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Analysis of *Salmonella enteritidis* Outer Membrane Proteins and Lipopolysaccharide Profiles with the Detection of Immune Dominant Proteins

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Abstract: Problem statement: *Salmonella* infection is a serious medical and veterinary problem world-wide and causes great concern in the human health and food safety. *Salmonella enteritidis* outer membrane proteins and lipopolysaccharide play important role in the virulence and immunological properties of bacteria. Prophylaxis of enterobacterial infection has proven difficult to achieve. Vaccination is an effective tool for the prevention of *Salmonella* infection. **Approach:** Therefore, a study was conducted on *S. enteritidis* isolates from chicken meat samples for Outer Membrane Proteins (OMPs) and Lipopolysaccharide (LPS) profile were analyzed by SDS-PAGE electrophoresis and immunodominent antigen was detected by western blot analysis. **Results:** *S. enteritidis* with different OMPs bands were exhibited with molecular weight ranging from 5-90 kDa. The major outer membrane protein profiles of all *S. enteritidis* isolates were homogenous with different expression in intensity of protein was observed. LPS of different *S. enteritidis* isolates exhibited doublet band was observed and identified as R type of strains. The immunoblotting results at 14.4 and 24 KDa proteins were good immunogen. **Conclusion:** The 14.4 and 24 KDa proteins were immune response protein. This proteins can use for vaccine development.

Key words: *Salmonella enteritidis*, outer membrane protein, immune-dominant antigen

INTRODUCTION

Salmonella enteritidis is a causative agent of major food-borne illness and life threading problem worldwide. Poultry products are known to be a significant reservoir for *Salmonella* and most important source in human infection (Maripandi and Al-Salamah, 2010). The World Health Organization (WHO) has estimated that annually 1.3 billion cases of acute gastroenteritis or diarrhea due to non typhoid salmonellosis occur with 3 million deaths. It is usually difficult to evaluate the situation of salmonellosis in developing countries because of the very limited scope of studies and lack of coordinated epidemiological surveillance systems (Acha and Szyfres, 2001). The relatedness of Salmonella isolates outer membrane proteins (Davies, 1991) and lipopolysaccharide profile (Threlfall and Chart, 1993) analyses have proved to be useful techniques in the characterization of these bacteria.

The currently available vaccines against salmonellosis can be divided into three major classes: Bacterins, attenuated and subunit vaccines. Protection induced by bacterins in poultry is generally modest (Barbour *et al*., 1993; Gast *et al*., 1993). In humans, killed vaccines elicit good antibody responses but induce poor cell-mediated immunity, are reactogenic and confer a moderate degree of protection (Collins, 1974). Live attenuated vaccines have multiple advantages over nonviable vaccines because of their ease of administration, ability to carry heterologous antigens and capacity to induce mucosal, cellular and humoral immune responses. Live attenuated *Salmonella* vaccines offer varying degrees of protection in chickens (Cooper *et al*., 1996; Zhang-Barber *et al*., 1999). Acellular vaccines containing immune-dominant protein components of the bacteria may offer an alternative. The components of such subunit vaccine should be founded on the empirical data indicating that in animals and humans exposed to live *Salmonella*, long-lasting immunological memory are directed towards a broad spectrum of antigens, including lipo-polysaccharide, outer-membrane proteins, flagellae epitopes and fimbriae. The present study was to

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investigate *Salmonella* OMP and LPS profiling and to evaluate the antigenicity of the outer membrane proteins by western blot as an effective identification method for potent antigen protein detection. In future acellular vaccine development would be carried out with the help of immune-dominant proteins against poultry salmonellosis.

MATERIALS AND METHODS

Bacterial cultures and maintenance: Salmonella cultures were isolated from poultry meat samples. *S. enteritidis* isolates were identified by standard biochemical and serological methods (Cox and Williams, 1976). The cultures were stored at 4°C as stationary cultures on nutrient agar slants (Hi-Media, India) maintained at Microbiology Laboratory, KS Rangasamy College of Arts and Science College, Tiruchengode, Namakkal District, Tamilnadu, India for experimental studies. A total of twelve *S. enteritidis* isolates were used based on the antibiotic resistant patterns. For outer membrane protein and lipopolysaccharide profiles analyses, the isolates were subcultured on Luria Bertanii medium at 37°C for overnight on orbital shaker incubator.

