Pak. J. Biochem. Mol. Biol. 2011; 44(1): 8-11

Molecular characterization of *Salmonella* species isolated from patients at a local hospital in Islamabad

Farida Iftikhar and Khalida Sultana*
Honeybee Research Institute, NARC, Islamabad, Pakistan

Abstract: The present study is based on molecular characterization of 17 clinical isolates of *Salmonella* obtained from Children's Hospital, PIMS, Islamabad, Pakistan. Whole cell protein profile analysis of all the isolates was similar and did not reveal any difference within the population. However, analysis of outer membrane protein profiles showed that the isolates fell into two groups on the basis of level of expression and presence or absences of two peptide, i.e. 48 Kd and 43 Kd. A 27 Kd outer membrane protein was expressed strongly only in one isolate. Analysis of purified lipopolysaccharide fractions obtained from *Salmonella paratyphi* showed a lower optical density/ absorption, i.e. 1.003 as compared to the fractions obtained from *Salmonella typhi* i.e. 2.001-2.224. Western blot analysis of cell lysate fraction against sera from a typhoid patient indicated that four major outer membrane protein bands were immunogenic. The data revealed a 46 Kd outer membrane protein to be highly immunogenic which was not reported earlier.

Keywords: *Salmonella,* whole cell protein, outer membrane protein, molecular characterization.
Received: August 10, 2010 **Accepted:** January 30, 2011
***Author for Correspondence:** farida_hbrp@yahoo.com

INTRODUCTION

Salmonella species are members of *Enterbateriaceae* family and have been divided into different subclasses. Only few are medically important like *Salmonella typhi* which causes typhoid fever. These organisms are noncapsulated, nonsporulating, facultative anaerobic bacilli, which have characteristic flagellar, somatic and outer coat antigens[1]. The characteristic lesions of typhoid fever are in the lymphoid tissues of the intestinal wall and mesenteric lymph nodes. Typhoid fever may also be accompanied by disseminated intravascular coagulations leading to haemorrhage[2,3]. A variety of complications may occur including laryngeal ulcer, infection in gall bladder and inflammatory process in different parts of the body, disease of bone-marrow and joints.

Typhoid fever is an unsolved health problem in the world[4]. There are approximately 16 to 33 million cases globally with over 500,000 deaths in the WHO report[5]. Ninety three percent of these cases are reported in Asia[6,7]. The number of human *Salmonella* isolates reported to the Centre for Disease Control, USA has been increasing since 1970[8]. *Salmonella paratyphi* A and B cause paratyphoid fever which is like typhoid fever but not as much severe. *Salmonella paratyphi* C causes septicaemia and is uncommon cause of infections in humans[9]. Non-typhoidal *Salmonella* are major causes of diarrhoeal disease that is gastroenteritis and bacteraemia in children[2,10,11].

Biochemical properties, serotyping and phage typing are routinely used in the reference laboratories for the identification and characterization of *Salmonella* isolates, but their overall low discriminative power mean that these methods are of limited use as discriminative tools in epidemiological studies[12-14]. A number of genotyping and genetic methods represent the major techniques for the characterization of bacteria. Electrophoratic separation of whole cell and outer membrane proteins, or lipopolysacchrides by sodium dodicylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunobloting are the molecular techniques used for the characterization of the bacteria[12,14-18].

The outer membrane protein (OMPs) of *Salmonella* have been considered possible candidates for conferring protection against typhoid. Over the past years, several *Salmonella* OMPs have been investigated as potential vaccine candidates, virulence factors and diagnostic antigens[19]. A mojor OMP with a molecular mass of 49 Kd[20] from *Salmonella typhimurium* that is antigenically conserved[21] has been purified and characterized. Its antigenic evaluation has been reported by Nowsheen and Jain[22].

MATERIALS AND METHODS

Salmonella isolates (17) were obtained from Pakistan Institute of Medical Sciences (PIMS), Islamabad. These pathogens were isolated from the stool sample of patients suffering from typhoid and paratyphoid fever. These isolates were identified in the Quaid-i-Azam University, Department of Microbiology, on the basis of routine serological tests as described in the Medical laboratory manual for tropical countries[23].

