# Comparison of live and inactivated *Salmonella* Typhimurium vaccines containing different combinations of SPI-1 and SPI-2 antigens in poultry

K. Papezova, H. Havlickova, F. Sisak, V. Kummer, M. Faldyna, I. Rychlik

Veterinary Research Institute, Brno, Czech Republic

**ABSTRACT**: *Salmonella enterica* subsp. *enterica* originating from poultry and poultry products is responsible for the vast majority of human gastrointestinal disorders in Europe. For this reason different measures that seek to decrease its incidence in poultry including vaccination with inactivated vaccine continue to be tested. In this study we compared four different inactivated vaccines of *S.* Typhimurium in chickens which were enriched by SPI-1 or SPI-2 proteins that are central to *Salmonella* virulence. Six-week-old chickens were intramuscularly vaccinated, revaccinated at 9 weeks and challenged at 12 weeks of age. For two weeks post challenge faecal shedding was monitored. There was no significant difference in the performance of the four compared inactivated vaccines and all of them decreased faecal shedding during the first weeks post infection by 10–1 000× when compared with non-immunized control chickens. However, the level of protection provided by inactivated vaccines was much lower when compared with a live vaccine based on a *phoP rpoS* double deletion *S.* Typhimurium mutant which was included as an additional control.

**Keywords**: serological response; faecal shedding; attenuated vaccine

In developed countries, poultry and poultry products are considered as a major source of *Salmonella enterica* subsp. *enterica* (*S. enterica*) which is one of the leading causes of human gastrointestinal disorders. Several strategies such as increased hygiene, use of specific feed additives or competitive microflora can be applied to reduce the prevalence of *S. enterica* in poultry flocks and subsequently in humans. Another method which can be used to decrease *S. enterica* incidence in poultry flocks is vaccination (Anonymous, 2006).

*S. enterica* is a facultative intracellular parasite which activates the cell-mediated immune response. Such an immune response is usually stimulated by live attenuated vaccines due to the specific cytokine response of dendritic cells during antigen presentation (Norimatsu et al., 2004). However, the selection and construction of live vaccines requires the consideration of several issues. The vaccine must be sufficiently attenuated so as to be suitable for immunocompromised individuals while still remaining immunogenic. Also, the vaccine should not spread and survive in the environment. Furthermore, if any live vaccine is to be newly introduced, it will most likely need to contain genetically modified *S. enterica*, a scenario that is not generally acceptable to the public.

Although live vaccines usually have better protective effects when compared with inactivated vaccines (Silva et al., 1981; Robertsson et al., 1983), the humoral response, highly inducible by inactivated vaccines, is especially important for the control of faecal shedding (Desmidt et al., 1998). For this reason different vaccination schemes (Nassar et al., 1994), modes of application (Thatte et al., 1995) or adjuvans (Giannati-Stefanou et al., 1999; Barbou et

Supported by the Ministry of Agriculture of the Czech Republic (Grant No. MZE 0002716201) and the Ministry of Education, Youth and Sports of the Czech Republic (Grant No. LC06030).

al., 2000) have been tested with the aim of further improving their efficacy. Another property of inactivated vaccines which impacts on their efficacy is their composition. That is why vaccines containing outer membrane proteins, fimbriae, or proteins induced by iron limitation have been produced and tested (Barbou et al., 2000; Clifton-Hadley et al., 2002; Woodward et al., 2002; Khan et al., 2003).

*Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) expresses multiple proteins which allow it to successfully colonise the gut, invade epithelium and survive and multiply inside the eukaryotic cells of a host. Genes which facilitate successful infection are clustered at particular parts of chromosomes called *Salmonella* Pathogenicity Islands (SPI). Most of the genes required by *S.* Typhimurium to invade epithelial cells (e.g. *invJ, sipA, sipB, sipC, hilA* or *invF*) are localised to SPI-1 (Darwin and Miller, 1999; Eichelberg and Galan, 1999) while genes essential for the intracellular survival of *S.* Typhimurium (e.g. *ssrA, ssaB* or *sseA*) are clustered in SPI-2 (Cirillo et al., 1998; Hensel et al., 1998). The expression of SPI-1 genes is known to be suppressed by bile salts, acidification or nutrient limitation and *S. enterica* grown in such an environment exhibits a reduced ability to invade tissue culture cells (Prouty and Gunn, 2000; Boddicker and Jones, 2004). Positive signals inducing SPI-1 gene expression are decreased oxygen supply and transition from an exponential to a stationary phase of growth (Bajaj et al., 1996). SPI-2 genes are, on the other hand, expressed at a high level in the stationary phase of growth when the availability of nutrients become limiting. This is consistent with the intracellular conditions encountered by *S.* Typhimurium (Deiwick et al., 1999; Lee et al., 2000). Since SPI-1 and SPI-2 encoded proteins are expressed *in vivo*, these proteins may represent important antigens for the immune system of a host. Recently, we have described in the vitro conditions under which either SPI-1 or SPI-2 genes are expressed (Papezova et al., 2007). Using transcriptional promoter fusions we found that it is relatively easy to stimulate *S.* Typhimurium strain F98 to either simultaneously express SPI-1 or SPI-2 proteins, one set of proteins at a time, or none at all. *S.* Typhimurium was grown under these conditions and different inactivated vaccines enriched by SPI-1 or SPI-2 antigens were produced and tested in a preliminary way in poultry. As a control we also included a recently described attenuated *S.* Typhimurium live vaccine based on deletions in *phoP* and *rpoS* genes (Methner et al., 2004).

## MATERIAL AND METHODS

### Bacterial strains and growth conditions

*S.* Typhimurium strain F98, a clone spontaneously resistant to nalidixic acid, was used in this study (Barrow et al., 1990). When necessary this strain was transformed with the promoter fusion reporter plasmid pCS26. Strains transformed with the reporter plasmids were grown in the presence of kanamycin (10 μg/ml). However, when the strains were inoculated onto microplates for luminometry (see below), the broths were antibiotic-free. The previously described live attenuated strain *S.* Typhimurium F98 Δ*phoP* Δ*rpoS* (Methner et al., 2004) was included in the vaccination trial for the purposes of comparison.

The promoter fusion strains were grown in LB broth (Gibco) with or without supplementation with 0.25% bile salts or 25mM butyrate. After the addition of butyrate, the pH of the broth was re-adjusted to pH 7.4 by 2M NaOH. The promoter fusion strains were also grown in M9 medium with 0.01% casamino acids and 0.2% glucose as the carbon source. All experiments were performed at 37°C. The same media and growth conditions were used for antigen preparation.

### Construction of promoter fusions and luminometry

The construction of *sopB* and *sifA* promoter fusions has been described by us previously (Papezova et al., 2007). For luminometry, 18-hour-old cultures harbouring the reporter fusions were diluted 1 000× in 200 μl of LB broth (or M9 medium when the expression in M9 medium was monitored) in individual wells of the microplate. The microplate was then incubated in a microplate luminometer (BMG Fluorstar Galaxy) at 37°C for 48 h and during the incubation period, luminescence was recorded automatically every 15 min.

### Antigen preparation and immunisation

*S.* Typhimurium F98 with the reporter pCS-26sopB or pCS26sifA plasmid was grown until the maximal expression of *sopB* and *sifA* promoters was achieved (see also Figure 1). To obtain antigen enriched for both the SPI-1 and SPI-2 pro-

Table 1. List of primers used for promoter cloning in this study. The *luxCR* primer was used in combination with forward gene promoter primers to verify the promoter-luciferase gene junction

| Gene promoter | Primer forward/reverse* | Function |
|---|---|---|
| *sopB* | ATCGGTGAATTTGATCTGAG/ GTAGGCTTTTAAAAGCCTCC | SPI-5, secreted by SPI-1 encoded type III secretion system |
| *sifA* | CCTGCTGATTGGTAACGTCA/ ACCATGCTTCTTTCGTATTC | SPI-2 associated |
| *luxCR* | TTCATTTTTCCATCTTTGCC | control primer |

*5´end extensions containing restrictions sites enabling cloning are not shown

teins, *S.* Typhimurium pCS26sopB was collected after 16-hour growth in LB broth. For the SPI-1 antigen only, *S.* Typhimurium pCS26sopB was grown for 19 h in LB supplemented with 25mM butyrate. For the antigen free of any SPI proteins, *S.* Typhimurium pCS26sopB fusion strain was grown for 24 h in LB supplemented with 0.25% bile salts, and to obtain the SPI-2-enriched antigen, *S.* Typhimurium transformed with pCS26sifA was grown for 44 h in M9 minimal medium. At appropriate time, cultures from multiple wells of the microplate were collected, washed with phosphate-buffered saline (PBS) and resuspended in 0.4% formaldehyde in PBS for inactivation. After an 18 hour inactivation, the antigens were spun and resuspended in PBS to OD 1.5. Before the immunization, inactivated *Salmonella* antigens were mixed with an equal volume of complete Freund adjuvant (BACTO – Adjuvant complete H37 Ra, Difco, USA) and thoroughly homogenized. In parallel, the expression of SPI proteins was also verified by their precipitation from bacterial culture supernatants with trichloracetic acid, separation by polyacrylamide gel electrophoresis and staining with Comassie Blue.

