNOLOGICAL CHARACTERIZATION OF 37.81 kDa COMMON IMMUNODOMINANT SURFACE PROTEIN OF SOME *SALMONELLA* SEROVARS

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ABSTRACT

The research was performed for the immunological characterization of the 37.81 kDa common immunodominant surface protein of some *Salmonella* serovars in order to examine the potency of that particular protein in terms of immunity production as well as withstood challenge exposure against virulent homologous and heterologous organism. The protective efficacy study in chicks gave protective levels of 40-50% in the heterologous challenge experiments and 50-60% in the homologous virulent challenge experiments. Therefore, the 37.81 kDa surface protein might be useful at a certain level in control of salmonellosis in chicks.

Key words: Characterization, surface protein, Salmonella, chickens

INTRODUCTION

Salmonella infection is a serious medical and veterinary problem world-wide and causes great concern in the food industry. Meat of *S. typhimurium* DT104-infected cattle and pigs as well as poultry and eggs contaminated with *S. enteritidis* and S. *typhimurium* are responsible for acute gastroenteritis in humans (Carraminana *et al*., 1997, Solano *et al*., 1998, Schwartz, 1999). Fowl typhoid (*Salmonella gallinarum*) and Pullorum disease (*S. pullorum*) remain to cause economic losses in many countries where the poultry industries are continuing to develop and where open-sided housing is common (Mastroeni and menager, 2003). Prevention of the disease by implementation of hygienic measures is possible but difficult. Antibiotic treatment of the infection has been successful in the past, but new multi-drug-resistant *Salmonella* strains are rapidly emerging (Mastroeni and menager, 2003). Vaccination is an effective tool for the prevention of *Salmonella* infection. However, efficacy of currently available vaccines is not always enough (Mastroeni and menager, 2003).

Exposure to *Salmonellae* can result in antibody responses to a large number of antigens including lipopolysaccharide determinants (O-polysaccharide and core regions), porins, outer membrane proteins, lipoproteins, heat-shock proteins, flagella and fimbriae (Kuusi *et al*., 1979, Brown and Hormache, 1989, Matsui and Arai, 1989, Muthukkumar and Muthukkaruppan, 1993, Cooper and Thorns, 1996, Harrison *et al*., 1997, McSorley *et al*., 2000). The chemical and immunological characterization of a low molecular weight outer membrane protein (Omp-28) with 28-kDa and pl 4.6 which was isolated from *S. typhi* cells was reported (Andrade *et al*., 1998). The antisera from mice immunized with Omp-28 kDa gave a bactericidal activity to kill 50% of *S. typhi* cells in serum dilution of 1/80 (Andrade *et al*., 1998). These studies demonstrated that immunological responses to *Salmonella* species dramatically varies on the basis of antigenic source and nature.

The interest of the present study was characterization of outer surface proteins of a wide variety of *Salmonella* serovars which possessed immunological activity to develop a suitable common vaccine for control Salmonellosis with a wide variety of *Salmonella* serovars and in various hosts. To achieve this purpose, a well-characterized and immunologically dominant common surface protein was important. The presence of the common surface protein of 37.81 kDa among fifty four *Salmonella* serovars (Begum, 2005) raised a possibility that the 37.81 kDa protein induced homologous or heterologous protective efficacy against the infection of *Salmonella* serovars. The common surface protein was characterized and used for immunization for a protective efficacy study.

MATERIALS AND METHODS

The whole research work was performed in the Animal Health Laboratory, School of Agriculture, Ibaraki University, Ibaraki, Japan during the period of January 2004 to December 2004. Two hundred-day-old specific pathogen free (SPF) White Leghorn chicks (Average body weight: 33.91±3.32g) were obtained from the National Institute of Animal Industry, Tsukuba, Japan. The chicks were divided into 4 groups, each group containing 50 chicks, and again subdivided into 5 groups, each group containing 10 chicks, of which one group was used as control. Each chick in each group was individually marked by tag in leg. The chicks were individually weighed and assigned to electrically heated pens. The pens were in a plastic isolator (A device for filtering the air for taking breath of chicks). The chicks were housed in a quiet and undisturbed room which was previously fumigated with formaldehyde. The chicks were reared with the supplement of antibiotic-free starter feed and tap water ad libitum.

