JOURNAL OF PURE AND APPLIED MICROBIOLOGY, APRIL 2014. Vol. **8**(2), p. 1569-1577

Evaluation of Protective Efficacy of *Salmonella flagellin* as Vaccine Antigen

Mithilesh Singh[1], Tapas Kumar Goswami[1] and Kuleep Dhama[2]

[1]Scientist, Immunology Section, I.V.R.I, Izatnagar - 243 122, India.
[2]Avian Disease Section, I.V.R.I, Izatnagar - 243 122, India.

(Received: 11 October 2013; accepted: 29 November 2013)

Salmonellosis is a disease complex of man and animals caused by various serovars of *Salmonella enterica* subspecies *enterica. Salmonella* spp. are facultative intracellular pathogens capable of causing localized and systemic disease of significant morbidity and mortality. The present study was aimed to investigate the immune response elicited in mice following subcutaneous immunization with purified flagellin derived from *Salmonella enterica* serovar Typhimurium. Humoral immune response in mice was analyzed by indirect enzyme linked immunosorbent immunoassay (ELISA) wherein a significantly greater antibody response was noticed in immunized group of mice compared to the control group. Further confirmation by western blotting showed immunoreactivity of the isolated flagellin against sera obtained from flagellin immunized group on 21st day post immunization. Bacterial colony count in spleen from the mice immunized with flagellin and challenged with *Salmonella* intra-peritoneally showed complete clearance of the bacteria. In conclusion, bacterial flagellin demonstrated the seroconversion and protective efficacy against homologous bacterial challenge post immunization. Further explorative studies are suggested to unravel its potential application to be used as vaccine antigen or adjuvant.

Key words: *Salmonella,* Flagellin, Mice, Vaccine, Adjuvant.

The genus *Salmonella* are an antigenically diverse group (>2400 serovariants) of Gram-negative facultative intracellular pathogens responsible for a variety of enterica and systemic diseases in human and animals[1,2]. *Salmonella* serovars Enteritidis and Typhimurium are the key pathogen behind most of the food-borne diseases reported worldwide [3,4,5]. Bacterial flagellin is the most abundant monomeric surface protein (50-60 kDa) component of the flagella, encoded mainly by two genes named as *fliC* and *fljB*, that is necessary for motility and survival in a wide variety of prokaryotes [6,7]. This surface protein is the major virulence factor of *Salmonella* and it acts through chemotaxis, adhesion and invasion of host surfaces. *Salmonella flagellins* are able to elicit a great degree of humoral immune response and thus the immunogenicity of flagellin has been exploited in a variety of vaccine strategies[8,9].

In addition to being a target of adaptive immune system; flagellin can also be recognized as pathogen associated molecular pattern (PAMPs) molecules through toll like receptor-5 (TLR-5) and thus able to directly activate innate immune responses by involving various immune cells like monocytes[10], immature dendritic cells[11] and epithelial cells[12]. Recognition of this evolutionary conserved molecule through TLR-5 which is also a highly conserved structure localized on various immune cell surfaces may results into the activation of various potent inflammatory responses and secretion of some effector molecules[13] (eg., cytokines). A number of scientific reports have demonstrated the use of flagellin either as fusion

* To whom all correspondence should be addressed.
Tel.: +91-581-2302692(O); +91-9319092686 (M)
E-mail: drmithileshsingh@yahoo.com

protein[14,15] or even using flagellin alone as vaccine candidate antigen[16].

To develop a safe and effective vaccine antigen/adjuvant it is essential to generate a very high degree of purified product. The current study was undertaken to evaluate the immunogenic potential of *Salmonella* Typhimurium flagellin using two steps procedures involving extraction, purification and characterization of flagellin followed by evaluation of protective immune-response in mice.

MATERIALS AND METHODS

Bacterial strain

An indigenous isolate of *Salmonella* enterica subbspecies *enterica* serovar Typhimurium designated as E-2391 was procured from National *Salmonella* Centre, Indian Veterinary Research Institute, Izatnagar for use in present study. This isolate was originally isolated from the 2 week old broiler chicks suffering from diarrohea from Tanajii Poultry Farm, Pune, Maharashtra. The isolate was confirmed to be as *Salmonella* by biochemical reaction and serotyping according to Kauffmann[17]. To confirm the motility of the bacterial isolate (swim phenotype) semi-solid agar test was done with its inoculation into soft agar (0.5%) containing antibiotic at a final concentration of 20 µg/ml and incubated over night at ambient temperature. Finally, the ability to move through the agar was recorded as per the method described by Clegg and Hughes[18].

