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VACCINE POTENTIAL OF A NONFLAGELLATED, VIRULENCE-PLASMID-CURED (*fliD*–, pSEVΔ) MUTANT OF *SALMONELLA* ENTERITIDIS FOR CHICKENS

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The aim of these studies was to assess residual virulence and early protective capacity of a negatively markered live attenuated vaccine candidate *Salmonella* Enteritidis mutant against a highly virulent *S.* Enteritidis strain using a day-old chicken model. Nonflagellated FliD negative mutants of *Salmonella* Enteritidis 11 (SE11) with and without the virulence plasmid proved to be sufficiently attenuated (limited invasiveness *in vitro*/*in vivo*) without reduced ability to colonise chicken gut. The early protective activity of a nonflagellated, virulence-plasmid-cured (*fliD–,* pSEVΔ) mutant against organ invasion, caecal colonisation and faecal shedding by the highly virulent challenge strain *S.* Enteritidis 147 Nal^R proved to be effective and safe. The innate and adaptive immunity was demonstrable during the first four weeks of life, and the serological response was clearly distinguishable from the response induced by the wild parental strain. In conclusion, we provided data for the first time about a virulence-plasmid-cured, nonflagellated mutant of *S.* Enteritidis to serve as a basis for development of a negatively markered potential live oral vaccine against virulent *S.* Enteritidis in chicken.

Key words: *Salmonella* Enteritidis, *fliD* flagellin, vaccine, virulence plasmid, chicken

Poultry products contaminated with *Salmonella* are a major cause of foodborne zoonoses, and certain non-host-restricted serovars like *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*S.* Enteritidis) have become a worldwide public health concern, arising primarily from poultry and eggs (Rodrigue et al., 1990; EFSA, 2010). This worldwide veterinary public health problem has led to an increasing demand for effective vaccines to control *S.* Enteritidis infection in the poultry industry. Vaccination has been recognised as an important preventive

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measure against salmonellosis of poultry, for which killed parenteral and orally applicable live attenuated vaccines have been widely used, as reviewed by Barrow (2007) and Van Immerseel et al. (2005). Killed vaccines confer strong protection primarily against host-restricted *Salmonella* serovars such as *S*. Gallinarum in poultry (Barrow and Wallis, 2000) but also against the *in ovo* transmission of *S*. Enteritidis (EFSA, 2007). This is probably due to the fact that killed vaccines induce good humoral immune responses which may be sufficient against septicaemia and related clinical disease. However, antigens present in killed vaccines could be more rapidly eliminated from the host as compared to live bacteria in orally applied vaccines without effectively stimulating cytotoxic T cells, especially in unprimed hosts (Nagaraja and Rajashekara, 1999). These facts may explain why the use of killed *Salmonella* vaccines against non host-restricted *Salmonella* serovars like *S*. Enteritidis had varying success in poultry (Barrow, 2007). Live attenuated *Salmonella* vaccines have several advantages over killed vaccines. They effectively stimulate both cell-mediated and humoral immune responses and express appropriate protective antigens *in vivo* (Van Immerseel et al., 2005). Live vaccines have been shown to be more effective in inducing lymphocyte proliferation in response to *S*. Enteritidis antigens (Babu et al., 2003). Furthermore, in the case of oral application on the first day of life the newly designed live *Salmonella* vaccine candidate strains may also protect birds by inhibiting colonisation by wild *Salmonella* strains at a very young age (Barrow et al., 1987; Nógrády et al., 2003; Methner et al., 2011*a*).

Although experimental data related to the recently developed and marketed vaccines against *Salmonella* in poultry are generally favourable (Barrow, 2007; EFSA, 2004, 2007), there are differing regulations for the use of 'live' versus 'killed' vaccines in different countries, indicating that there are still several aspects to be considered for the further development of such vaccines. One of them is the need for serological markers, so that naturally infected and vaccinated flocks could be distinguished by simple serological assays. For that purpose, three recent studies have reported the use of nonflagellated *fliC* (H1 flagellin) deletion mutants of *S*. Enteritidis (Adriaensen et al., 2007; Methner et al., 2011*b*; Matulova et al., 2013), produced by the method reported by Datsenko and Wanner (2000). None of these *fliC*–, nonmotile vaccine candidates were reported to be devoid of the *S*. Enteritidis virulence plasmid.

Earlier we produced nonflagellated (*fliD*–) and plasmidless mutants of strain *S*. Enteritidis 11 (Imre et al., 2006, 2011). Here we aimed to test the nonflagellated *(fliD–)* mutants of *S.* Enteritidis 11 with and without the virulence-plasmid (pSEV) for *in vitro* and *in vivo* verification of the attenuation. We also aimed to test the ability of a double-attenuated (*fliD*–, pSEVΔ) strain to protect very young chicks against intestinal colonisation and organ invasion by a highly virulent *S*. Enteritidis challenge strain and to prove that the lack of flagellin of the *fliD*– mutant could be utilised as a negative serological marker.