Isolation of outer membrane proteins: The bacterial culture (10 mL) was taken and centrifuged at 3000 rpm for 10 min at 4°C. The pellet was resuspended in 1000 μL of phosphate buffer saline and washed twice. The washed pellet was resuspended in 1000 μL of HEPES-KOH (0.25 M Sucrose 30 mM HEPES 0.1 mM $MgCl_2$14 Mm β-(1.2 mL) 0.1 mM PMSF-Phenyl methane sulfonyl fluoride.) buffer. It was homogenized by sonication for 30 min, centrifuged at 7000 rpm for 15 min at 4°C and the supernatant was transferred to fresh tube, centrifuged 20,000 rpm at 4°C for 30 min. The supernatant was discarded and the membrane pellet was air dried. The membrane pellet was resuspended in 300 μL of membrane extraction buffer (50 mM Tris HCL; 1 mM NaCl 0; 1 mM PMSF; 8 M Urea). It was incubated at room temperature for 1-2 h with occasional shaking. About 700 μL of potassium buffer (50 mM KH_2PO_4; 1 mM EDTA; 50 mM NaCl pH 10.7) was added for lysis of the mixture. The samples was incubated at room temperature for 30 min and centrifuged at 10,000 rpm for 5 min at room temperature supernatant was transferred to fresh tube. By adding saturated $(NH_4)_2SO_4$, mixed well and allowed to precipitate at 4°C for 30 min and centrifuged at 12,000 rpm for 20 min at 4°C thereby pellet was resuspended in 50 mM sodium acetate (pH 4.5-5.5). Protein concentration was estimated by the method of Lowry *et al.* (1951). SDS-PAGE was performed under reducing conditions with the discontinuous buffer systems of Laemmli (1970).

Isolation of Lipopolysaccharides (LPS): The lipopolysaccharide extractions were prepared from whole cell lysates and also from outer membrane fractions. Whole cell lysates were prepared by adapting the methods of Hitchcock and Brown (1983). The *Salmonella* culture was inoculated in 50 mL of LB broth supplemented with 3% of NaCl and incubated at 37°C for 18 h on orbital shaker incubator. After the incubation the culture was centrifuged at 12000 rpm for 15 min, supernatant was discarded and pellet was allowed to dry. The dried pellet was washed with Phosphate Buffer Saline (PBS) for several times, after that the pellet was resuspended with 50 μL of PBS. This resuspended pellet was mixed with equal volume of double concentrated electrophoresis (SDS-PAGE) sampling buffer and boiled for 10 min at 100°C in water bath. The sample were digested with 30 μL of Proteinase K, electrophoresis sample buffer (0.25% (w/v)) (Laemmli, 1970) and boiled for 15 min at 60°C in water bath. After that the sample was loaded on to 1.5 mm 12.5% SDS-PAGE gel. The gel was stained for visualizing the lipopolysaccharides bands by the methods described by Tsai and Frasch (1982). The gel was removed from the plate and immersed in the fixative solution (Ethanol: Glacial acetic acid: Water) and incubated for 4-12 h. After the incubation, the fixative solution was discarded and the gel was transferred to 30% ethanol solution and incubated for 30 min. The gel was put into distilled water and gently shaken for about 10 min. Later the gel was put into a freshly prepared silver nitrate solution (0.25%) and incubated in dark condition for 30 min. After that the solution was discarded and the gel was rinsed with distilled water for several times. Then, the gel was kept in 2.5% sodium hydroxide solution and 0.02% formaldehyde solution till the bands of desired contrast were obtained.

Immunoblotting: Resolved antigens were electrohporetically transferred to nitrocellulose paper (0.45 mm pore size) using a blot apparatus (Towbin *et al*., 1979). The gels were soaked in transfer buffer (0.025 M Tris, pH 8.3 containing 0.192 Mg lycine and 20% methanol) for 30 min and transfer was carried out at 50V for overnight. The nitrocellulose sheets were used immediately or were stored at 4°C.

Incubating with blocking solution (2% Skimmed milk powder) blocked non reactive sites of the nitrocellose. After washing with blocking buffer for 4 times at 3 min interval, the study was incubated overnight at 4°C with sera diluted in phosphate buffer saline tween. Antibodies bound were detected with Horseradish peroxides conjugated anti rabbit immunoglobulin's diluted 1:1000 at room temperature for 1 h. After repeated washes, the strip were developed using TMB/H_2O_2 as the substrate and was stopped by washing the membrane in distilled water. The molecular weights of the recognized bands were calculated with reference to the standard molecular weight marker.