Extraction and purification of flagellin

Bacterial flagellin was isolated and purified according to the method described by Strindelius and coworkers[19] with slight modifications. Briefly, *S.* Typhimurium strain was grown under aerobic conditions on semi-solid swarm agar (0.5%) for some passages, inoculated in 5 ml Brain-Heart Infusion (BHI) broth at 37 °C for 16 h. Thereafter, it was further cultivated in fresh 500 ml of BHI broth for 16 h at 150 rpm. The bacterial pellet was harvested following centrifugation at 5000 x g for 30 min at 4°C and re-suspended in normal saline solution (NSS), pH adjusted to 2.0 with 1 M HCl. The bacterial flagellar component was extracted by continuously stirring of the suspension at room temperature for 30 min, centrifugation at 5,000 x g for another 30 min at 4°C and the supernatant obtained being further centrifuged at 100,000 x g for 1 h at 4°C. After final centrifugation, the pH of the supernatant containing soluble monomeric flagellin was adjusted to 7.2 with 1M NaOH followed by precipitation with ammonium sulfate (2/3rd saturation) at 4°C overnight. The precipitate was then centrifuged at 17,400 x g for 15 min at 4°C and dialyzed against PBS, pH 7.4. Isolated protein was filtered through 0.22 µM membrane filter and its concentration was estimated by dye binding method[20].

Characterization of *S.* Typhimurium flagellin by SDS-PAGE and Blotting

Electrophoresis of *S.* Typhimurium flagellin proteins was done in Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) in the Tris/glycine discontinuous buffer system according to Laemmli[21]. Immunoblot analysis was carried out using mice anti-flagellar hyperimmune serum according to Towbin *et al*.[22].

Immunization studies

The extracted *Salmonella* flagellin protein was evaluated for its potential to induce immune-responses and providing protection against homologus challenge in mice. For this purpose, a total of 24 Swiss albino mice (6 to 8 weeks old) of either sex free from *Salmonella* infection were housed under hygienic condition with good management practices. Approval for the experimentation on the mice was granted by Institutional Animal Ethics Committee. Mice were divided into two groups, each comprising of 12 mice. First group was injected subcutaneously (s/c) with 20 µg of flagellin /100 µl NSS while the second group (control) received only NSS. Blood samples were collected from the retro-orbital venous plexus at weekly intervals following flagellin immunization for five successive weeks and the respective serum samples were harvested and stored at -20 °C for further use.

Subclass typing of flagellin specific serum IgG antibodies

Characterization of flagellin specific serum IgG antibodies in the serum samples of immunized mice was done employing indirect ELISA as previously described by Karlsson and coworkers[23] with slight modifications, by analyzing the systemic immunoglobulin G (IgG) and it's subclasses at 0, 1, 2, 3, 4 and 5th week post flagellin

immunization. Briefly, flat-bottom, high binding capacity microtiter plates (Nunc, Denmark) were coated with flagellin (5 µg /ml) and test serum samples were diluted to 1:1600 in PBS with 0.05% Tween-20 (PBS-T); for determination of IgG, goat-anti-mouse IgG peroxidase conjugate (Promega, USA) diluted at 1:5,000 while for IgG1 and IgG2a, goat-anti-mouse IgG1 and IgG2a peroxidase conjugates (Santacrutz, CA), diluted at 1:5,000 were applied. After final three-wash steps with PBS-T, bound conjugate was detected using o-phenylenediamine (OPD)/H_2O_2 substrate solution (Sigma, USA) before the reaction was arrested with 4M H_2SO_4 in a volume of 25 µl /well. Absorbance values were measured at 492 nm wavelength using Multiskan-Ex Microplate ELISA reader (Thermo Scientific, USA).

Western-blotting

Immunoblot analysis of the sera from the experimental mice was done on 21st day post immunization for assessing the sero-reactivity against purified flagellin. Briefly, flagellin was resolved by SDS-PAGE (12.5%) and transferred to polyvinyl difluoride (PVDF) membrane, treated with 1:500 dilutions of test serum and then reacted with goat anti-mouse IgG HRPO conjugate. Finally, diamino benzedene tetrahydro chloride (DAB)/ H_2O_2 substrate solution (Sigma, USA) was added and the colour development was observed indicative of positive sero-reactivity.

