CELLULAR RESPONSE AND PROTECTIVE EFFECT IN HENS IMMUNISED WITH *SALMONELLA ENTERITIDIS* RECOMBINANT FIMBRIAL SefA, FimA AND AgfA PROTEINS

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**Abstract**

Fimbrial proteins of *Salmonella* Enteritidis: SefA and FimA, in addition to strong humoral response, can induce cellular response. Highly increased number of CD+4 T cells present in the spleen just day 2 after challenge suggests the presence of CD+4 memory cells in immunised birds. Our data clearly show that SefA and FimA fimbrial proteins are highly immunogenic in hens, however, such response seems to affect weakly or to reduce significantly the organ colonization and persistence of *S*. Enteritidis.

**Key words:** hens, *Salmonella* Enteritidis, fimbrial proteins, vaccine, cellular response.

*Salmonella* Enteritidis emerged during the last twenty years as the major infectious agent in poultry farms in Europe and North America. Such infections are both a source of significant production losses in poultry industry and a serious public health problem. Poultry meat, eggs and processed products are considered as major source of food-borne infection by *Salmonella* in humans (22, 23, 35). In order to reduce the number of *Salmonella*-derived gastroenteritis in humans diversified programmes are required, including serological and bacteriological monitoring of chicks and hens at each production stage, regular and effective poultry unit disinfection, prevention of feed infections, competitive exclusion by nonpathogenic bacteria and vaccination (31, 32, 34).

Immunisation reduces the *Salmonella* infection rate in poultry, and may lead to the limitation or elimination of chemical therapeutics in the treatment of salmonellosis (4). In view of the fast growing resistance of microorganisms, including *Salmonella*, to chemical therapy and the risk of drug residue presence in food (e.g. meat, eggs) as well as in the environment, immunisation is an effective infection control measure (30, 32).

Presently, in poultry production units both attenuated and inactivated bacteria-based vaccines are commonly used (16, 26, 31, 34). However, progress in molecular biology techniques creates opportunities of development of new, so called subunit vaccines containing only well-defined repertoire of immunogenic proteins. Such vaccines do not contain unwanted antigens that are neither required to induce specific immunity nor desired due to toxic or allergic side effects (10). For these reasons much effort is put to identify antigens that may be the most effective components of the subunit vaccines.

Adhesion of *S*. Enteritidis to the surface of the intestine epithelium is the first and critical step in the successful infection of the host. The adhesion is mediated by proteinaceous, filamentous structures called fimbriae, present on the surface of bacterial cells. The main fimbriae of the *S*. Enteritidis serovar are SEF14, SEF17 and SEF21 which are built from SefA, AgfA and FimA fimbrial proteins, respectively (28, 33). *S*. Enteritidis fimbriae, representing long, rod-shaped projection surrounding the bacterial cell, seem to be the perfect target for the immune system of the host. Thiagarajan *et al.* (29) have shown that *S*. Enteritidis 14 kDa fimbrial protein induced strong immunological response in hens. Indeed, egg yolk antibodies directed against SefA fimbrial protein protected mice to *S*. Enteritidis infections (21). Later, it was shown that SefA protein induced delayed-type hypersensitivity in *S*. Enteritidis primed animals and stimulated *in vitro* proliferation and production of cytokins by T lymphocytes (19). The effects observed after administration of fimbriae was comparable to that following a whole bacteria cell-based vaccine immunisation. Recently, it was shown that immunisation of chickens with liposome-associated purified SEF14
and SEF21 induced effective systemic and mucosal humoral responses (12). In our previous work we have studied the humoral response in hens immunised with purified recombinant fimbrial proteins of _S. Enteritidis_ (11). With the ELISA assay it was shown that immunisation with SefA and FimA induced strong humoral response in contrast to the low immunogenic AgfA protein. However, the results obtained to-date do not provide comparative information on cellular response of hens or chickens to various types of _S. Enteritidis_ fimbrial proteins, and there is very little information on their usefulness as components of subunit vaccines preventing _Salmonella_ infections of animals and humans. So, the aim of the present study was to evaluate the effects of immunisation of hens with SefA, AgfA and FimA recombinant fimbrial proteins of _S. Enteritidis_ on the cellular response and to assess the protective effect of such immunisation against experimental infection with _S. Enteritidis_.

**Material and Methods**

**Hens.** All the hens used were SHAVER 579. They were purchased from a commercial breeder at the age of 5 weeks. Birds were provided with water and pelleted feed _ad libitum_. They were free from _Salmonella_ according to plate agglutination assay and bacterial culture.