## Biological experiment

Six week old specific pathogen free ISA Brown chickens were divided into six groups of four birds. Chickens in Groups 1–4 were immunised intramuscularly with 0.3 ml of inactivated *S.* Typhimurium antigens enriched with either SPI-1, both SPI-1 and SPI-2, no SPI, or with SPI-2 proteins, respectively. Birds in Group 5 were immunised orally into the crop with $5 \times 10^8$ CFU in 0.1 ml of the live attenuated *S.* Typhimurium F98 Δ*phoP* Δ*rpoS* strain and chickens in Group 6, the negative controls, were not immunised at all. Three weeks later, the birds were revaccinated with either the inactivated vaccines or the live vaccine in the same dose and by the same route as in the first vaccination. After an additional three weeks, all the chickens in all six groups were orally challenged with $5 \times 10^8$ CFU of *S.* Typhimurium F98 Nal. For 11 days thereafter *S.* Typhimurium faecal shedding was quantified by plating serial dilutions of fecal homogenates in PBS on Rambach agar (Merck, Germany) supplemented with 20 μg of nalidixic acid per ml. At twelve days post infection the chickens were euthanised and *S.* Typhimurium counts per gram of caecum, liver and spleen were determined by plating the serial dilutions of tissue homogenates in PBS on Rambach agar supplemented with 20 μg of nalidixic acid. In the case of no growth, the homogenates were processed by the pre-enrichment procedure according to the ISO 6579 protocol for *Salmonella* isolation.

Prior to the first vaccination, the revaccination, before the challenge and after the challenge (during *post mortem* analysis after the termination of the experiment), blood samples were taken from a wing vein to obtain serum for serological analysis. The presence of anti-*Salmonella* antibodies was determined using a commercial *Salmonella* Typhimurium Antibody ELISA Kit according to the instructions of the manufacturer (Guildhay, UK).

For the comparison of antibody titers the nonparametric Mann-Whitney test was used. Comparisons of the faecal shedding between each of the vaccinated groups and the non-vaccinated control was performed by paired *t*-tests. All the experiments involving animals were approved by an ethical committee and were performed according to current Czech legislation.

## RESULTS

### Preparation of antigens

The expression profiles of *sopB* and *sifA*, the two representative promoter fusion strains, are shown in Figure 1. In LB broth both *sopB* and *sifA* genes were expressed, in LB supplemented with butyrate, only the *sopB* gene was expressed, in LB supplemented with bile salts neither *sopB* nor *sifA* genes were expressed and in M9 minimal medium only the *sifA* gene was expressed. Similar expression profiles have been observed for other SPI-1 and SPI-2 promoter fusions (Papezova et al., 2007). This indicated that SPI-1 and SPI-2 antigens were specifically expressed or suppressed under given experimental conditions in *S.* Typhimurium strain F98. In addition, we assessed the secretion of SPI-1 proteins into culture medium and as expected we observed that SipA, SipB, SipC and InvJ proteins were secreted in essentially the same amounts in LB and LB supplemented with butyrate, but that their secretion was diminished in the presence of bile salts and was virtually absent in culture supernatants obtained from minimal medium (Figure 2). However, since the expression of other proteins could also have been influenced by the growth conditions used in this study, and because some of them might influence the immune response too, we refer to the different antigens as SPI-1 or SPI-2 enriched.