Materials and methods

Bacterial strains

Salmonella Enteritidis 11 (SE11) PT1 is a wild-type strain isolated from poultry and designated as E296 in an earlier study on flagellar systems (Imre et al., 2005). Its spontaneous nalidixic-acid-resistant derivative (SE11 Nal^R) was used for invasion and colonisation tests. Its spontaneous spectinomycin-resistant derivative (SE11 Spe^R) was used for the early protection experiments reported here. The two mutants of SE11 used here were as follow: *S.* Enteritidis 2102 (SE2102) a nonmotile, FliD-negative (*fliD*:pFOL1069), chloramphenicol-resistant mutant containing a 55 kb virulence plasmid, and its nonmotile virulence plasmid cured (*fliD–,* pSEVΔ) derivative of SE2102 (SEΔ155) (Imre et al., 2006, 2007, 2011). *Salmonella* Enteritidis 147 Nal^R (SE147) PT4, a wild-type virulent reference strain with a high capability to colonise the intestine and internal organs of day-old chicks, was used for challenge in protection experiments as described (Methner et al., 1995). *Escherichia coli* C600 (Sambrook et al., 1989) served as negative control in testing *in vitro* invasion. All strains used here were proven to be sensitive to kanamycin (Table 1).

Table 1

Organ invasion and caecal colonisation by *S.* Enteritidis 11 and its nonmotile mutant SE2102 and by the nonmotile-plasmidless mutant SEΔ155 in day-old White Leghorn chicks, five days after oral inoculation in two independent experiments (A and B)

Strains used for inoculation and experimental groups	Exp.	Infective dose (log_{10} CFU/chick)	Caecal colonisation (log_{10} CFU/g)	Liver invasion (+/tested)	Spleen invasion (+/tested)
S. Enteritidis 11 wt	A	7.44	6.3	6/6	6/6
	B	8.39	8.5	6/6	6/6
S. Enteritidis 2102*	A	7.34	5.7	2/6	1/6
	B	8.17	7.8	3/6	5/6
S. Enteritidis Δ155**	A	7.53	7.0	3/6	2/6
	B	8.43	8.2	6/6	3/6
Intact control	A	Not infected	Not applicable	0/4	0/4
	B			0/4	0/4

*nonmotile mutant of *S.* Enteritidis 11 Nal^R; **nonmotile-plasmidless mutants of *S.* Enteritidis 11: SE2012 (*fliD–*: Cm^R) and SEΔ155 (*fliD–,* pSEVΔ: Cm^R). Statistically significant ($P < 0.05 – P < 0.001$) differences were detected between the wild-type parent strain SE11 and either of the two mutants SE2102 and SEΔ155 respectively, regarding organ invasion. No statistically significant difference was detectable between mutants SE2102 (*fliD–)* and SEΔ155 (*fliD–*, pSEVΔ) regarding organ invasion, and no statistically significant difference was detectable between any of the strains regarding caecal colonisation

Microbiological techniques

For culturing bacteria the following media (Merck) were used: trypticase soy broth (TSB) for general purposes, bromothymol blue–lactose (BTB) agar for invasion, colonisation and protection assays, and Rappaport-Vassiliadis (RV) broth for the selective enrichment of *Salmonella*. Antibiotics (Sigma-Aldrich) were used in the following final concentrations: chloramphenicol (Cm): 20 μg/ml, nalidixic acid (Nal): 50 μg/ml, spectinomycin (Spe): 50 μg/ml, and kanamycin (Km): 250 μg/ml.

In vitro *fibroblast invasion*

Primary chicken embryo fibroblasts (CEFs) were obtained from 12-day-old specific pathogen free (SPF) embryos of the Leghorn breed. CEFs were cultured in MEM (Sigma-Aldrich) complemented with 5% fetal calf serum (FCS). One day prior to infection, fibroblasts were seeded into 36-mm Petri dishes (Nunc) and grown overnight at 37 °C under 5% CO_2.

The invasiveness of the *Salmonella* strains was tested as described by Barrow and Lovell (1989). Briefly, semi-confluent cell cultures were washed three times with PBS and cultivated in DMEM (Sigma-Aldrich) supplemented with 5% FCS and 1% D-mannose. Overnight bacterial cultures were incubated with fibroblasts at 1:200 dilutions for 2 h at 37 °C under 5% CO_2. The infectious dose was 4–6 × 10^6 CFU/ml. The number of bacteria in the supernatant was determined by plating serial dilutions on Bromothymol Blue (BTB) agar plates. CEFs were washed three times with PBS and incubated for 1.5 h at 37 °C and 5% CO_2 in MEM (Sigma-Aldrich) containing kanamycin 250 μg/ml to eliminate extracellular bacteria. Finally, cells were washed three times with PBS and digested with 0.025% trypsin (Sigma-Aldrich) and 1% Tween 20 (Sigma-Aldrich) in 0.01M NaH_2PO_4 (pH 8) for 30 min at 37 °C. Intracellular *Salmonella* counts were determined by plating serial dilutions on BTB plates. *Salmonella* invasion was tested three times, with 2–3 replicates each time.