RESULTS

Salmonella enteritidis OMPs analysis revealed that the different isolates of *S. enteritidis* showed uniform patterns and intensity of protein bands expression differ in different isolates. In isolate number 1, 2, 10 and 11 the OMP expression has been unique when compared to existing others *S. enteritidis* protein profiles. These isolates have been observed more expressed protein on 14 KDa. Whereas culture number 4 and 7 they have 90% similarities with the earlier mentioned isolates but they have been dissimilar in quantum of 14 KDa protein appearances on the protein gel. On the other hand isolates 6 and 9 they expressed same protein profiles and rest of the *S. enteritidis* number 5, 3, 8 and 12 they have been differ from other isolated in the pattern of protein expression Fig. 1. All the isolates of *S. enteritidis* homogenous protein bands as intense protein bands in the range from 14 and 45 KDa was a general character of the OMP preparation while fainter bands were revealed at the higher molecular weight end (higher than 45 KDa) as well as at the lower molecular weight end (lower than 14 KDa).

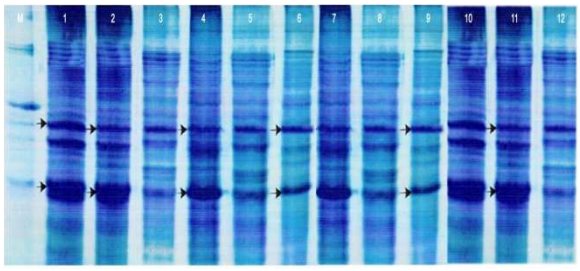

Fig. 1: Outer membrane protein profiles of *Salmonella enteritidis* isolates Lane M: Protein marker with molecular weight 94, 66.2, 45, 31, 21.5 and 14.4 KDa and Lane 1-12 different isolates of *S. enteritidis* OMP expression profiles

The chicken isolates used for the OMP isolation were subjected to LPS analysis. The LPS were extracted from whole cells (grown at 37°C) treated with proteins-K and results were doublet from Fig. 2, which was identified as all the isolates were R type strains. The western blot analysis was carried out against OMP of *S. enteritidis*, serum antibodies from chicken infected with *S. enteritidis* reacted with protein band at 14.4 and 24 KDa while non-infected chicken with *S. enteritidis* were non-reactive with any protein band Fig. 3, based on which the reacted proteins were identified as immuno-dominant antigen.

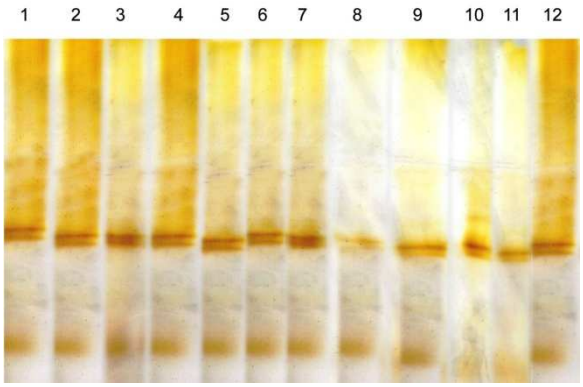

Fig. 2: Lipopolysaccharide expression profile of *S. enteritidis* with doublet appearance by silver staining

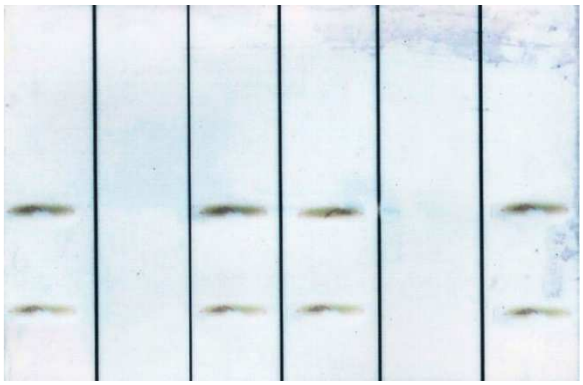

Fig. 3: Immunoblot analysis of antisera from immunized chicken with OMPs of *S. enteritidis.* Lane 1, 3, 4 and 6 OMPs of *S. enteritidis* reacted with immunized chicken sera at 14 and 24 KDa. Lane 2 and 5 uninfected chicken sera not reacted with OMPs of *S. enteritidis*

DISCUSSION

Salmonella infection is a serious medical and veterinary problem world-wide and causes great concern in the food industry. Fowl typhoid (*Salmonella gallinarum*) and Pullorum disease (*S. pullorum*) remain to cause economic losses in many countries (Mastroeni and Menager, 2003). Prevention of the disease by implementation of hygienic measures is possible but difficult. Now-a-days *S. enteritidis* develop a multidrug resistance to many of the antibiotics. In future, application of antibiotics for treatment will become less common. The possible treatment and preventive approach may include recombinant vaccines, peptide library screening and adhesion receptor interaction studies. Prophylaxis of enter bacterial infection has proven difficult to achieve. 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). Despite numerous attempts at vaccine development, the only recognized vaccine still is the *Salmonella* vaccine consisting of whole killed bacteria. Even this vaccine is not satisfactory the protections given is incomplete and of short duration and adverse effects due to toxicity of lipopolysaccharide are always same. Development of vaccine based on defined non-toxic, components of these bacteria would therefore seem desirable.