**Production of fimbrial proteins.** The production and purification of SefA, FimA and AgfA fimbrial proteins was performed essentially as it was described by Kisiela _et al._ (11). PCR-cloned fimbrial genes were cloned into pTrcHis2b plasmid (Invitrogen). Resulted expression vectors: sefA/pTrcHis2b, agfA/pTrcHis2b and fimA/pTrcHis2b were used to transform _E. coli_ DHα bacteria. Recombinant fimbrial proteins were purified by using a 6xHis affinity tag located at the carboxy terminus of the proteins on Ni-nitrilotriacetic acid resin (Qiagen). Proteins were quantified by the bicinocochinonic acid protein assay kit (Sigma).

**Preparation of the vaccine and immunisation of hens with fimbrial proteins.** Samples of fimbrial proteins in water-phase containing 0.05% Tween 20 (Sigma), corresponding to 75 µg of protein, were mixed with oil-phase (92% mineral oil and 8% SPAN80) at a ratio of 1:5. Samples corresponding to 0.5 ml of homogenous oil emulsion per bird were administered intramuscularly (i.m.). The hens (70 in each group) were immunised with 75 µg of SefA or FimA or AgfA at 6 weeks of age and boosted with identical doses of antigens at 12 weeks of age. The non-immunised birds were used as control.

**Challenge with _S. Enteritidis_.** The _S. Enteritidis_ strain that was used to infect hens had been originally isolated from poultry infection. Both the immunised and non-immunised hens, all at 16 weeks of age, were infected orally with 5 x 10^7 CFU/ml of _S. Enteritidis_.

**Flow cytometry analysis.** The spleen was removed from hens, which were sacrificed by decapitation at one day before and 2, 4, 6 and 14 d after challenge with _S. Enteritidis_. Animal experiments were performed according to the International Laboratory Animal Care Convention. Single cell suspension was obtained by pressing the spleen through the nylon sieve and suspending in PBS without Mg^2+ and Ca^2+. Lymphocytes were purified on LymphoFlot (Biotest) and washed twice with PBS/1% BSA. Subpopulations of T cells were detected with monoclonal antibodies directed against chicken CD4 and CD8 cell surface antigens (Southern Biotech Associated Inc., Birmingham). Cells, after incubation with primary antibody for 1 h at 4°C, were stained with FITC-conjugated goat F(ab')2 fragment against mouse immunoglobulins (DAKO). Afterwards, the samples were immediately subjected to fluorescence analysis using flow cytometer (FACS Calibur - Becton Dickinson). Five thousand cells were acquired for each datafile. Data were processed, and the mean fluorescence intensity calculated using of the CellQuest 3.1f programme.

**Bacteriological examination.** At one day before the challenge and 2, 4, 6, 14 and 42 d postinoculation with _S. Enteritidis_ the hens were sacrificed as above. The liver, spleen, duodenum with pancreas and caecum with tonsils from each bird were removed aseptically, and small pieces (1 cm x 1 cm) of each organ were transferred to selenite F-broth (SF) and Rappaport-Vassilliadis broth (RV) (bioMérieux) and incubated for 24 h at 37°C for SF and 42°C for RV. Then, the obtained bacterial cultures were grown aerobically on McConkey and Brilliant green agar (BGA) (bioMérieux) for 24 h at 37°C. The isolates were identified as _S. Enteritidis_ by biochemical typing with the use of API-20 E (bioMérieux) and serotyping with diagnostic antisera for rapid agglutination (Biomed, Kraków).

**Statistical analysis.** The results from flow cytometric analysis were analysed by one–way analysis of variance (ANOVA) and differences between the mean values were calculated by the Tukey’s test. The results were classified as statistically significant when P<0.01. For bacteriological isolation rate from organs, significant differences between groups were determined by Fisher’s exact propability.

**Results**

**Flow cytometric analysis of cellular response against _S. Enteritidis_ infection in hens immunised with recombinant SefA, FimH and AgfA proteins.** The _S. Enteritidis_ challenge of SefA or FimA fimbrial protein - immunised hens induced changes within the population of CD4+ and CD8+ splenocytes.

The results obtained from hens immunised with SefA protein are shown in Fig. 1a. A significant increase in the number of CD4+ cells, up to 36.6%
infected hens, the group of FimA immunised hens the percentage of compared with the non-immunised group (Fig. 2a). In was much lower during all period after challenge when positive samples in the group of SefA-immunised hens were analysed for the presence of AgfA protein (data not shown) (Figs 2a, 2b, 3a, 3b).