In vivo *organ invasion and intestinal colonisation studies*

Specific pathogen free (SPF), day-old White-Leghorn chickens of mixed sex (Dabas Hatchery, Hungary) were housed in a room in well-distanced plastic boxes (six chickens/box forming one group). In the three experimental groups, birds were inoculated by the oral route using a sterile plastic gavage on the day of hatch with 0.5 ml of the 20 times diluted stationary-phase TSB culture of *S.* Enteritidis 11 Nal^R or with one of its two mutants: SE2012 (*fliD*–: Cm^R) or SEΔ155 (*fliD*–, pSVEΔ: Cm^R). Five days after inoculation, chickens from each group were killed humanely and 0.2 g of the caecal contents as well as of the liver and spleen were removed aseptically from each bird. Caecal content was resuspended in a ratio of 1:10 in RV broth containing nalidixic acid in the case of

the SE11-inoculated group, or chloramphenicol in the case of chicks inoculated with the mutants. From these homogenates, decimal dilutions were made and 10 µl from each dilution were plated onto BTB agar plates supplemented with one or the other of the above antibiotics in order to determine the *Salmonella* CFU/g in the caecal contents. Liver and spleen samples were incubated for 48 h at 41 °C in 1.8 ml RV broth containing either nalidixic acid (for isolation of the parent SE11) or chloramphenicol (for isolation of the mutants), from which BTB plates containing one or the other of the appropriate antibiotics were inoculated by loops to determine the presence of *Salmonella* in the parenchymal organs. The experiment was performed twice using a lower dose (approx. 5×10^7 CFU/chick) (Experiment A) and a higher dose (approx. 5×10^8 CFU/chick) (Experiment B) in order to study the dose response. The noninfected control groups were kept in a separate room in complete isolation. The *Salmonella*-free status of these control birds was monitored through parallel testing of the caecal content of all 6 chicks on the 5th day after arrival (ISO 6579). The license for the above pathogenicity testing and for the assessment of early protection was granted by the Animal Health and Food Control Station of Capital Budapest (No. 273/003/2004).

Assessment of early protection of day-old chicks against S. Enteritidis challenge

One-day-old *Salmonella*-free male Ross 308 broiler breeder grandparent chickens (Bábolna Hatchery, Hungary) were randomly divided into 6 groups of 40 chickens/group in two independent experiments. Groups of chickens were placed into separate isolation rooms. The chickens were fed an antibiotic-free 'finisher' feed previously tested for the absence of *Salmonella* (ISO 6579) and of the antimicrobial compounds (Ács and Simonffy, 1984). Feed and drinking water were made available *ad libitum*. The animal work was organised so as to prevent cross contamination.

Inoculations were done on the day of hatch (day 1) as follows. One-day-old birds in the groups 'Principal 1' and 'Principal 2' were vaccinated by an oral gavage with *S.* Enteritidis 11 (Spe^R) or with its nonmotile-plasmidless (*fliD*–, pSEVΔ: Cm^R) mutant (SEΔ155) respectively in a dose of approx. 1×10^8 CFU/chick. Both groups were challenged 24 h later with virulent *S.* Enteritidis 147 (Nal^R) by oral gavage with a 1000 times diluted overnight TSB culture (approx. 1×10^5 CFU/chick). The challenge control group was only inoculated with challenge strain SE147 (Nal^R) (approx. 1×10^5 CFU/chick) at one day of age. As a safety control, a 'vaccine control group 1' and a 'vaccine control group 2' were inoculated with SE11 (Spe^R) or SEΔ155 (*fliD*–, pSEVΔ: Cm^R) respectively at one day of age in a dose of approx. 1×10^8 CFU/chicken, enabling verification of the attenuation level. Birds were inoculated individually by oral gavage. A noninfected intact control group was used to monitor the *Salmonella*-free status of the birds.

The first sampling was done 5 days after the inoculation (in the case of the vaccine control and challenge control groups) or 5 days after challenge (in the case of the Principal groups). Samplings of that order were repeated weekly until the 4th week (Tables 2 and 3). The experiment was performed twice (Experiment 1 and Experiment 2).

In Experiment 1 the presence of the challenge SE147 Nal^R in the liver and spleen and its CFU/g in the caecal contents were selectively determined as described above in the section '*In vivo* organ invasion and colonisation studies'. In Experiment 2, besides the presence of *Salmonella* in organs, the bacterial counts of liver and spleen were also determined as follows: 0.2 g samples of liver and spleen were taken aseptically, homogenised and diluted in a 50-fold volume of RV broth supplemented with the appropriate antibiotics (Nal, Spe or Cm) in a sterile plastic bag, using a Stomacher blender (Seward Stomacher 80, Biomaster). Homogenised liver and spleen samples were decimally diluted and dilutions plated out on BTB agar containing matching antibiotics for the respective determination of CFU/g of *Salmonella*. For assessing *Salmonella* shedding of chicks in the Principal and control groups, cloacal swab samples were taken weekly, and incubated in RV broth at 41 °C for 48 h. Subsequently a loopful of culture from each selective enrichment RV broth was streaked onto BTB agar plates containing the appropriate antibiotics and incubated for further 24 h at 37 °C. The *Salmonella*-free status of the birds was monitored through parallel testing of the caecal content of three chicks at each sampling time from a noninfected intact control group (ISO 6579:2002).