Bacterial cultures were stored in the freezing medium and skimmed milk medium at -70^{o}C. In routine experiments the cultures were maintained on LB-Agar slants at room temperatures and sub-

cultured bimonthly. All chemicals used in this study were of molecular biology grade and were obtained from E. Merck and Sigma Chemical Company. Culture media were purchased from Oxide limited and Difco Laboratories.

Whole cell protein preparation

The cultures were grown in 10 ml LB-broth overnight at 37°C. The cells from overnight cultures were harvested by spinning in a microcentrifuge at 8,000 rpm for 2 minutes. The pellet was suspended in 600 µl of Tris-HCl buffer (pH 6.8) and spun again at 8,000 rpm for 2 minutes. The pallet was resuspended in Tris-HCl buffer (400 µl). The cells were then lysed by sonication for 30x6 seconds with a break of 5 seconds in between. The whole sonicate was spun at 8,000 rpm for 2 minutes to remove the unbroken cells. The supernatant of about 40 µl was taken in a fresh ependroff and 4 µl of sample buffer+2-5 µl of B-mercaptoethanol were added. The samples were boiled for 3 minutes in waterbath and quickly placed on ice. These were ready to load on SDS-PAGE for electrophorsis by which the protein fractions were analysed after staining.

Outer membrane protein preparation

Outer membrane proteins (OMPs) were obtained according to the method of Miller Mekalanos[24].

SDS-PAGE

Protein fraction were analysed by SDS-Polyacrylamide Gel Electrophorsis according to the method of Laemmli[25]. Electrophorsis was performed in vertical slab gel apperatus (Bio-RAD Mini Protean-II). The gels were fixed in fixative solution for one hour, stained in staining reagent for two hours and destained for 3-4 hours at 50°C in the detaining reagent.

Isolation of lipopolysaccharides

Lipopolysacharaides were isolated by a modification of the method of Goldman[26]. The samples were analysed for carbohydrates and optical density/absorption was obtained at 485 nm.

Immunoblotting/western blotting

SDS-PAGE was performed in a vertical slab gel apparatus (Bio-RAD mini PROTEAN-II). Electrophoretic transfer of proteins, i.e. whole cell proteins and outer membrane proteins, from polyacrylamide gels to nitrosellulose paper was accomplished in electrophoretic transfer cell (Mini Trans-Blot, BIO-RAD) by means of the transfer buffer.

RESULTS AND DISCUSSION

Whole cell protein profiles

The whole cell protein profiles of all the *Salmonella* isolates are shown in figure 1. The overall protein profile for all isolates are similar and five major protein bands can be identified as is seen due to their strong staining. These are of approx. 37 Kd, 40Kd, 46 Kd,68 Kd and 72 Kd molecular weights. However in isolate number FI-3 the bands of 68 Kd and 72 Kd are lighltly stained despite the fact that other strongly strained bands common to all are equally expressed. In addition, 9 protein bands of approximately molecular weights of 82 Kd, 59 Kd, 48 Kd, 43 Kd, 34 Kd,24 Kd, 22 Kd, 20 Kd and 17 Kd are present in all the isolates. These are less strongly stained. A protein band of 27 Kd molecular weight is strongly expressed in the isolate FI-4 in contrast to all the other strains. Four bands of lesser intensity with molecular weight of 16 Kd, 15 Kd, 14 Kd and 12.7 Kd are also detectable in all the *Salmonella* strains.

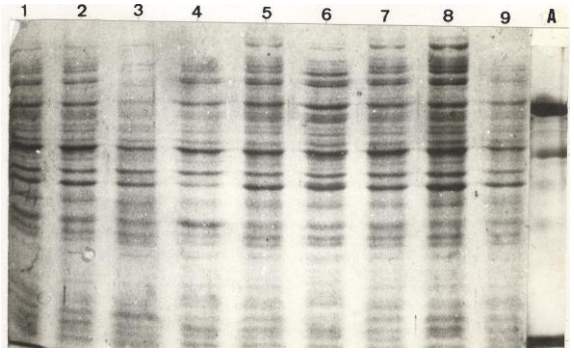


Figure 1: SDS-PAGE of whole cell proteins of *salmonella.* Lane 1-9 (FI-1 to FI-9), lane A 45 kd marker and 14.3 kd lysozyme.