### Antibody response in ELISA test

At the beginning of the experiment, anti *S.* Typhimurium antibodies were not produced by any of the birds (not shown). Three weeks after the first vaccination, birds immunised with the inactivated vaccines produced high amount of antibodies. After the second vaccination, the amount of antibodies further increased both in the group of inactivated and live vaccine immunised birds although antibody levels in live vaccine immunised chicken never exceeded those obtained for inactivated vaccine immunised birds. Although there were differences in antibody response to different formula of

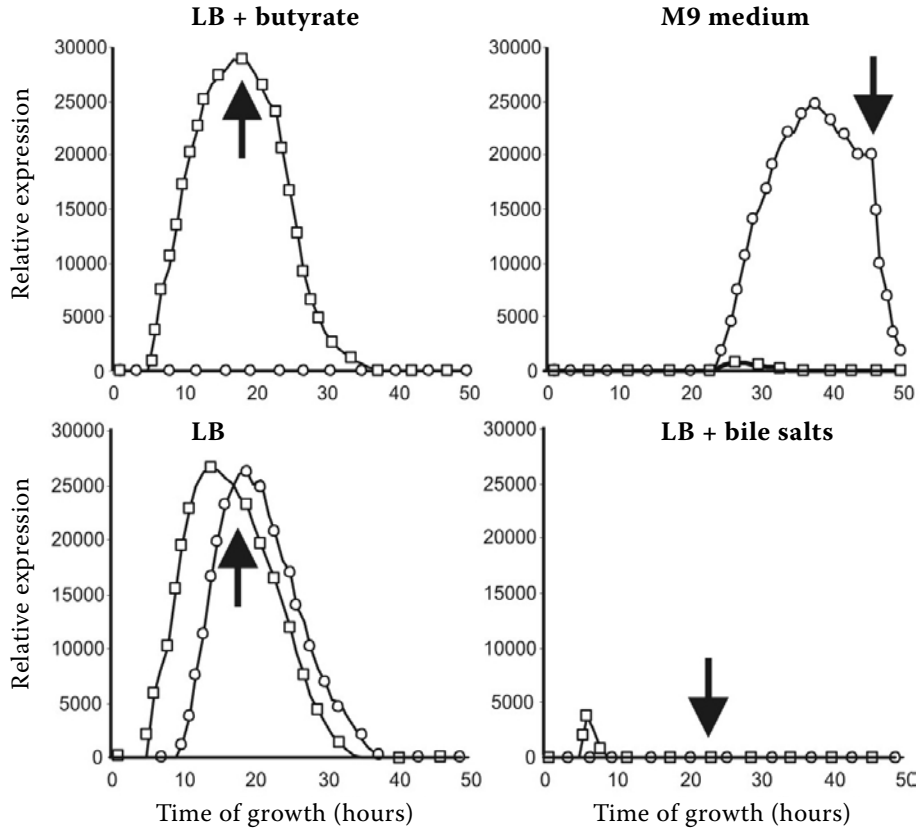


Figure 1. Expression of *sopB* (squares) and *sifA* (circles) in *S.* Typhimurium grown in media as indicated. Arrows indicate the time points of culture collection for antigen production. X-axis = time of population growth in hours, Y-axis = relative luminescence

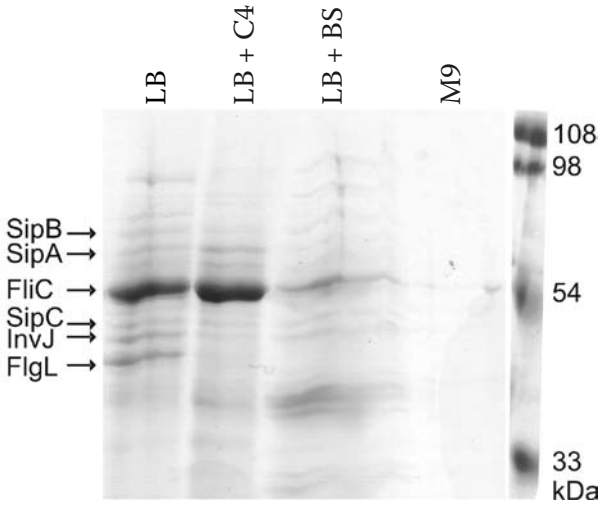


Figure 2. *S.* Typhimurium proteins secreted into the culture supernatants in LB, LB supplemented with butyrate (LB + C4), LB supplemented with bile salts (LB+BS) and M9 minimal medium

inactivated vaccines, these were not statistically significant. After the challenge, levels of antibodies in immunised birds essentially did not further increase. An increase in antibody response after the challenge was observed only among the non-immunised control birds which had remained free of antibodies until this time point (Figure 3).