NA-resistant *Salmonella typhimurium* L1338, *Salmonella enteritidis* A19, *Salmonella cerro* A12, and *Salmonella johannesburg* A28 *w*ere used for virulent challenge as well as a source of 37.81 kDa protein. The 37.81 kDa protein bands of *Salmonella* serovars described above were excised from the SDS-PAGE gels for purification according to the method of Miyazaki *et al*. (1994). Prior to immunization, the chick sera were checked for antibodies to *Salmonella* antigens by agglutination test. The chicks which were negative for *Salmonella* antibody were used for raising antibodies against the surface protein of 37.81 kDa.

Chicks were immunized according to the method of Tabaraie et *al*., (1994), with some modifications. Briefly, chicks were given intraperitonial (i/p) injection of 150 µg of the 37.81 kDa surface protein of the NA-resistant 4 *Salmonella* serovars. Primary and secondary immunizations were performed in 2-day-and 21-day-old chicks respectively. Blood samples of control and immunized chicks were collected at 2, 7, 14 and 21days of primary immunization. Similarly, blood samples were also collected from chicks at 7 and 14 days of secondary immunization. Control and immunized sera were used for agglutination test. The control and immunized chicks were challenged with challenge dose of virulent homologus and heterologus *Salmonella* serovars after 14 days of the secondary immunization.

The challenge dose was prepared according to the method of Nakamura *et al*., (2002) and Seo *et al*., (2002). Briefly, one colony of *S. typhimurium* L1338, *S. enteritidis* A19, *S. cerro* A12 and *S. johannesburg* A28 from the DHL agar containing 25 µg/ml NA, was transferred into 100 ml of TSB and incubated for 24 h at 37ºC. The culture was then diluted 100 times with sterile TSB and 0.5 ml of the diluted culture was inoculated orally into each group of chick. Instead of TSB only 0.5 ml physiological saline was inoculated orally into control group. Thereafter, the 0.5 ml TSB inocula were diuluted in saline (0.85% NaCl), and spread on DHL agar. After 24 h incubation at 37ºC, the colony forming units were counted. The challenge dose was *S. typhimurium* = 5.8×10^6; *S. enteritidis* =7.5×10^6; *S. cerro* =7.6×10^6; *S. johannesburg* A28 =7.5×10^6.

Agglutination test was performed according to the method of Kashiwazaki *et al*., (1980), with slight modification. One colony of *Salmonellae* from DHL agar containing 25µg/ml NA was cultured in 100 ml of TSB. After incubation for 24 h at 37ºC, the culture was centrifuged at 9,500 rpm for 15 min at 5˚C. The precipitate was resuspended in 25 ml of formalized saline (0.85% NaCl and 1% formaldehyde) for 4-h at room temperature. Agglutination test was carried out as follows: 0.25 ml of the formalized cell suspension was mixed with an equal volume of serially twofold dilution of a serum. After incubation for 2 h at 37ºC, agglutination was observed with a dark-field microscope at magnification of ×200. Antibody titres were expressed as the maximum dilution of the serum giving 50% microscopic agglutination of the organism. Instead of a serum, physiological saline was used as negative control.

RESULTS AND DISCUSSION

The purity of protein was checked by SDS-PAGE using a 10% separation gel (Fig. 1) and IB was performed for further confirmation (Fig. 2). Fig. 1 shows 10% SDS-PAGE electrophoretic pattern of the 37.81 kDa pure proteins from *S. typhimurium* L1338, *S.* Enteritidis A19, *S*. Cerro A12, *S. johannesburg* A28. Fig. 2 shows IB patterns of whole cell lysates and the pure 37.81 kDa protein of *S. typhimurium* L1338, *S. enteritidis* A19, *S. cerro* A12, and *S. johannesburg* A 28. Fig. 1 showed the 37.81 kDa pure protein was used for immunological response. Immunological responses after immunization of the pure 37.81 kDa protein were detected by agglutination test with 4 *Salmonella* serovars (Table 1).