Challenge studies

Six mice each from both the flagellin immunized and control group were challenged with homologous strain of *S. enterica* serovar Typhimurium (1×10^8 CFU per mouse per 0.2 ml) on 40th day post immunization through intra-peritoneal (I/P) route. Three mice from both the groups were sacrificed on day 7 of post challenge. Spleen was collected separately for each mice in 5 ml of sterile PBS, homogenized using tissue homogenizer and in appropriate concentrations plated onto MacConkey's lactose agar plate. After overnight incubation at 37^{0}C overnight, the number of bacterial colonies (CFU) was counted separately for all three mice and finally average count was considered for final interpretation for assessing the level of clearance of *Salmonella* in flagellin immunized mice.

Gross pathology

From the above sacrificed mice of both the groups, internal organs (liver, spleen and intestine) were examined to record the gross pathological changes following bacterial challenge, if any.

Statistical analysis

All the data were analyzed by GraphPad ver. 4 software. Results from ELISA were analyzed by one way ANOVA and Bonferroni post hoc tests. Differences in variation in the analyzed data were expressed as standard deviation and considered significant at a P value of <0.01.

RESULTS AND DISCUSSION

The purpose of this study was to investigate if the flagellin protein alone could function as an effective immunogen in parentral vaccination against a bacterium that is known to adhere to and pass through the intestinal epithelium. Thus, we have taken *S. enterica* serovar Typhimurium, which is a gram-negative, facultative intracellular bacterium that proliferates inside macrophages in liver and spleen. In present study, the secreted flagellin protein extracted following short cultivation of the bacteria was used to evaluate the potential of flagellin as a vaccine candidate, which is not in parallel with many other vaccination studies, in which attenuated *Salmonella* whole-cell vaccines or secreted proteins in combination with suitable adjuvant have been tested.

Bacterial strain

The isolated *S.* Typhimurium remained localized to the point of the inoculums and was unable to swim when kept at 37^{o}C for overnight period in the presence of antibiotic. However, in the absence of antibiotic, the bacteria expanded from the inoculums and rapidly migrate through the agar. This result indicated that antibiotic might influence the release of flagella[18].

Characterization of *S.* Typhimurium flagellin by SDS-PAGE and Blotting

SDS-PAGE analysis of isolated flagellin of *S.* Typhimurium has permitted the identification of protein component in 50 to 55 kDa (major band) and 2 minor bands as doublet 35 to 40 kDa (Fig. 1). The doublet was not so prominent even in extended staining condition indicating its low detectability. Low detectability of this doublet along with the prominence of the major polypeptide of 50 to 55

Table 1(a). Overall mean of indirect ELISA IgG levels (OD_{492}) in the immunized and control mice. The values are shown as means ±S.D (n= 3) from the mice receiving the flagellin and placebo control

Weeks post immunization	A_{492} nm (mean± SE) values of immunized mice	A_{492} nm (mean±SE) values of control mice	Level of Significance
0	0.16±0.02	0.12±0.01	ns
1	0.38±.01	0.10±0.01	ns
2	0.81±0.01	0.13±0.02	**
3	1.22±0.05	0.1±2.0	***
4	1.41±0.01	0.12±0.01	***
5	1.55±0.02	0.52±0.38	***

*Level of statistical significance ns= not significant ** P<0.01 *** P<0.001

Table 1(b). The flagellin specific IgG1/IGg2a ratio shown as means ± S.D from the mice receiving the flagellin, (n=3)

Weeks post immunization	A_{492} nm (IgG1/IgG2a) values of immunized mice
0	1.00±0.02[a]
1	1.13±0.02[b]
2	1.71±0.04[c]
3	2.42±0.015[d]
4	2.42±0.07[d]
5	2.12±0.02[e]

[a-e]means bearing different superscripts in a column which differs significantly (P<0.001)

kDa was observed repeatedly during our extraction procedure. These findings are in agreement with those of Strindelius *et al*.[19] who pointed that SDS-PAGE of the purified flagellin corresponds to a single dominating band at about 56 kDa, which match with the molecular mass of flagellin (50-60 kDa). Furthermore, immunoblotting was done to confirm the profile of electrophoretically separated proteins and it was concluded that the chief immunogen is around 50 to 55 kDa polypeptide as mentioned by Strindelius *et al*.[19]. Moreover, Alexan *et al*.[16] and Namba *et al*.[24] also found that flagellin is a 55 kDa monomeric protein, extending from the outer membrane of Gram-negative bacteria that propels the organism through its aqueous environment.