Detection of antibodies against Salmonella *Enteritidis flagellin by double-antibody sandwich (DAS) blocking ELISA*

Levels of anti-flagellar antibodies were determined by double-antibody sandwich blocking enzyme-linked immunosorbent assay (DAS-ELISA) as described by van Zijderveld et al. (1993). For the specific blocking of serum antibodies the monoclonal antibodies 9G3 were used. These were produced and tested against *S*. Enteritidis flagellar antigen H:g,m, and for specific detection of these flagellar antibodies in poultry sera by our group (Szmollény et al., 1999). Serum inhibitory values > 40% of the absorbance (A_{450}) in wells to which only the conjugate was added, were regarded as positive. In these studies sera from 20 birds of the vaccine control group 1 (inoculated with parental SE11 only) and 20 birds from the vaccine control group 2 [inoculated with nonflagellated-plasmidless (*fliD*–, pSEVΔ) mutant SEΔ155 only] were tested at 4 weeks post inoculation. Sera of four uninoculated SPF broiler breeder (Ross 308) chicks were used as negative control.

Table 2

Numerical results of the early protection of day-old chicks against *Salmonella* Enteritidis 147 Nal^R in Experiment 1

		Challenge control SE147 only		Principal 1 SE11 + SE147		Principal 2 SEΔ155 + SE147		Vaccine control 1 SE11		Vaccine control 2 SEΔ155	
		+/ total	lg CFU/ ml	+/ total	lg CFU/ ml	+/ total	lg CFU/ ml	+/ total	lg CFU/ ml	+/ total	lg CFU/ ml
Week 1	Caecal count		8.34		5.25		5.60		8.77		7.85
	Cloacal swab	6/6		3/6		2/6		6/6		6/6	
	Spleen	6/6		0/6		4/6		6/6		6/6	
	Liver	6/6		0/6		4/6		6/6		6/6	
Week 2	Caecal count		5.82		< 3.00		3.59		5.63		5.25
	Cloacal swab	2/6		0/6		2/6		6/6		6/6	
	Spleen	6/6		2/6		5/6		6/6		6/6	
	Liver	6/6		0/6		2/6		5/6		5/6	
Week 3	Caecal count		4.12		< 3.00		< 3.00		4.20		3.68
	Cloacal swab	4/6		0/6		0/6		5/6		6/6	
	Spleen	5/6		0/6		0/6		4/6		6/6	
	Liver	5/6		0/6		0/6		0/6		0/6	
Week 4	Caecal count		< 3.00		< 3.00		< 3.00		< 3.62		< 3.00
	Cloacal swab	2/6		0/6		0/6		4/6		4/6	
	Spleen	5/6		0/6		2/6		4/6		1/6	
	Liver	2/6		0/6		0/6		0/6		0/6	

Challenge control*:* group receiving *S.* Enteritidis 147 Nal^R pathogenic strain (10^5 CFU/chick); Principal 1: group receiving *S.* Enteritidis 11 wild parental strain (10^8 CFU/chick) on day 1, and challenged with *S.* Enteritidis 147 Nal^R (10^5 CFU/chick) on day 2 of life; Principal 2: group receiving *S.* Enteritidis Δ155 plasmidless-nonflagellated mutant (10^8 CFU/chick) on day 1, and challenged with *S.* Enteritidis 147 Nal^R (10^5 CFU/chick) on day 2 of life; Vaccine control group 1 and Vaccine control group 2 were inoculated with SE11 (Spe^R) or SEΔ155 (*fliD*–, pSEVΔ: Cm^R), respectively, at one day of age in a dose of approx. 1×10^8 CFU/chicken; For each experiment, the first columns of data show the presence of the challenge *S.* Enteritidis 147 Nal^R in cloacal swabs, spleen and liver (positive/total tested), while the second columns present data on $\log_{10}$ CFU/g of these challenge bacteria in caecal, spleen and liver samples; <: *Salmonella* count was below the given detection limit

Table 3

Numerical results of the early protection of day-old chicks against *Salmonella* Enteritidis 147 Nal^R in Experiment 2