Isolation of S. Enteritidis from organs of infected hens The liver, spleen, duodenum and caecum were analysed for the presence of S. Enteritidis. In the case of the duodenum, the percentage of Salmonella-positive samples in the group of SefA-immunised hens was much lower during all period after challenge when compared with the non-immunised group (Fig. 2a). In the group of FimA immunised hens the percentage of S. Enteritidis-positive birds was much lower on days 6 and 14 (Fig. 2b). Figure 3a shows the recovery of S. Enteritidis from the caecum of SefA-immunised hens after the challenge. Again, significantly lower number of caecum-infected birds was found in the group of SefA-immunised and S. Enteritidis-infected hens when compared with non-immunised and challenged hens on days 2, 4, and 6 after the infection. Interestingly, after immunisation with FimA protein, the recovery of S. Enteritidis was higher in the infected birds on days 2 and 4 than in the non-immunised and challenged ones. But from the day 14 after the infection there was no presence of S. Enteritidis in the caecum of immunised hens (Fig. 3b). No significant differences in the recovery of S. Enteritidis from the spleen as well as from the liver were found between immunised and non-immunised hens after challenge (data not shown). No differences in the number of Salmonella-positive samples were observed in the analysed organs after immunisation with AgfA protein (data not shown) (Figs 2a, 2b, 3a, 3b).

The data obtained for the individual organs were combined and expressed as the percentage of Salmonella-positive hens. Immunisation with SefA markedly reduced the number of colonised birds on day 2 (P<0.01) and reduced it further until day 42 after challenge with S. Enteritidis, in comparison to non-immunised ones (Fig. 4a). However, these differences from day 4 to day 42 p.i. were not statistically significant. In contrast, the percentage of Salmonella-positive cases in the group of FimA-immunised hens was even higher on days 2 and 4 p. i. when compared with non-immunised birds. However, S. Enteritidis was recovered from significantly (P<0.01) fewer number of vaccinated birds than from control hens at days 6 and 14 post-challenge (data not significant in comparison with non-immunised hens). There was no presence of S. Enteritidis in organs on day 42 p.i. (Fig. 4b). There were no significant differences in the number of Salmonella-positive birds, when hens were immunised with AgfA protein (Fig. 4c). In each group of hens, immunised with fimbral proteins and non-immunised, S. Enteritidis was isolated from very few birds on day 42 p. i. (Figs 4a, 4b, 4c).

Discussion
Both live attenuated and killed vaccines as well as certain subunit vaccines may protect poultry against Salmonella infections. The shedding of bacteria in bird faeces is effectively reduced, also the contamination of organs and eggs with Salmonella is lower. One of the highly immunogenic structures elaborated by Enterobacteriaceae are fimbriae, proteinaceous filaments present on the surface of bacterial cells (33), which were successfully used for the production of subunit vaccines against E. coli (1, 5, 6, 18, 27). However, in spite of this information there is surprisingly little data available on the immunogenic properties of Salmonella fimbriae, including S. Enteritidis, especially in poultry. Thiagarajan et al. (29) showed that hens infected with S. Enteritidis strain expressing SEF14 and SEF21 fimbriae had specific anti-SefA antibodies until the 9th week after infection. Recently, specific antibody response was observed in chickens immunised with liposome-associated purified SEF14 and SEF21 fimbriae (12). In our previous work we have analysed the humoral response of hens immunised with purified recombinant fimbral proteins: SefA, FimH and AgfA. It was found that SefA and FimH are highly immunogenic and therefore can be the components of subunit vaccine, as strong antibody response seems to be important in mediating resistance to Salmonella (7, 13, 16). However, protection against intracellular pathogens, including Salmonella infections, is mostly dependent on T cell-mediated immunity (9). It has been shown that CD4+ and CD8+ T lymphocytes cooperate to control infections with virulent Salmonella serovars (8, 14, 15). In spite of this knowledge, there is even less information available on the cellular response induced by S. Enteritidis fimbriae. It was only shown that SefA fimbrial protein induced in mice delayed-type hypersensitivity, stimulated proliferation of T cells, and production of cytokines (20).