Although we have not specifically studied the active epitopes of flagellin in present work, but it can be speculated that they remain preserved through a whole process of purification due to their ability to induce a strong immune response in subsequent studies. Thus, it is evident that the procedure adopted for the extraction and purification of flagellin from *Salmonella* in present study was quite effective and promising in terms of no significant loss in immunogenic epitopes and sufficient protein yield.

Evaluation of humoral immune response by indirect-ELISA and Western blotting

In present study, the kinetics, IgG subtypes and specificity of serum in response to subcutaneous immunization of mice with purified *Salmonella* flagellin @ 20µg/ mouse (without exogenous adjuvant and any booster dose) was investigated. Flagellin-specific antibodies were measured at weekly interval upto 5th wk post immunization. An impressive systemic, humoral response in all flagellin immunized animals was observed. The serum antibody response to flagellin was undetectable at 2 days post-immunization (OD at 1/1600 dilution was not above background, i.e., secondary antibody only) but started increasing significantly from 2nd wk and maintained the same kinetics upto the 5th wk post immunization (Table 1a). The level of response from this single immunization became maximal at 5th week post flagellin administration and thereafter it remained more or less same or started declining (data not shown), but still remained well above the untreated mice. Contrary to our present findings, Alexan *et al*.[16] has reported maximal antibody response against *Salmonella* flagellin upto 6th week post administration. This may be possible because they performed booster inoculation on 3rd wk of primary immunization which may further extend the course of immune response.

The IgG1/IgG2a subclass ratio in the

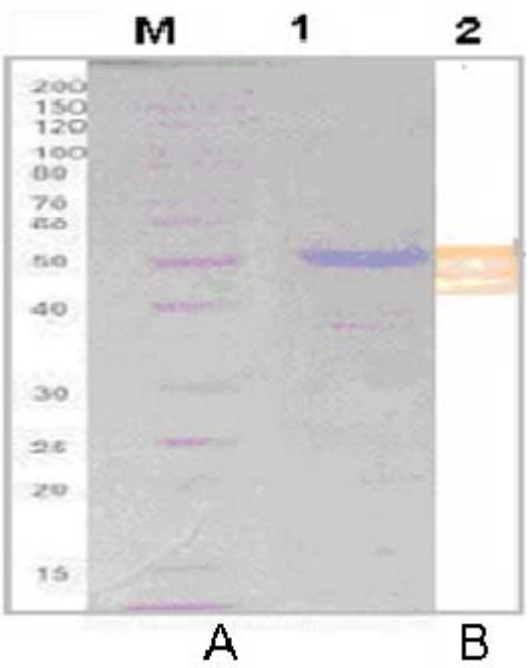
group of mice immunized with flagellin was found to be in the range of 1.13 to 2.42 (Table 1b). The profile of the isotype class switching post immunization demonstrated that flagellin enhanced both the Th1 and Th2 responses as shown by the increases of IgG1 and IgG2a in serum gradually from 1st wk of immunization compared to the control group but it is evident from present finding that the immune response is biased towards the Th_2, although the Th1-type response is known to be an important component of the protective defense against *Salmonella*[25]. Interestingly, Strindelius *et al*.[19] has analyzed this subclass ratio of the systemic humoral immune response in mice by oral immunization with flagellin along with starch microparticles as adjuvant and detected a relatively low ratio of IgG1*/(*IgG2a + IgG2b*)*, which indicates a dominating Th1-type immune response. But the current findings supported the previous report which postulated that soluble flagellin induces Th2-biased responses which are sufficient enough to protect the mice against *Salmonella* infection[26]. This might be due to the fact that divergent flagellin-specific polarization to either Th1 or Th2 subset of CD4+ cells solely depends on the context in which this antigen is encountered to antigen presenting cells rather than its intrinsic immunostimulatory properties which determines the direction of Th polarization.