## Fecal shedding

The highest rates of faecal shedding were observed in the group of non-vaccinated chickens. In birds immunised with inactivated vaccines, an approximately 1–3 log decrease in faecal shedding was observed during the first week after the challenge and the protection induced by different inactivated vaccines was quite similar (Figure 4). However, when the feacal shedding between Days 4 and 8 after infection in the non-vaccinated group of chickens was compared by the paired *t*-test with the groups of chickens immunised with the inactivated vaccines, a statistically significant difference was observed only between the groups immunised with the vaccine enriched in either both SPI-1 and SPI-2 antigens or containing no SPI antigens ($P < 0.05$). Chickens immunised with the vaccine enriched in

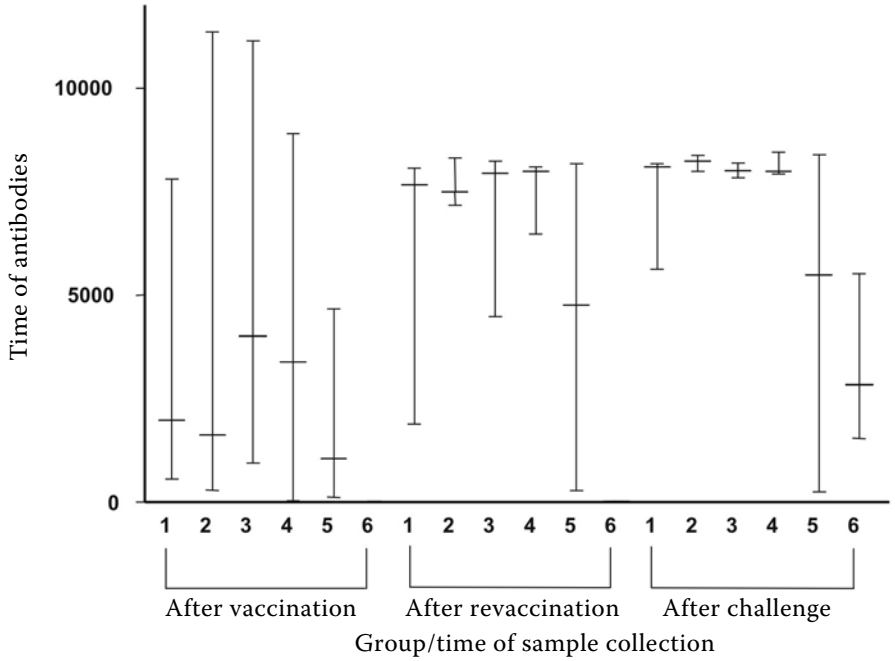


Figure 3. Antibody responses in experimental groups of chickens. Group 1 = chickens immunised with SPI-1 enriched antigen, Group 2 = chickens immunised with both SPI-1 and SPI-2 enriched antigen, Group 3 = chickens immunised with the antigen containing no SPI proteins, Group 4 = chickens immunised with SPI-2 enriched antigen, Group 5 = chickens immunised with live vaccine, Group 6 = non-vaccinated control. Data are presented as median, minimal and maximal values in respective groups. After the first vaccination and the revaccination, antibody titres in Groups 1–5 significantly differed from the non-vaccinated birds. After the challenge, only in birds in Groups 1–4 did antibody titres differ from the negative control group as determined by the parametric Mann-Whitney test ($P < 0.05$)

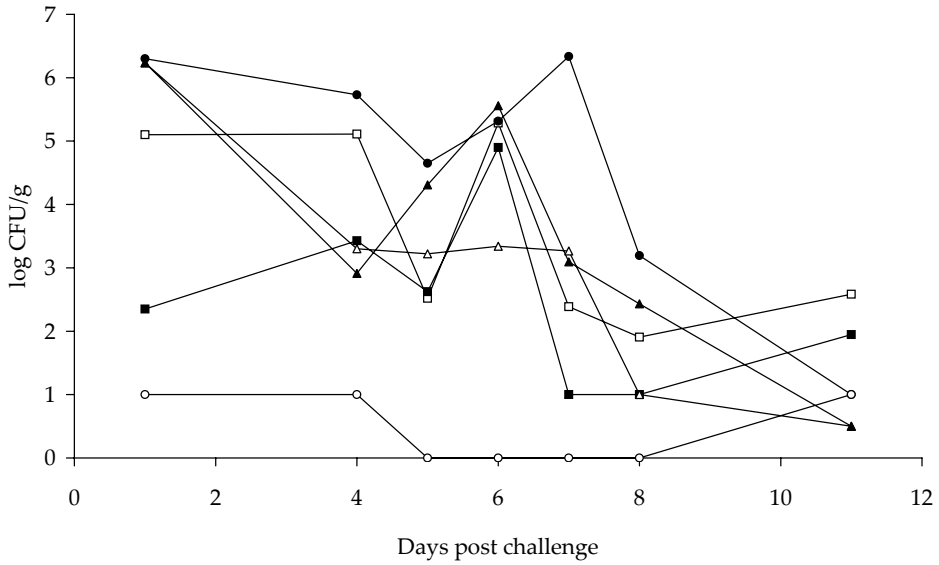


Figure 4. Faecal shedding of *S.* Typhimurium in experimental groups of chickens vaccinated with different inactivated and live vaccines and challenged with wild type *S.* Typhimurium