		Challenge control SE147 only		Principal 1 SE11 + SE147		Principal 2 SEΔ155 + SE147		Vaccine control 1 SE11		Vaccine control 2 SEΔ155	
		+/ total	lg CFU/ ml	+/ total	lg CFU/ ml	+/ total	lg CFU/ ml	+/ total	lg CFU/ ml	+/ total	lg CFU/ ml
Week 1	Caecal count		6.23		< 3.00		< 4.60		6.64		6.32
	Cloacal swab	6/6		0/6		1/6		6/6		6/6	
	Spleen	6/6	4.16	0/6	< 2.69	2/6	< 4.19	6/6	4.72	6/6	< 3.77
	Liver	6/6	3.98	0/6	< 2.69	1/6	< 2.69	6/6	3.63	6/6	< 2.69
Week 2	Caecal count		6.75		< 3.00		< 3.00		5.98		4.40
	Cloacal swab	6/6		0/6		0/6		6/6		6/6	
	Spleen	6/6	4.35	0/6	< 2.69	0/6	< 2.69	6/6	3.87	6/6	< 2.92
	Liver	6/6	3.57	0/6	< 2.69	2/6	< 2.69	6/6	2.85	4/6	< 3.00
Week 3	Caecal count		4.35		< 3.00		< 3.00		4.89		< 3.12
	Cloacal swab	5/6		0/6		0/6		5/6		2/6	
	Spleen	6/6	3.14	1/6	< 2.69	0/6	< 2.69	6/6	3.87	3/6	< 2.69
	Liver	6/6	2.69	0/6	< 2.69	0/6	< 2.69	3/6	2.85	1/6	< 2.39
Week 4	Caecal count		< 3.00		< 3.00		< 3.00		< 3.00		< 3.00
	Cloacal swab	3/6		0/6		0/6		5/6		0/6	
	Spleen	4/6	< 2.39	0/6	< 2.69	1/6	< 2.69	2/6	< 2.39	2/6	< 2.39
	Liver	2/6	< 2.39	0/6	< 2.69	0/6	< 2.69	1/6	< 2.39	1/6	< 2.39

Challenge control: group receiving S. Enteritidis 147 NalR pathogenic strain (105 CFU/chick); Principal 1: group receiving S. Enteritidis 11 wild parental strain (108 CFU/chick) on day 1, and challenged with S. Enteritidis 147 NalR (105 CFU/chick) on day 2 of life; Principal 2: group receiving S. Enteritidis Δ155 plasmidless-nonflagellated mutant (108 CFU/chick) on day 1, and challenged with S. Enteritidis 147 NalR (105 CFU/chick) on day 2 of life; Vaccine control group 1, and Vaccine control group 2 were inoculated with SE11 (SpeR) or SEΔ155 (fliD–, pSEVΔ: CmR), respectively, at one day of age in a dose of approx. 1 × 108 CFU/chicken; For each experiment, the first columns of data show the presence of the challenge S. Enteritidis 147 NalR in cloacal swabs, spleen and liver (positive/total tested), while the second columns present data on log10 CFU/g of these challenge bacteria in caecal, spleen and liver samples; <: Salmonella count was below the given detection limit

Data analysis

Data from the fibroblast invasion experiments were analysed by Student's *t*-test. *Salmonella* isolation (percentage of *Salmonella*-positive samples from the organ invasion and protection experiments) was compared between each group by G-test. Data analysis of the second protection experiment (with CFU of *Salmonella* in organs) was made by ANOVA. We used a significance level of P = 0.05 for all statistical tests.

Results

In vitro *invasiveness of the SE11 mutants: SE2102 (fliD–) and SEΔ155 (fliD–, pSEVΔ)*

The mutants of SE11 described above designated as 'nonmotile' (SE2102) and 'nonmotile-plasmidless' (SEΔ155), respectively, have proved to be significantly (P < 0.005) less invasive than the wild-type parental strain SE11 in CEF cell cultures *in vitro*. On the other hand, there was no significant difference between the nonmotile SE2102 and the nonmotile plasmid-cured SEΔ155 (P = 0.415) mutants in this respect (Fig. 1).

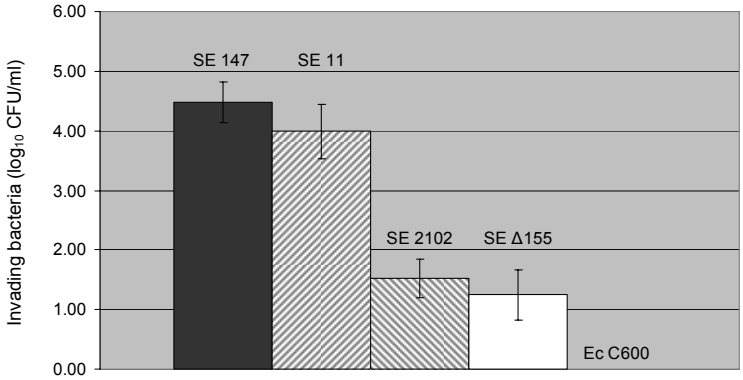

Fig. 1. In vitro invasion properties of the wild-type parental *S.* Enteritidis 11 (SE11), its nonmotile (*fliD–*) mutant SE2102 and its nonmotile-plasmidless (*fliD–*, pSEVΔ) derivative SEΔ155 in comparison with the virulent wild-type *S.* Enteritidis 147 (SE147) strain in chicken embryo fibroblast (CEF) cells. *Escherichia coli* strain C600 served as negative control. There is no statistically significant difference between wild SE strains or between mutant strains. Both wild strains are significantly (P < 0.005) more invasive than the mutant strains