The specificity of the humoral immune response assessed by Western blotting demonstrated that essentially three major antigens

Table 2. Evaluation of protective immunity in immunized and control mice which were challenged by i.p. inoculation of 1x 10^8 CFU of the homologous strain of *S*. Typhimurium strain. One week post-challenge, three mice from each group were sacrificed and the *Salmonella* CFU in their spleen were counted

Group	Animal ID	Organ (spleen)
Mice	F1	-
immunized	F2	-
with flagellin	F3	-
Mice immunized	C1	+
with PBS	C2	+
(Control)	C3	+

F1, F2 & F3 indicates flagellin received group while C1, C2 & C3 indicates PBS control group which were sacrificed on 7th day post-challenge.



Lane M: Molecular weight marker,
Lane 1, 2: The purified flagellin protein

Fig. 1. SDS-PAGE (Panel A) and Western blot (Panel B) analysis of purified flagellar protein

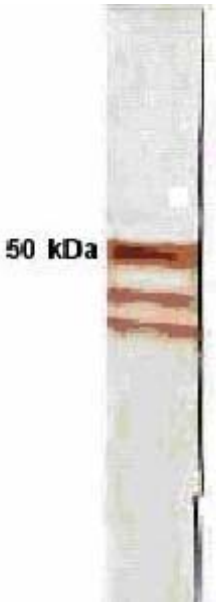


Fig. 2: Western blot analysis of pooled sera from mice immunized with purified flagellin on 3rd wk post immunization against extracellular antigens from *Salmonella* Typhimurium

had triggered the antibody response which resembled the findings of Strindelius *et al*. [27] (Fig. 2). They have analyzed these protein bands by electrospray-mass spectroscopy and concluded that this has originated from the flagellum, namely, flagellin and the hook-associated protein 2.

Thus, it is apparent that majority of antibody responses induced following immunization in mice were directed against flagellin which is a strong immunogen and it could be considered as a potent stimulator of adaptive immune responses even when given alone without any booster and adjuvant support. The present findings are in similar line to some of the previous study carried out in BALB/c mice with secreted antigens from *S. enterica* serovar Enteritidis, which indicated that the anti-flagellin response was an important component of the host's defense against *Salmonella* infections[27,28].

Challenge studies

Challenge studies in animals are limited due to animal protection guidelines, which state that the survival rate should not be a parameter for evaluation of protection. Therefore, in present study, after assessing the virulence of the bacterium strain, 1×10^8 CFU/mouse was used as a quantitative parameter to evaluate protection in terms of bacterial clearance from spleen, as the main sites for multiplication of *Salmonella* species during invasive infection are the liver and spleen[29]. Interestingly, there was direct correlation between the challenge results and the immunological data obtained. Mice immunized with flagellin were protected against homologous *S.* Typhimurium