The whole cell protein profile analysis has been used for the subtyping of various bacterial strains[27,28]. All the *Salmonella* strains used in the present study have similar protein profiles. Only one protein band of 27 Kd showed variability which could be due to the difference in level of expression. It is strongly stained in nine isolates and weakly stained in eight out of seventeen isolates. Such a difference has not been reported earlier.

Outer membrane proteins

Outer membrane proteins profile of *Salmonella* isolates are represented in figure 2. Two major outer protein bands can be observed in all the isolates as is seen by their apparent strong intensity of staining. These bands are of molecular weights of 37 Kd and 40 Kd. Further in isolates FI-1, FI-2, FI-5 and FI-6, these appear to be over-expressed as all of these isolates analysed represent similar sample concentrations. In addition two outer membrane proteins bands of 46 Kd and 48 Kd are present in all the isolates. However, these are strongly expressed only in FI-1 and FI-2 strains. The 48 Kd outer membrane band is very weakly stained in FI-6. An

outer membrane protein of 43 Kd is present in all the isolates except FI-6. A 27 Kd outer membrane protein band is more prominent in FI-4 as compared to the other isolates used.

Outer membrane protein profile analysis of various human pathogens can be used for their subtyping[29] and has applied for studying variability in *Salmonella*[30]. The OMPs from *Salmonella typhi* have molecular sizes in the range from 17 to 80 Kd[19]. The isolates used in the present study show the overlapping results. OMPs bands of 46Kd and 48 Kd have not been reported earlier. Differences in the OMPs of both *Salmonella typhi* and *Salmonella paratyphi* strains, used in the present study, were evident especially with reference to the two protein bands. One OMP of 48 Kd was very weakly expressed in the *Salmonella paratyphi* strain. In addition an OMP band of 43 Kd was totally absent from it. On the basis of these results it can be suggested that subtyping of *Salmonella* isolates into *Salmonella typhi* and *Salmonella paratyphi* may be possible by using this approach.

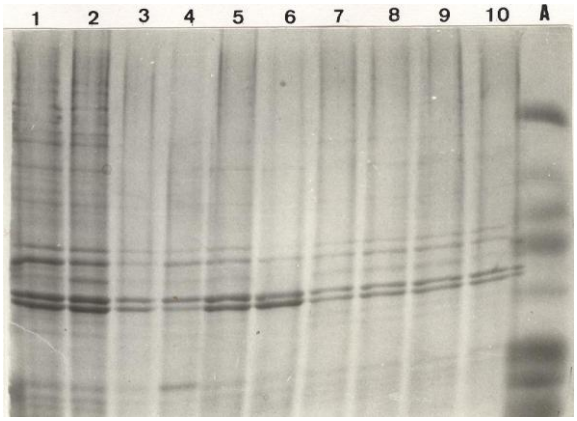


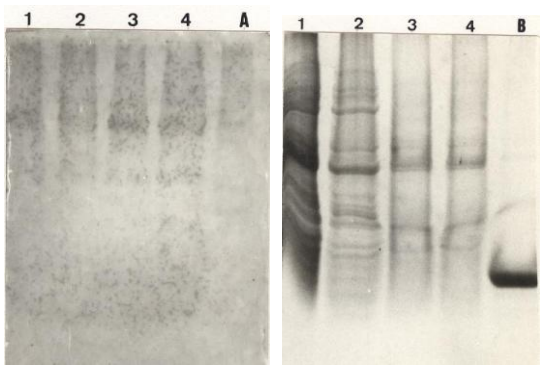
Figure 2: SDS-PAGE of outer membrane proteins of *Salmonella.* Lane 1-10 (FI-1 to FI-10), lane A- daltons marker VI.