Vaccines prepared from outer membrane proteins of *S. enterica* have been promising in experiment. The outer membrane of gram negative bacteria, in addition to lipopolysaccharides and lipids contains several proteins which represent integral proteins of the bacterial outer membrane. The OMP electrophoresis analysis of *S. enteritidis* showed that many protein bands which constitute the common genus antigen. The intense protein region which occupied the range from 14 and 45 KDa constituted the *Salmonella* specific OMP bands. The fainter bands which were revealed at the higher molecular weight region (higher than 45 KDa) and at the lower molecular weight region (lower than 14 KDa) were bands related or associated to the OMP or residues of flagella and pilus protein (lower than 20 KDa) or could be residues of *Salmonella* toxins (higher than 67 KDa). OMP preparation from *S. enteritidis* showed greater similarities in their electrophoretic patterns and expression of protein intensity bands were different Fig. 1. Similar type of results reported by Chart and Rowe (1991) demonstrated three major OMPs of 33, 35 and 36 KDa in three *S. enteritidis* strains and one *S. typhimurium* strain. The results revealed that the majority of *S. typhimurium* isolates (74.3%) contained two OMPs of 30.6 and 34.6 kD. Six isolates (17.1%) carried three OMPs of 27.2, 30.6 and 34.6 KDa and three isolates (8.6%) contained only a 30.6 KDa OMP. On the other hand, Helmuth *et al*. (1985) who found that *S. typhimurium* strains generally contained OMPs of 37 and 40 and 41.7 KDa.

In our study *S. enteritidis* lipopolysaccharde analysis showed golden yellow doublet bands when performed silver staining and characteristic of R type strains. Although similar type of results were observed by Hitchcock and Brown (1983) has classified this type of strain as R type (with doublet band) in addition to another *S. typhi* with ladder like lipopolysaccharde. *S. enteritidis* the diversity exhibited by the isolates in terms of OMP should be considered. In the western blot analysis carried out against OMP of *S. enteritidis*, serum antibodies from chicken infected with *S. enteritidis* reacted with protein band at molecular weight 14.4 and 24 KDa. Similar findings were reported by Zamora *et al*. (1999). Antibodies raised against *S. typhimurium* reacted with protein bands at molecular weights of 17, 24 and 31 KDa. This range of protein bands have been claimed as the major outer membrane protein of *S. typhimurium*. In another study involving natural infection of hen with *S. enteritidis* reported by Javier *et al*. (2004) they observed that the higher frequency of reactants of naturally infected hen sera against OMP of 21.1 KDa (66.2%). A reaction against the other OMP defined by its molecular weight was observed between 50.0 and 63.5%, in contrast to low percentage of seropositivity against rest of the components (14, 6.6%; SEF 21, 3.3%; OMP A, 6.7%; Flagella, 0.0%) These results suggest that there is a specific reactivity of sera from naturally infected hens with *S. enteritidis* against SEFs, OMP A and Flagella. The unidentified OMPs with apparent molecular masses of 30.5, 35.8, 41 and 55, may correspond with some of the major antigenic protein from whole cells of *S. enteritidis* described by Barbour *et al*. (2000). The immunogenicity of these components during the natural infection would indicate their possible role as inductive antigens in a sub cellular vaccine for its use in poultry.

In order to control and stop the losses incurred by *Salmonella* in chicken formalin killed *Salmonella* bacterins has been used commercially in the field in many areas of the world (Wray and Sojka, 1984). Yet out of these result we could conclude that *Salmonella* OMP can play an essential role in the induction of immune response in the animals and can be employed as an effective candidate vaccine which can confer solid and active immunity and evade the hefty expenses of treatment of *Salmonella* infected which

lately has acquired multiple potent antibiotic resistance (Carlson *et al*., 2002; Donkersgoed *et al*., 1999; Galland *et al*., 2000).

CONCLUSION

In conclusion, our finding highlighted the OMPs of 14.4 and 24 KDa protein of S. *enteritidis* could immune-response proteins. This proteins can use for vaccine preparation in future. It containing immune-dominant protein components of the bacteria may offer an alternative to an existing vaccine which control the salmonellosis in human and animals.

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