Organ invasion and intestinal colonisation properties of the SE11 mutants

The nonmotile (*fliD–*) SE2102, the nonmotile-plasmidless (*fliD–,* pSEVΔ) SEΔ155 mutants and the parental *S.* Enteritidis 11 strains were tested for liver and spleen invasion as well as for caecal colonisation in day-old SPF chicks in

two independent oral infection experiments (A and B) using a lower and a higher infective dose (Table 1). None of the doses of any of the strains resulted in the death of inoculated chicks. Both the SE2102 and the SEΔ155 mutants proved to have significantly ($P < 0.05$) reduced liver and spleen invasiveness as compared to the wild-type parental strain SE11. On the other hand, there was no reduction in caecal colonisation by these mutants in comparison to the parental strain, and there was no statistically significant difference between mutants SE2102 and SEΔ155 in terms of organ invasion and caecal colonisation either. The results of caecal colonisation and organ invasion for all the test strains proved to be dose dependent (Table 1).

Protection of day old chicks by the nonmotile-plasmidless mutant SEΔ155 against organ invasion, caecal colonisation and shedding of the virulent challenge S. Enteritidis 147 Nal^R strain

Oral inoculation of day-old chicks with *S.* Enteritidis 11 Spe^R parental strain or its nonmotile-plasmidless (*fliD–,* pSEVΔ) SEΔ155 mutant and the 2nd day challenge with virulent *S.* Enteritidis 147 Nal^R were performed in two subsequent experiments. Liver and spleen invasion and caecal colonisation as well as the results of *Salmonella* shedding were determined weekly until the 4th week after challenge. Combined results of the two experiments (proportions of organs and cloacal samples with SE147 challenge strain, and CFU/g of SE147 in caecum) are presented in Fig. 2. Detailed numerical results of the two experiments are shown in Table 2 and Table 3, respectively. The data of Experiment 2 presenting CFU/g of *Salmonella* in organs indicate that the application of the vaccine candidate nonmotile-plasmidless (*fliD–,* pSEVΔ) mutant SEΔ155 significantly ($P < 0.05$) reduced the counts of SE147 challenge strain in the liver during the first two weeks post challenge, and there was a significant reduction ($P < 0.05$) of the challenge strain in the spleen for three weeks post challenge. This reduction proved to be at least as effective as that induced by the SE11 parental strain as there was no significant difference between the parental SE11 and its nonmotile-plasmidless (*fliD–,* pSEVΔ) mutant SEΔ155 (Table 3). Besides, there was a significantly ($P < 0.01$) reduced caecal colonisation in groups Principal 1 and Principal 2 relative to the challenge control in both experiments (Table 2, Table 3 and Fig. 2). Furthermore, a strong reduction ($P < 0.01$) of cloacal shedding of the challenge strain was clearly demonstrable with no detectable challenge strain in faecal samples from the third week post infection both in Principal group 1 (vaccinated previously with the SE11 parental strain) and in Principal group 2 [vaccinated previously with the nonmotile-plasmidless (*fliD–,* pSEVΔ) mutant SEΔ155], as shown in Table 2, Table 3 and Fig. 2.