In our present study, we have analysed the cellular response in hens immunised with recombinant S. Enteritidis fimbrial proteins after challenge with S. Enteritidis. Significant increase in the number of CD4+ T lymphocytes was found in the spleen of hens immunised with SefA and FimH proteins on day 2 post-challenge. Our data are in good agreement with those obtained by Sasai et al. (25). These authors showed that the percentage of CD4+ T cells in the spleen of chickens that were challenged twice with S. Enteritidis increased significantly on day 2 to drop on day 4 after the second challenge.
Fig. 1. a) The percentage of CD4+ T lymphocytes in the spleen of hens immunised with SefA recombinant fimbrial protein and challenged with *S. Enteritidis*.

![Graph](image1)

- **Black**: hens immunised twice with fimbrial protein SefA; percentage of CD4+ cells
- **White**: non-immunised hens; percentage of CD4+ cells
- **Dotted Line**: hens immunised twice with fimbrial protein SefA; index CD4+/CD8+
- **Dotted Line**: non-immunised hens; index CD4+/CD8+

*significant differences between vaccinated and non-immunized groups P<0.01.

Fig. 1. b) The percent of CD4+ T lypositive cells in the spleen of hens immunised with FimA recombinant fimbrial protein and challenged with *S. Enteritidis*.

![Graph](image2)

- **Black**: hens immunised twice with fimbrial protein FimA; percentage of CD4+ cells
- **White**: non-immunised hens; percentage of CD4+ cells
- **Dotted Line**: hens immunised twice with fimbrial protein FimA; index CD4+/CD8+
- **Dotted Line**: non-immunised hens; index CD4+/CD8+

Fig. 1. c) The percent of CD4+ lymphocytes cells in the spleen of hens immunised with AgfA recombinant fimbrial protein.

*significant differences between vaccinated and non-immunized groups P<0.01.
a) SefA

\[ \text{Fig. 2. Recovery of } \textit{Salmonella} \textit{Enteritidis} \text{ from the duodenum after challenge of vaccinated and control hens. Six birds per time point.} \]

b) FimA

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**Fig. 3.** Recovery of *Salmonella* Enteritidis from the caecum after challenge of vaccinated and control hens. Six birds per time point.

**a) SefA**

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**b) FimA**

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<th>Days post infection with S.E.</th>
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c) AgfA

Fig. 4. Recovery of *Salmonella* Enteritidis from hens after challenge of vaccinated and control hens. The bars represent combined date for the duodenum, caecum, liver and spleen. Six birds per time point.

*significant differences between vaccinated and control groups (P<0.01).

Similarly, the experiments performed on 18-week-old laying hens revealed that birds immunised three times with an attenuated vaccine or killed vaccine based on *S*. Enteritidis showed both highly increased number of CD4+ splenocytes and value of the CD4+/CD8+ index in 2 weeks after the final challenge (2). In accordance with these observations, as it was shown recently by McSorley *et al.* (17), CD4+ T lymphocytes seem to be of critical importance in the protection against *S*. Typhimurium infections. However, in contrast to Berndt and Methner (3) and Babu *et al.* (2) we did not observe the increase in the number of CD8+ T cells. These authors found highly increased number of CD8+ T lymphocytes in the thymus and peripheral blood of immunised chickens after challenge with *S*. Enteritidis. Babu *et al.* (2) who obtained similar results propose that such discrepancy could be due to differences in the age of experimental birds.

As fimbrial protein SefA and FimA turn out to highly immunogenic, we have studied their protective affect as vaccines against *S*. Enteritidis infections. Hens were inoculated twice i.m. with oil-emulsion of each protein and challenged with field-strain of *S*. Enteritidis. Studies on the colonisation of different organs of hens immunised with recombinant SefA fimbrial proteins by *S*. Enteritidis revealed no great differences when compared with non-immunised and challenged birds (only on day 2 p.i. significantly highly reduced the number of colonised organs). Interestingly, Rajashekara *et al.* (24), found that vaccination of chickens with recombinant SEF17 and SEF21 did not significantly reduce coecal colonisation or persistance of *S*. Enteritidis. However, in contrast to this result, the elimination of bacteria from organs of the hens immunised with FimA protein was quicker (on days 6 and 14 p.i.) and there was no *S*. Enteritidis in the organs on 42 d p.i..

In conclusion, we have shown that fimbrial proteins of *S*. Enteritidis, SefA and FimA, in addition to strong humoral response (11), can induce cellular response against *S*. Enteritidis infection. Highly increased number of CD+4 T cells present in the spleen just 2 d after challenge suggests the presence of CD+4 memory cells in immunised birds. Our data clearly show that SefA and FimA fimbrial proteins are highly immunogenic in hens, however, such response seems to affect weakly or to reduce significantly the organ colonization and persistance of *Salmonella* Enteritidis.

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**References**