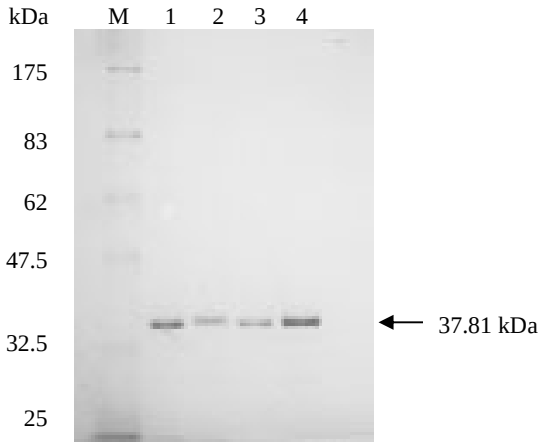

Fig. 1. SDS-PAGE of several pure 37.81 kDa proteins. Lane 1, the 37.81 kDa protein of *S. typhimurium* L1338; Lane 2, the 37.81 kDa protein of *S. enteritidis* A19; Lane 3, the 37.81 kDa protein of *S. cerro* A12; Lane 4, the 37.81 kDa protein of *S. johannesburg* A28. Arrow indicates the 37.81 kDa. M, molecular size markers.

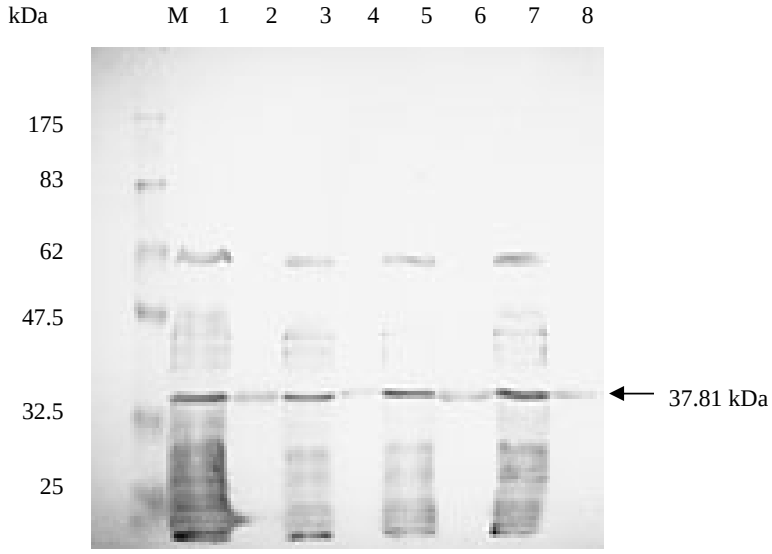

Fig. 2. IB reaction profiles of the pure 37.81 kDa protein and several whole cell lysates of *Salmonella* serovars using the sera of chicks infected with *S. typhimurium*. Lane 1, *S. typhimurium* L1338; Lane 2, the 37.81 kDa protein of *S. typhimurium* L1338; Lane 3, *S. enteritidis* A19; Lane 4, the 37.81 kDa protein of *S. enteritidis* A19; Lane 5, *S. cerro* A12; Lane 6, the 37.81 kDa protein of *S. cerro* A12; Lane 7, *S. johannesburg* A28; Lane 8, the 37.81 kDa protein of *S. johannesburg* A28. Arrow indicates 37.81 kDa. M, molecular size markers.

Table 1. Immunological response (Homologous reaction) by agglutination test after primary and secondary immunization with the pure 37.81 kDa protein

Sera of	Days (Observation)	Antigens			
		ST	SE	SC	SJ
2 dpi of primary immunization	2	–	–	–	–
7 dpi of primary immunization	7	–	–	–	–
14 dpi of primary immunization	14	–	–	–	–
21 dpi of primary immunization	21	±	±	±	±
7 dpi of secondary immunization	28	±	±	±	±
14 dpi of secondary immunization	35	+	+	+	+

–, no agglutination; +, more than 50% agglutination; and ±, less than 50% agglutination; ST, *S. typhimurium* L1338; SE, *S. enteritidis* A19; SC, *S. cerro* A12; SJ, *S. johannesburg* A28; dpi-days post immunization; Serum agglutination titre1:200, which indicate the highest dilution of the serum using agglutination test and gave more than 50% agglutination after secondary immunization.