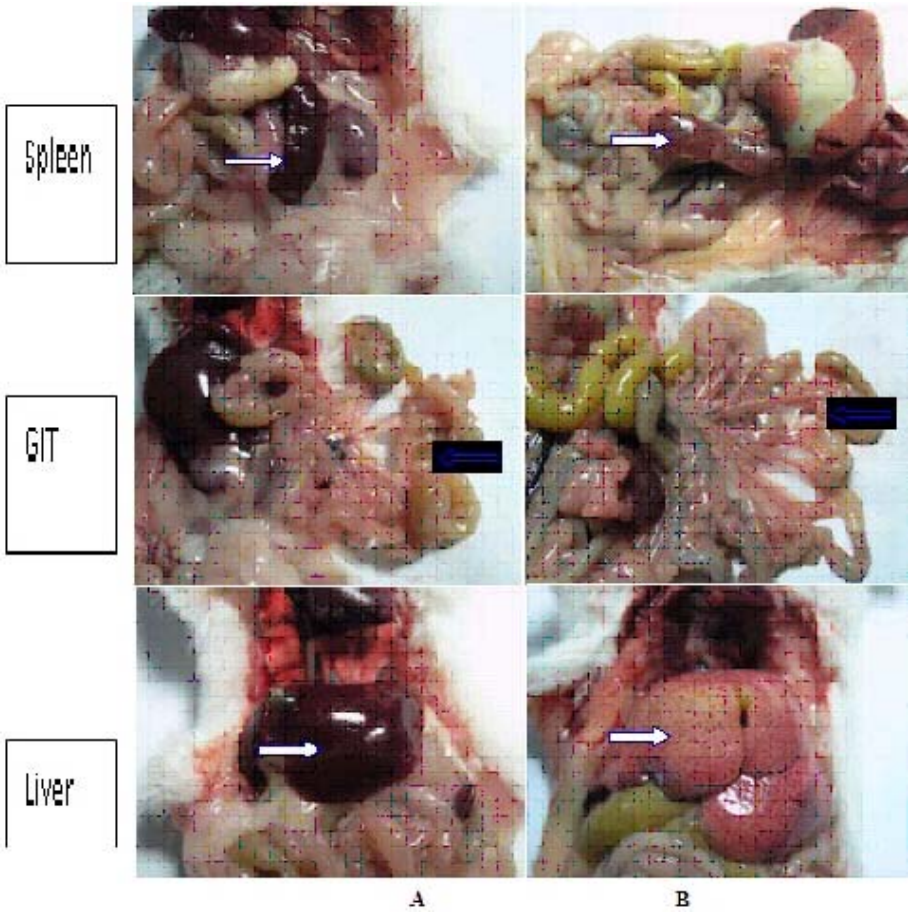


Fig. 3. Gross pathological changes observed in spleen, intestine (GIT) and liver of immunized (Panel A) and control mice (Panel B) post one week challenge with the homologous strain of *S.* Typhimurium strain revealing necrotic lesions in all examined internal organs in addition to the petichial haemorrhages in intestine

challenge showing no characteristic lesion of salmonellosis on necropsy, and their spleen were negative for *Salmonella* in contrast to control group in which pathogen was still persisted (Av. 1x 10^6 CFU/ml) (Table 2 and Fig. 3), which is indicative of 100% bacterial clearance post flagellin immunization in challenged mice. This suggests the role of flagellin in prevention of the multiplication of *Salmonella* in internal organs. It also indicates that the protective responses in terms of bacterial clearance observed were primarily due to immune responses directed against the flagellin protein. These observations are consistent with the hypothesis that the humoral antibodies are largely directed towards bacterial surface antigens such as flagellin, the major subunit of the bacterial flagella[30-32]. Moreover, antibodies may be important in controlling bacterial replication by acting as opsonins[33,34]. The important role of antibody-producing B cells in protection against *Salmonellosis* has been already shown by various workers[35,36]. Furthermore, Yokoyama *et al*.[37] showed that egg yolk antibodies specific for *Salmonella* flagellin, LPS, and outer membrane proteins such as porin, protect mice from Salmonellosis when administered orally. This is in parallel to the present study, in which it has been demonstrated that parentral administration of secreted proteins primarily gives protective antibodies against *Salmonella* infection.

CONCLUSION

Taken together, the evidence to date suggests that flagellin or fragments thereof could be a promising and potent component of a vaccine against *Salmonella* or as an adjuvant for other antigens. In conclusion, present study revealed that the purified *Samonella* flagellin possessed the ability to effectively trigger an effective immune response following immunization in mice and has potential to combat homologous bacterial challenge. This indicates the utility of *Salmonella* flagellin to be exploited as a potent vaccine candidate for this economically important pathogen having public health concerns or to be used as a vaccine adjuvant. However, further explorative studies are suggested for its potential applications as a vaccine antigen or adjuvant.