□ – birds vaccinated with SPI-1 enriched antigen
■ – birds vaccinated with antigen containing both SPI-1 and SPI-2 proteins
△ – birds vaccinated with antigen containing neither SPI-1 nor SPI-2 proteins
▲ – closed triangles – birds vaccinated with SPI-2 enriched antigen
○ – birds vaccinated with live attenuated *S.* Typhimurium *ΔphoP Δrpo*S
● – non-vaccinated birds

either only SPI-1 or only SPI-2 antigens did not exhibit significant protection in comparison to the non-vaccinated control ($P = 0.078$ and $P = 0.117$, respectively). Similarly, no difference was found when the chicken groups immunised with different inactivated vaccines were compared among themselves. In the chickens vaccinated with the live vaccine, the challenge strain was shed in very low quantities and could be detected only after the pre-enrichment procedure (Figure 4). The protection induced by the live vaccine was significantly higher when compared with the non-vaccinated chickens ($P < 0.01$) or with any chicken group immunised with any of the inactivated vaccines ($P < 0.05$).

### *Post mortem* analysis

Chicken with normal gut microflora are relatively resistant to *Salmonella* infection. In agreement

Table 2. *Post mortem* analysis and *S.* Typhimurium presence in caecum, liver and spleen of immunised and challenged birds. Data show number of negative chicken/number of chickens positive for *S.* Typhimurium after pre-enrichment/number of chicken positive for *S.* Typhimurium after direct plating (high level of colonization)

| Group | Chickens immunised with | Caecum | Liver | Spleen |
|---|---|---|---|---|
| 1 | SPI-1 | 3/1/0 | 2/2/0 | 3/0/1 |
| 2 | SPI-1 and SPI-2 | 2/1/1 | 3/1/0 | 0/4/0 |
| 3 | none SPI | 3/1/0 | 3/1/0 | 3/1/0 |
| 4 | SPI-2 | 3/1/0 | 2/1/1 | 1/2/1 |
| 5 | live vaccine | 1/3/0 | 4/0/0 | 2/2/0 |
| 6 | non vaccinated control | 1/2/1 | 2/2/0 | 0/3/1 |

with this fact, only low numbers of *S.* Typhimurium were detected in both immunised and non-immunised chickens after *post mortem* analysis (Table 2). The highest numbers of positive samples were observed among non-vaccinated chickens. Among the immunised birds the most effective was the live vaccine and the antigen prepared from *S.* Typhimurium grown in LB supplemented with bile salts, i.e., without any SPI proteins. The lowest efficacy was observed in the vaccine obtained from *S.* Typhimurium grown in minimal medium, i.e., containing SPI-2 antigen only.

## DISCUSSION

In this study we constructed four inactivated vaccines containing different antigens closely associated with different stages of *S. enterica* infection and tested them for the protection of poultry against *S.* Typhimurium infection. A similar approach has been employed by Khan et al. (2003) who used outer membrane proteins for immunisation or Clifton-Hadley et al. (2002) who tested an inactivated vaccine from *S. enterica* grown under iron restriction.

All four inactivated vaccines induced strong humoral immune response, stronger than that observed in the chickens immunised with the live vaccine, and were similar to the results of others who have compared the antibody response induced by inactivated and live vaccines (Silva et al., 1981; Nassar et al., 1994; Babu et al., 2004). Also in keeping with earlier studies, we observed a considerable variation in antibody response to the first vaccination in individual birds which decreased after the revaccination (Clifton-Hadley et al., 2002). It is obvious that a stronger humoral response to the inactivated vaccines is not only associated with the viability status of the vaccine but also with the route of vaccine application and the presence of adjuvans in the inactivated vaccines.