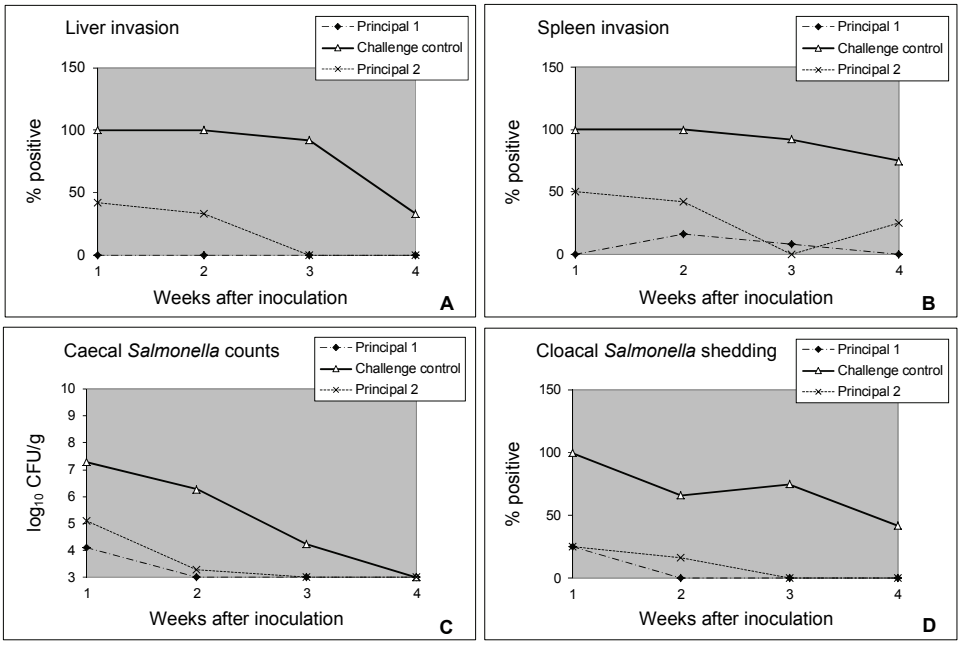

Fig. 2. Early protective capacity of the *S.* Enteritidis 11 strain and its nonmotile-plasmidless (*fliD*–, pSEVΔ) mutant SEΔ155 in day-old chicks. Combined results of Experiments 1 and 2. Percentages of organs and cloacal samples containing the challenge strain SE147, and caecal counts (CFU/g) of the challenge strain SE147. One-day-old *Salmonella*-free Ross chicks were orally inoculated with parental strain SE11 Spe^R and challenged 24 h later with SE147 Nal^R in group Principal 1. The group Principal 2 was inoculated at the same age with the nonmotile-plasmidless (*fliD*–, pSEVΔ) mutant SEΔ155 Cm^R and challenged 24 h later with SE147 Nal^R. The challenge control group was only inoculated with SE147 Nal^R at one day of age. The first sampling was done 5 days after challenge and it was repeated weekly until the 4th week after challenge. *Salmonella* positivity of liver (A) and spleen (B) and bacterial counts of caecal samples (C) were determined. Faecal shedding of experimental strains was determined by testing cloacal swabs (D). There was a significant reduction ($P < 0.05$) of the challenge strain in the spleen for 3 weeks post challenge. There was a significantly ($P < 0.01$) reduced caecal colonisation in the groups Principal 1 and Principal 2 relative to the challenge control in both experiments. A strong reduction ($P < 0.01$) was demonstrable in the cloacal shedding of the challenge strain

Serological distinction of vaccinated flocks by DAS-ELISA

In these studies, sera of 20 birds each from the vaccine control groups infected only with the parental SE11 or with the nonmotile-plasmidless (*fliD*–, pSEVΔ) mutant SEΔ155 were compared. The average inhibitory capacity of anti-flagellin antibodies detectable in sera of the birds inoculated with the parental strain SE11 was 48.1% in contrast to the birds inoculated with the mutant SEΔ155 (10.8%) (Fig. 3). In the group infected with the parental strain SE11 the inhibitory potential of the anti-flagellin antibodies exceeded the 40% threshold in 12 out of the 20 birds tested. In contrast, no individual serum of the chickens in-

fected with the nonmotile-plasmidless (*fliD–,* pSEVΔ) mutant SEΔ155 exceeded this inhibitory threshold, and the inhibitory values of the four negative control sera of intact birds were ≤ 10.0%. The difference in anti-flagellar antibody response between the SE11-inoculated and the mutant SEΔ155 inoculated chickens was significant (P < 0.001).

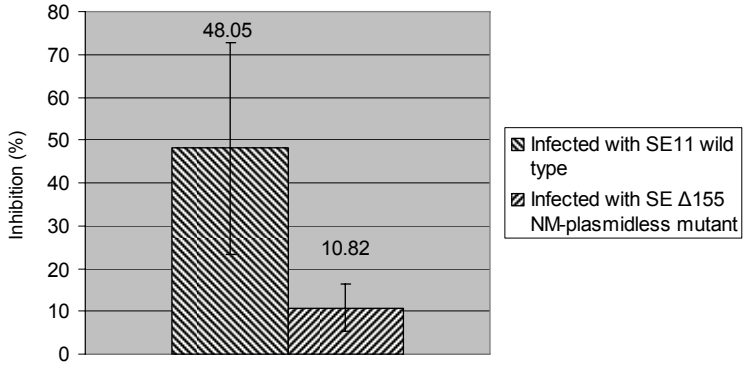

Fig. 3. Average inhibitory capacity of anti-flagellin antibodies detectable in the sera of 20 birds inoculated with SE11 wild-type strain and of 20 birds inoculated with its nonmotile-plasmidless (*fliD–*, pSEVΔ) mutant SEΔ155 four weeks post infection. A significant (P < 0.01) increase was detected at 4 weeks post inoculation in SE11-inoculated chickens as compared to those inoculated with the nonmotile-plasmidless (*fliD–*, pSEVΔ) mutant SEΔ155

Discussion

An important requirement for the new generation of live oral vaccines is to be distinguishable from field isolates by inducing a different serological host response (Barrow and Wallis, 2000). The most straightforward solution to provide a negative serological marker for a *Salmonella* strain is the elimination of flagellae. A nonflagellated *S.* Enteritidis vaccine candidate strain (*ΔguaB, ΔfliC*) has been produced and proven to be promising in the protection of chicks against homologous challenge (Adriaensen et al., 2007). Further *fliC–* S. Enteritidis vaccine candidates have been reported by Methner et al. (2011*a*) and Matulova et al. (2013). In these studies nonflagellated mutants were produced by Lambda-Red recombination mutagenesis (Datsenko and Wanner, 2000). Here we have used a nonflagellated (*fliD–*) mutant with transposon insertion in the flagellar capping gene generated by a novel method based on IS*30* mediated site-directed mutagenesis (Imre et al., 2011).