In primary immunization, 2dpi (days post immunization), 7dpi, 14dpi sera showed no agglutination with *S. typhimurium* L1338, *S. enteritidis* A19, *S. cerro* A12, and *S. johannesburg* A28. But, 21dpi of primary immunization showed less than 50% agglutination. In secondary immunization, 7dpi sera showed less than 50% agglutination but 14dpi showed more than 50% agglutination. The agglutination titre of sera was 1:200. Cross reaction of immunological responses after immunization of the pure 37.81 kDa protein were detected by agglutination test with 4 *Salmonella* serovars (Table 2). The antisera against the pure 37.81 kDa protein of , *S. typhimurium* L1338, *S. enteritidis* A19, *S. cerro* A12, and *S. johannesburg* A 28 also showed cross reaction after secondary immunization by agglutination test. In cross reaction, the highest dilution of sera was used as 1:50 (Table 1). In each group of sera showed cross agglutination test and the highest dilution of sera were 1:50.

The protection level of 37.81 kDa protein of 4 *Salmonella* serovars was shown in Table 2. According to the Table 2, group-1**,** ST/ST, ST/SE, ST/SC, and ST/SJ indicated 37.81 kDa protein of *S. typhimurium* immunized chick group. This chick group was challenged by ST, (*S. typhimurium*) SE, (*S. enteritidis*) SC, (*S. cerro*) and SJ (*S. johannesburg*). The challenge doses were *S. typhimurium* = 5.8×10^6; *S. enteritidis* = 7.5×10^6; *S. cerro* =7.6×10^6; *S. johannesburg* A28 = 7.5×10^6. In case of homologous virulent challenge (ST/ST), the protection level was 60%. But in case of heterologous virulent challenge (ST/SE, ST/SC, and ST/SJ), protection level was 40-50%. The same results were found in case of group-2, SE/SE, SE/ST, SE/SC and SE/SJ indicated the 37.81 kDa protein of *S. enteritidis* immunized chick group. This chick group was challenged by SE, (*S. enteritidis*) ST, (*S. typhimurium*) SC, (*S. cerro*) and SJ (*S. johannesburg*). In group-3, SC/SC, SC/ST, SC/SE and SC/SJ indicated 37.81 kDa protein of *S. cerro* immunized chick group. This chick group was challenged by SC, (*S.* Cerro) ST, (*S. typhimurium*) SE, (*S. enteritidis*) and SJ (*S. johannesburg*). In case of homologous virulent challenge (SC/SC) the protection level was 50%. But in case of heterologous virulent challenge (SC/ST, SC/SE and SC/SJ), the protection level was 40%. More or less similar results were found in case of group-4, SJ/SJ, SJ/ST, SJ/SE and SJ/SC indicated 37.81 kDa protein of *S.* Johannesburg immunized chick group. This chick group was challenged by SJ (*S.* Johannesburg), ST (*S. typhimurium*), SE (*S. enteritidis*) and SC (*S. cerro*).

In each case same challenged doses were used. The protection level of 4 *Salmonella* serovars were 50-60% recorded in case of homologous virulent challenge and 40-50% recorded in case of heterologus virulent challenge (Table 3).

Table 2. Cross reaction of antisera against the pure 37.81 kDa protein by agglutination test with some *Salmonella* serovars

Name of antisera	No. of test	Reciprocal of the agglutination titres to these antigen			
		ST	SE	SC	SJ
S. typhimurium	1	200	<50	<50	<50
	2	200	<50	<50	<50
S. enteritidis	1	<50	200	<50	<50
	2	<50	200	<50	<50
S. cerro	1	<50	<50	200	<50
	2	<50	<50	200	<50
S. johannesburg	1	<50	<50	<50	200
	2	<50	<50	<50	200

ST, *S. typhimurium* L1338; SE, *S. enteritidis* A19; SC, *S. cerro* A28; SJ, *S. johannesburg* A28; 1 and 2 indicate test number.