Since *S. enterica* is an intracellular parasite, serum antibodies are usually of lower importance for the protection of vaccinated animals. To test the efficacy of inactivated vaccines against salmonellosis, a challenge experiment has to be done. In our study the live vaccine, based on *phoP* and *rpoS* gene deletions, was more efficient in decreasing faecal shedding than any of the inactivated vaccines, a phenomenon which has been described previously (Robertsson et al., 1983; Barrow et al., 1990). Despite this, a significant decrease in faecal shedding after the challenge was observed also in birds immunised with the inactivated vaccine enriched either in both SPI-1 and SPI-2 antigens or containing none of these antigens, when compared with the non-immunised chickens. These results indicate that a more efficient enrichment for SPI-1 and SPI-2 proteins might be needed but also that the role of other antigens, lying outside the Salmonella pathogenicity islands, must not be overlooked. The results of the *post mortem* detection of *S.* Typhimurium in caecum, liver and spleen were also consistent with the faecal shedding. The non-vaccinated birds were frequently positive for *S.* Typhimurium, while the birds vaccinated by either of the vaccines were protected. However, the counts of *S.* Typhimurium were quite low in all of the chicken groups, most probably due to the already established natural resistance of older chickens which were 12-week-old at the time of challenge.

In this study we have shown that similar to others, inactivated vaccines are efficient in moderately protecting chickens against colonisation by *S. enterica* (Gast et al., 1993; Nakamura et al., 1994; Khan et al., 2003) but that their effect is weaker when compared with the effect of live vaccines. However, in contrast to some previous reports (Timms et al., 1990; Nakamura et al., 1994; Giannati-Stefanou et al., 1999; Clifton-Hadley et al., 2002) we did not observe a significant difference in inactivated vaccine efficacy depending on its composition. In this study we have therefore demonstrated that the vaccination of chickens with live vaccine reduces faecal shedding of *S.* Typhimurium significantly more than vaccination with inactivated vaccines. Although no significant differences in the performance of different inactivated vaccines were identified, due to the limited number of chickens used in this study, this comparison must be considered as a preliminary one.

## REFERENCES

Anonymous (2006): Preliminary report on the analysis of the baseline study on the prevalence of *Salmonella* in laying hens. EFSA Journal, 81**,** 1–71.

Babu U., Dalloul R.A., Okamura M., Lillehoj H.S., Xie H., Raybourne R.B., Gaines D., Heckert R.A. (2004): *Salmonella enteritidis* clearance and immune responses in chickens following *Salmonella* vaccination

and challenge. Veterinary Immunology and Immunopathology, 101, 251–257.

Bajaj V., Lucas R.L., Hwang C., Lee C.A. (1996): Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. Molecular Microbiology, 22, 703–714.

Barbou E.K., Bejjani N.E., Daghir N.J., Faroon O.M., Bouljihad M., Spasojevic R. (2000): Induction of early immunopotentiation to fimbriae of *Salmonella* Enteritidis (SE) by administering thymulin and zinc to SE-vaccinated chicken breeders: relationship to protection. The Journal of Veterinary Medical Science, 62, 1139–1143.

Barrow P.A., Hassan J.O., Berchieri A., Jr. (1990): Reduction in faecal excretion of *Salmonella typhimurium* strain F98 in chickens vaccinated with live and killed *S. typhimurium* organisms. Epidemiology and Infection, 104, 413–426.

Boddicker J.D., Jones B.D. (2004): Lon protease activity causes down-regulation of Salmonella pathogenicity island 1 invasion gene expression after infection of epithelial cells. Infection and Immunity, 72, 2002–2013.

Cirillo D.M., Valdivia R.H., Monack D.M., Falkow S. (1998): Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. Molecular Microbiology, 30, 175–188.

Clifton-Hadley F.A., Breslin M., Venables L.M., Sprigings K.A., Cooles S.W., Houghton S., Woodward M.J. (2002): A laboratory study of an inactivated bivalent iron restricted *Salmonella enterica* serovars Enteritidis and Typhimurium dual vaccine against Typhimurium challenge in chickens. Veterinary Microbiology, 89, 167–179.

Darwin K.H., Miller V.L. (1999): InvF is required for expression of genes encoding proteins secreted by the SPI1 type III secretion apparatus in *Salmonella typhimurium*. Journal of Bacteriology, 181, 4949–4954.

Deiwick J., Nikolaus T., Erdogan S., Hensel M. (1999): Environmental regulation of Salmonella pathogenicity island 2 gene expression. Molecular Microbiology, 31, 1759–1773.

Desmidt M., Ducatelle R., Mast J., Goddeeris B.M., Kaspers B., Haesebrouck F. (1998): Role of the humoral immune system in *Salmonella enteritidis* phage type four infection in chickens. Veterinary Immunology and Immunopathology, 63, 355–367.