REFERNCES

1. Fàbrega A., Vila, J. *Salmonella* enterica serovar Typhimurium skills to succeed in the host: Virulence and regulation. *Clin. Microbiol. Rev*., 2013: **26**: 308-341.
2. Lai, M.A., Quarles, E.K., Lopez-Yglesias, A.H., Zhao, X., Hajja, A.M., Smith, K.D. Innate immune detection of flagellin positively and negatively regulates S*almonella* infection. *PLOS One*, 2013: **8**: e72047.
3. Perugini, A.G., Carullo, M.R., Esposito, A., Caligiuri, V., Capuano, F., Galiero, G., Lovane, G. Characterization of antimicrobial resistant *Salmonella* enterica serovars Enteritidis and Typhimurium isolates from animal and food in Southern Italy. *Vet. Res. Commun.*, 2010; **34**: 387-392.
4. Hur, J., Jawale, C., Lee, J.H. Antimicrobial resistance of *Salmonella* isolated from food animals: A review. *Food Res. Int.*, 2012: **45**: 819-830
5. Dhama, K., Rajagunalan, S., Chakraborty, S., Verma, A.K., Kumar, A., Tiwari, R., Kapoor, S. Food-borne pathogens of animal origin-diagnosis, prevention, control and their zoonotic significance: a review. *Pak. J. Biol. Sci*., 2013: **16**(20): 1076-1085.
6. Neish, A.S. TLRS in the Gut. II. Flagellin-induced inflammation and anti- apoptosis. *Am J. Physiol-Gast Liv. Physiol.*, 2007; **292**: 462-466.
7. Oliveira, B.H., Silva, M.R., Braga, C.J.M., Massis, L.M., Ferreira, L.C.S., Sbrogio-Almeida, M.E., Takagi, M. Production of native flagellin from *Salmonella* Typhimurium in a bioreactor and purification by tangential ultrafiltration. *Braz. J. Chem. Engin*., 2011: **28**: 3726-1505.
8. Pan, Z., Cong, Q., Geng, S., Fang, Q., kang, X., You, M., Jiao, X. Flagellin from recombinant attenuated *Salmonella* enterica serovar Typhimurium reveals a fundamental Role in chicken innate immunity. *Clin. Vaccine Immunol*., 2012: **19**: 304-312.
9. McSorley, S.J., Ehst, B.D., Yu, Y., Gevirtz, A.T. Bacterial flagellin is an effective adjuvant for CD_4^+ T-cells *in vivo. J. Immunol.*, 2002; **169**: 3914-3919.
10. Muzio, M., Bosisio, D., Polentarutti, N., Amico, G.D., Stoppacciaro, A., Marcinelli, R., Veer, C., Penton, G., Ruco, L.P., Allavcara, P. Differential expression and regulation of Toll-like receptor (TLR) in human leukocytes: Selective expression of TLR3 in dendritic cells. *J. Immuol.*, 2000;

164: 5998-6004.