Table 3. Protective effect of the 37.81 kDa protein immunization, after homologous and heterologous virulent challenge with *Salmonella* serovars

Challenge in immunized chick groups		Mortality	Survivals	% of protection
ST/ST	Group-1	4	6	60
ST/SE		6	4	40
ST/SC		5	5	50
ST/SJ		6	4	40
Control		10	0	0
SE/SE	Group-2	4	6	60
SE/ST		6	4	40
SE/SC		5	5	50
SE/SJ		6	4	40
Control		10	0	0
SC/SC	Group-3	5	5	50
SC/ST		6	4	40
SC/SE		6	4	40
SC/SJ		6	4	40
Control		10	0	0
SJ/SJ	Group-4	5	5	50
SJ/ST		6	4	40
SJ/SE		6	4	40
SJ/SC		6	4	40
Control		10	0	0

ST, *S. typhimurium* L1338, SE, *S. entritidis* A19, SC, *S. cerro* A12, SJ, *S. johannesburg* A12. Group-1 indicates the 37.81 kDa protein of ST (*S. typhimurium*) immunized chick group. This chick group was challenged by ST (*S. typhimurium*), SE (S. *enteritidis*), SC (S. *cerro*), and SJ (*S. johannesburg*). In case of other groups, the same conditions were maintained.

In the present experiments, immunological characterization of the 37.81 kDa surface protein from some *Salmonella* serovars was performed to find the suitability of this protein to use as a vaccine candidate for the control of a wide variety of *Salmonella* serovars. *Salmonella* infection results in antibody responses to a large number of antigens including LPS determinants (O-polysaccharide and core regions), porins, outer membrane proteins, lipoproteins, heat-shock proteins, flagella and fimbriae (Kuusi *et al*., 1979, Brown and Hormaeche., 1989, Charles *et al*., 1993, Cooper, 1996, Harrison *et al*., 1997, McSorley *et al*., 2000). Like other Gram-negative bacteria, *S. typhimurium* possesses an outer membrane surrounding the periplasmic space. The outer membrane contains numerous proteins reffered to as OMPs (Meyer *et al*., 1998). A subset of them is known as porins which form water-filled channels across the outer membrane to facilitate the transport of small hydrophilic molecules (Nikaido, 1996). *S. typhimurium* expresses three porins: OmpD (34 kDa); OmpF (35 kDa); and OmpC (36 kDa) when grown under normal condition of Lennox broth at 37ºC (Lee *et al*., 1980, Nikaido, 1985, Singh *et al*., 1992). OmpD is found in *S. typhimurium* but is absent from other Gram-negative bacteria including *E. coli*. OmpD is homologous with the NmpC and Lc porins in *E. coli* K-12 (Singh *et al*., 1992), both of which can only be expressed in *E. coli* K-12 mutants which lack normal outer membrane proteins (Riley *et al*., 1987). Little is known about the OmpD porin, apart from the genomic location of the *ompD* gene and the immunochemical and topological structure of the porin itself (Singh *et al*., 1996 and 1992).

Here, it was tried to establish porin-like materials which induced humoral immunity to find new proteins as candidates of vaccine for Salmonellosis control. Toward a first step, the surface proteins of 37.81 kDa from *S. typhimurium* L1338, *S. enteritidis* A19, *S. cerro* A12 and *S. johannesburg* A28 were purified according to the information received from SDS-PAGE, IB and 2D-PAGE (Begum, 2005). The 37.81 kDa protein was immunized the chicks followed by the homologous and heterologous virulent challenge experiments (Table 2). In each case, the highest protection level was recorded with the homologous virulent challenge in comparison with the heterologous virulent challenge. The protection ranges of 40-60% were recorded. Moreover, field trial should be given in different age, sex and breed groups of chicks and also in immunization protocols for the determination of better protection level.

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