Eichelberg K., Galan J.E. (1999): Differential regulation of *Salmonella typhimurium* type III secreted proteins by pathogenicity island 1 (SPI-1)-encoded transcriptional activators InvF and *hilA*. Infection and Immunity, 67, 4099–4105.

Gast R.K., Stone H.D., Holt P.S. (1993): Evaluation of the efficacy of oil-emulsion bacterins for reducing fecal shedding of *Salmonella enteritidis* by laying hens. Avian Diseases, 37, 1085–1091.

Giannati-Stefanou A., Koptopoulos G., Hatzopoulou E., Sarris K. (1999): A *Salmonella abortusovis* inactivated vaccine protects mice from abortion after challenge. Veterinary Microbiology, 66, 285–290.

Hensel M., Shea J.E., Waterman S.R., Mundy R., Nikolaus T., Banks G., Vazquez-Torres A., Gleeson C., Fang F. C., Holden D.W. (1998): Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. Molecular Microbiology, 30, 163–174.

Khan M.I., Fadl A.A., Venkitanarayanan K.S. (2003): Reducing colonization of *Salmonella* Enteritidis in chicken by targeting outer membrane proteins. Journal of Applied Microbiology, 95, 142–145.

Lee A.K., Detweiler C.S., Falkow S. (2000): OmpR regulates the two-component system SsrA-ssrB in *Salmonella* pathogenicity island 2. Journal of Bacteriology, 182, 771–781.

Methner U., Barrow P.A., Gregorova D., Rychlik I. (2004): Intestinal colonisation-inhibition and virulence of *Salmonella phoP*, *rpoS* and *ompC* deletion mutants in chickens. Veterinary Microbiology, 98, 37–43.

Nakamura M., Nagamine N., Takahashi T., Suzuki S., Sato S. (1994): Evaluation of the efficacy of a bacterin against *Salmonella enteritidis* infection and the effect of stress after vaccination. Avian Diseases, 38, 717–724.

Nassar T.J., al Nakhli H.M., al Ogaily Z.H. (1994): Use of live and inactivated *Salmonella enteritidis* phage type 4 vaccines to immunise laying hens against experimental infection. Revue Scientifique et Technique, 13, 855–867.

Norimatsu M., Chance V., Dougan G., Howard C.J., Villarreal-Ramos B. (2004): Live *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) elicit dendritic cell responses that differ from those induced by killed *S.* Typhimurium. Veterinary Immunology and Immunopathology, 98, 193–201.

Papezova K., Gregorova D., Jonuschies J., Rychlik I. (2007): Ordered expression of virulence genes in *Salmonella enterica* serovar Typhimurium. Folia Microbiologica, 52, 107–114.

Prouty A.M., Gunn J.S. (2000): *Salmonella enterica* serovar Typhimurium invasion is repressed in the presence of bile. Infection and Immunity, 68, 6763–6769.

Robertsson J.A., Lindberg A.A., Hoiseth S., Stocker B.A. (1983): *Salmonella typhimurium* infection in calves: protection and survival of virulent challenge bacteria after immunization with live or inactivated vaccines. Infection and Immunity, 41**,** 742–750.

Silva E.N., Snoeyenbos G.H., Weinack O.M., Smyser C.F. (1981): Studies on the use of 9R strain of *Salmonella gallinarum* as a vaccine in chickens. Avian Diseases, 25, 38–52.

Thatte J., Rath S., Bal V. (1995): Analysis of immunization route-related variation in the immune response to heat-killed *Salmonella typhimurium* in mice. Infection and Immunity, 63**,** 99–103.

Timms L.M., Marshall R.N., Breslin M.F. (1990): Laboratory assessment of protection given by an experimental *Salmonella enteritidis* PT4 inactivated, adjuvant vaccine. The Veterinary Record, 127**,** 611–614.

Woodward M.J., Gettinby G., Breslin M.F., Corkish J.D., Houghton S. (2002): The efficacy of Salenvac, a *Salmonella enterica* subsp. *enterica* serotype Enteritidis iron-restricted bacterin vaccine, in laying chickens. Avian Pathology, 31**,** 383–392.

Received: 2008–01–17
Accepted after corrections: 2008–05–21

Corresponding Author:

Ivan Rychlik, Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic
Tel. +420 533 331 201, fax +420 541 211 229, e-mail: rychlik@vri.cz