Lipopolysacchride analysis

The absorption spectrum of purified lipopolysacchride samples was obtained at 485 nm. All the *Salmonella typhi* isolates have absorption value in the range of 2.001-2.224. However, isolate FI-6(*Salmonella paratyphi*) has an absorption value of 1.003. Jimenez-Lucho and Foulds[31] have reported that the existence of strain difference include LPS composition and amount that is relevant to variation among *Salmonella* isolates. On the basis of present results it can be suggested that subtyping of *Salmonella* isolates by LPS analysis may be possible.

Immunoblotting/western blotting

There are two prominent bands in the immunoblot (Figure 3). The antisera reacted with greater intensity against the protein bands of 27 Kd and 37 Kd. In addition, two more bands of 40 Kd and 46 Kd are also detectable. These show a weak reaction. The 37 Kd band was revealed prominently. Ortiz et al., (1989) have also reported the presence of four proteins to be immunogenic. They have reported the presence of a 28 Kd, a 36 Kd and a 41 Kd band which are close to our analysis i.e 27 Kd ,37 Kd and 40 Kd. The sera also showed the presence of a new band of 46 Kd to be immunogenic. The variation in immunogenicity of OMP's may be strain specific or these may elicit a differential response in different patients.



a) **b)**

Figure 3: a) Immunoblot/western blot of *Salmonella* proteins detected by antisera from a typhoid patient and peroxidise conjugate of anti-Human IgG. b) Whole cell and outer membrane protein of *Salmonella.*

REFERENCES

1. Shahane V, Muley V, Kagal A and Bharadwaj R. Non-typhoid *Salmonellosis*: emerging infection in Pune. *Indian J. Med. Microbiol*., 2007; 25**:**173-174.
2. Hadfield TL, Monson MH and Wachsmuth KI. An outbreak of antibiotic-resistant *Salmonella Enteritidis* in Liberia, West Africa. *J. Infect. Dis*., 1985; 151: 790-795.
3. Setiadharma S and Kho LK. Disseminated intravascular coagulation in typhoid fever in childhood. S. E. Asian. *J. Trop. Med. Publ. Hlth*., 1973; 4: 461-467.
4. Edelman R. Summary of an international workshop on typhoid fever. *J. Infect. Dis*., 1983; 147: 1108-1118.
5. WHO (World Health Organization). Initiative for vaccine research. Typhoid fever. http://www.who.int/vaccine_research/diseases/diarrheal/en/index7.2008.
6. Mercian I. Typhoid fever present and future. *Med. J. Malaysia*, 1997; 52: 299-308.
7. Siddiqui FJ, Rabbani F, Hasan R, Nizami SQ and Bhutta ZA. Typhoid fever in children: Pakistan. Int. *J. Infect. Dis*., 2006; 10: 215-222.