11. Kadowaki, H.S., Ho, S., Antonenko, R.W., Matefyl, R.A., Bazar, K.F., Liu, Y.J. Subsets of human dendritic cell precursors express different toll like receptors and respond to different microbial antigens. *J. Exp. Med.*, 2001; **194**: 863-869.
12. Gewirtz, A.T., Nava, T.A., Lyons, S., Godowski, P.J., Madera, J. L. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J. Immunol.*, 2001; **167**: 1882-1885.
13. Nyström, S., Bråve, A., Falkeborn, T., Devito, C., Rissiek, B., Johansson, D.X., Schröder, U., Uematsu, S., Akira, S., Hinkula J., Applequist. S.E. DNA-encoded flagellin activates toll-likerReceptor 5 (TLR5), nod-like receptor family CARD domain-Containing protein 4 (NRLC4), and acts as an epidermal, systemic, and mucosal-adjuvant. *Vaccines*, 2013: **1**: 415-443.
14. Jeon, S.H., Ben-Yedidia, T., Arnon, R. Intranasal immunization with synthetic recombinant vaccine containing multiple epitopes of influenza virus. *Vaccine*, 2002; **20**: 2772–2780.
15. Stocker, B.A., Newton, S.M. Immune responses to epitopes inserted in *Salmonella* flagellin. *Int. Rev. Immunol.*, 1994; **11**: 167-178.
16. Alexan, A.F., Mohamed, S.H., Ibrahim, A.M. Immune response elicited in mice after immunization with flagellin from *Salmonella enterica* serovar Enteritidis. *Glob. Vet.*, 2009; **3**: 465-471.
17. Kauffmann, F. Serological diagnosis of *Salmonella* species. Kauffmann White-Scheme, munksgaard, 1972; Copenhagen, Denmark
18. Clegg, S., Hughes, K.T. FimZ is a molecular link between sticking and swimming in *Salmonella* enterica serovar Typhimurium. *J. Bacteriol.*, 2002; **184**: 1209-1213.
19. Strindelius, L., Filler, M., Sjoholm, L. Mucosal immunization with purified flagellin from *Salmonella* induces systemic and mucosal immune responses in C3H/HeJ mice. *Vaccine*., 2004; **22**: 3797-3808.
20. Bradford, M.M. A rapid and sensitive method for quantitiation of microgram quantities of protein utilizing principle of protein-dye binding. *Ana. Biochem.*, 1976; **72**: 248-254.
21. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 1970; **227**: 115-120.
22. Towbin, H., Staehelin, T., Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedures and some applications. *PNAS*, 1979; **76**: 4350-4354.
23. Karlsson, K., Grastrom, M., Lindberg, A.A. *Salmonella* sp. antibodies. In: methods in enzymatic analysis. 3rd ed. (Burgmeyer, J. and Grabl, M., eds.) vol. XI, Antigens and antibodies, VCH, 1986; pp 85-98.
24. Nmaba, K., Yamashita, I., Vonderviszt, F. Structure of the core and central channel of bacterial flagella. *Nature*, 1989; 342: 648.
25. Mittrucker, H.W., Kaufmann, S.H. Immune response to infection with *Salmonella* Typhimurium in mice. *J. Leuk. Biol.*, 2000; **67**: 457-463.
26. Cunningham, A.F., Khan, M., Ball, J., Toellner K.M., Serre, K., Mohr, E., MacLennan, I.M. Responses to the soluble flagellar protein FliC are Th2, while those to FliC on *Salmonella* are Th1. *Eu. J. Immunol.*, 2004; **34**: 2986–2995.
27. Strindelius, L., Degling, W.L., Sjoholm, I. Extracellular antigens from *Salmonella enteritidis* induce effective immune response in mice after oral vaccination. *Infect. Immun.*, 2002; **70**: 1434-1442.
28. Salazar-Gonzalez, R.M., Srinivasan, A., Griffin, A., Muralimohan, G., Ertelt, J.M., Ravindran, R., Vella, A.T., McSorley, S.J. *Salmonella* flagellin induces bystander activation of splenic dendritic cells and hinders bacterial replication in vivo. *J. Immunol.*, 2007; **179**: 6169-6175.
29. Makela, P. H., Hovi, M., Saxen, H, *et al*. *Salmonella* as an invasive enterica pathogen, In Molecular pathogenesis of gastrointestinal infection T. Wadstrom, *et al*. eds. New York, 1991; pp: 175-184.
30. Brown, A., Hormaeche, C.E. The antibody response to *salmonella*e in mice and humans studied by immunoblots and ELISA. *Microb. Patho.*, 1989; **6**: 445-454.
31. Cawthraw, S.A., Feldman, R.A., Newell, G.G. Long-term antibody responses following human infection with *Campylobacter jejuni*. *Clin. Exp. Immunol.*, 2002; **130**: 101-106.
32. Barat., S., Willer, Y., Rizos, K., Claudi, B., Maze, A., Schemmer, A.K., Kirchhoff, D., Schmidt, A., Burton, N., Bumann, D. Immunity to intracellular *Salmonella* depends on surface-associated antigens. *PLOS Pathogens*., 2012; **8**: e1002966.
33. Rydstrom, A., Wick, M.J. Monocyte recruitment, activation and function in the gut-associated lymphoid tissue during oral *Salmonella* infection. *J. Immuol.*, 2007: **178**: 5789-5801.
34. Lindow, J.C., Fimlaid, K.A., Kirkpatrick, B.D. Antibodies in action: Role of human opsonins in killing *Salmonella enterica* serovar Typhi.

Infect. Immun., 2011: **79**: 3188-3194.

35. Mastroeni, P., Simmons, C., Fowler, R., Hormaeche, C.E., Dougan, G. Igh- "/" (B-cell-deficient) mice fail to mount solid acquired resistance to oral challenge with virulent *Salmonella* enterica serovar Typhimurium and show impaired Th1 T-cell responses to *Salmonella* antigens. *Infect. Immun.*, 2000; **68**: 46-53.
36. Nanton, M.R., Way, S.S., Shlomchik, M.J., McSorley, S.J. Cutting edge: B cells are essential for protective immunity against Salmonella independent of antibody secretion. *J. Immunol*., 2012: **189**: 5303-5307.
37. Yokoyama, H., Umeda, K., Peralta, R.C., Hashi, T., Icatlo, F.C., Kuroki, J.M., Ikemori, Y., Kodama, Y. Oral passive immunization against experimental salmonellosis in mice using chicken egg yolk antibodies specific for *Salmonella Enteritidis* and *S. Typhimurium*. *Vaccine*, 1998; **16**: 388-393.