8. Chalker RB and Blazer MJ. A review of human Salmonellosis III. Magnitude of *Salmonella* infection in United States. *Rev. Infect. Dis*., 1988; 10: 111-124.
9. Saphra I and Winter JWN. Clinical manifestations of Salmonellosis in man. As evaluation of 7779 human infection identified at the New York *Salmonella* Center. *N. Eng. J. Med.*, 1957; 256: 1128-1134.
10. Lindquist BL, Lebenthal E, Stinson MW and Merrick JM. Adherence of *S. typhimurium* to small intestinal entrecotes of the rat. *Infect. Immunol*., 1987; 55: 3044-3050.
11. Meadow L, Schneide H and Beem MO. *Salmonella* enteritidis bacteremia in childhood *J. Infect. Dis*., 1985; 152: 185-189.
12. Heir E, Lindstedt BA, Nygai I, Vardund T, Hasseltvedt V, Kappeerud G. Molecular epidemiology of *Salmonella typhimurium* islates from human sporadic and outbreak cases. *Epidemiol. Infect.*, 2002; 128: 373-382.
13. Rementeria A, Vivanco AB, Ramirez A. Charaterization of a monoclonal antibody directed against *Salmonella enteric a typhimurium. Appl. Environment. Microbiol.*, 2009; 75: 1345-1354.
14. Akasakal A. Analysis of whole cell protein profiles of *Salmonella* by SDS-PAGE. *Veterinarni Medicina*., 2010; 55: 259-263.
15. Mare L, Dicks LMT, van der Walt ML. Characterization of South African isolates of *Salmonella enteritidis* by phage typing, numerical analysis of RAPD-PCR banding patterns and plasmid profiles. *Int. J. Food Microbiol.*, 2001; 64: 237-245.
16. Ngwai YB, Ochi K, Ogawa Y and Adachi Y. Analysis of the protein profiles of the antibiotic resistant *Salmonella typhimurium* definitive phage type 104. *Afr. J. Biotechnol.*, 2005; 4: 727-737.
17. Durrani R, Abubakar M, Arshed JM, Saleha S, Ullah I and Ali Q. Biological characterization and protein profiles of two model bacteria by SDS-PAGE and FT-IR. *ARPN J. Agricult. Biol. Sci*., 2008; 3: 6-16.
18. Foley SL, Lynne AM and Nayak R. Molecular typing methodologies for microbial source tracking and epidemiological investigations of Gram-negative bacterial food borne pathogens. *Infect., Genet. Evol.*, 2009; 9: 430-440.
19. Isibasi A, Ortiz V, Vargas M, Paniagua J, Gonzalez C, Moreno J and Kumate J. Protection against *Salmonella typhi* infection in mice after immunization with outer membrane proteins isolated from *Salmonella typhi* 9, 12, dVi. *Infect. Immun.*, 1988; 56**:** 2953-2959.
20. Hamid, T. Biological characterization of theouter membrane proteins of *S. typhi* and *S. typhimurium* and studies on their role in protection against typhoid. Ph.D.thesis. Jamia Hamdard University, New Delhi, India. 2001.
21. Virji M, Zak K and Heckels JE**.** Monoclonal antibodies to gonococcal outer membrane protein IB: use in investigation of the potential protective effect of antibodies directed against conserved and type-specific epitopes. *J. Gen. Microbiol.*, 1986; 132**:** 1621-1629.
22. Nowsheen H and Jain SK. Charaterization of an outer membranr protein of *Salmonella tyhimurium*. *Clin. Vaccine Immun*., 2008; 15: 1461-1471.
23. Monica C. *Medical laboratory manual for Tropical Countries*, Vol II: Microbiology, Butterworth and Co Publishers Ltd.1985.
24. Miller VL, and Mekalanos J. A novel suicide vector and its use in construction of insertion mutation. Osmoregulation of OMP and virulence determinants in *V. cholera* requires toxin. *R. J. Bacteriol*., 1988; 170: 2575-2583.
25. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 1970; 227: 680-685.
26. Goldman, RC, Joiner K and Leive L. Serum-resistant mutants of *E. coli* contain increased lipopolysacchride, lacke an o-antigen containing capsule and cover more of their lipid A core with antgen. *J. Bacteriol*., 1984; 159: 877-882.
27. Nicolet J, Paroz P and Krawinkler M. Nordstom K. M. 1985. Polyacrylamide gel electrophoresis of whole cell proteins of cutaneous Propionibacterium spp. *J. Med. Microbiol*., 1980; 19: 14.
28. Pennington TH and Thomson-Carter FM. Chacterization of methicillin-resistant isolates of S. Aureus by analysis of whole cell and exported proteins. *J. Med. Micro*., 1989; 28: 25-32.
29. Taylor DV, Echeverria P, Hancely S, Slootman L and Piot P. Antimicrobial susceptibility and characterization of OMPs of *H. ducreyi* in Thailand. *J. Clin. Microbiol.*, 1985; 21: 442-444.
30. Ortiz V, Isibasi A, Garcia-ortigoza E and Kumate J. Immunoblot detection of class specific humoral immune response to OPM isolated from *S. typhi* in human with typhoid fever. *J. Clin. Microbiol.*, 1989; 27: 1640-1645.
31. Jimenez-Lucho V. And Foulds J . Hetrogenicity of LPS phenotype among *S. typhi* strains. *J. Infect. Dis*., 1990; 162: 763-764.