Another specific attribute of our nonmotile vaccine candidate mutant SEΔ155 is the lack of its serovar-specific virulence plasmid (*fliD–,* pSEVΔ). This is in contrast to the above mentioned non-motile *S.* Enteritidis vaccine can-

didates which are not reported to be devoid of pSEV. The role of such plasmids in the *in vitro* and *in vivo* invasion by *Salmonella* is less clear. Virulence plasmids of certain *Salmonella* serovars (*S*. Enteritidis, *S*. Gallinarum, *S*. Pullorum, *S*. Typhimurium, *S*. Choleraesuis, *S*. Dublin) are known to contribute to the propagation and survival of bacteria in the appropriate host. These plasmids contain several genes responsible for virulence-related traits. The most important among them is the *spv* (*Salmonella* plasmid virulence) region, assisting in bacterial survival within granulocytes and macrophages. Further important virulence determinants are the *pef* (plasmid-encoded fimbriae) operon, mediating adhesion to the intestinal cells, and the *rck* gene (resistance to complement killing) (Rychlik et al., 2006; Imre et al., 2007). The function of these genes may be important in the infection process, and in the long-time persistence of *Salmonella* in some animal hosts and in humans. Earlier Barrow and Lowell (1989) found that serovar-specific virulence plasmids of *S*. Typhimurium, *S*. Gallinarum and *S*. Pullorum are not essential for Vero cell invasion. The results of Halavatkar and Barrow (1993) and of Martin et al. (1996) indicate that such virulence plasmids of *S*. Enteritidis are important for virulence in mice, but not in chickens. These findings are in line with the *in vivo* results of Gulig and Curtiss (1987) and of Imre et al. (2007). However, based on the recent evidences and the potential public health aspects the elimination of the serovar-specific virulence plasmid should be regarded as an advantage for the development and registration of live oral *Salmonella* vaccines (Martin et al., 1996; Barrow and Wallis, 2000).

Invasion and virulence tests carried out on nonmotile (*fliD–*) and on nonmotile plasmid-cured (*fliD–,* pSEVΔ) mutants of *S.* Enteritidis 11 strain showed that the above mutants were equally much less invasive in a CEF model *in vitro* than the wild-type parental strain, and proved to have diminished organ invasiveness in day-old chickens as well. Our results are in harmony with the *in vitro* invasion results of previous publications comparing wild-type strains and nonflagellated (*fliC*-deleted) mutants of different *Salmonella* serovars, indicating that flagellae are necessary for the proper invasion of *S*. Enteritidis into human Caco-2 and Hep-2 tumour cells (Van Asten et al., 2000; La Ragione et al., 2003), but differ somewhat from those obtained on *fliC*-deleted mutants of different strains of *S*. Enteritidis (Adriaensen et al., 2007; Methner et al., 2011*b*). Adriaensen et al. (2007) found that the *fliCΔ* mutant of their wild-type *S*. Enteritidis 76Sa88 showed less reduced invasion of human (T84) or chicken intestinal epithelial cells. Unfortunately, these authors did not compare their double-deletion mutant (*ΔguaB ΔfliC)* in day-old chicks to either the *ΔfliC* single mutant or to the wild parent strain. Therefore, our and their results could not be directly compared. In general, it must be noted that not all kinds of 'nonflagellated' mutants of all *S*. Enteritidis strains may lose their invasiveness. The *fliCΔ* mutant of S.E147 of Methner et al. (2011*b*) did not prove to be sufficiently attenuated in chicks. As a further example, *flhD* deletion mutants of the SE-HCD strain remained invasive

in spite of their nonflagellar phenotype (Parker and Guard-Petter, 2001). This might be due to the temporary suppression of Class I regulators of flagellin biosynthesis, in contrast to Δ*fliC* flagellin gene mutants of the same strain that became significantly and permanently nonflagellated and less invasive when tested in orally inoculated day-old chicks. In our studies we partly confirmed and extended the above observations by proving the significantly reduced invasiveness of the *S*. Enteritidis nonflagellated *fliD*– mutants of SE11 *in vitro* and *in vivo*. Our results also suggest that flagellae *per se* may act as a virulence factor. At the same time, here we confirm that the flagellar protein FliD itself is not required for the efficient vaccination of chickens. Although there are several data supporting the role of flagellae in cellular and humoral immune responses to *Salmonella* (Salazar-Gonzalez and McSorley, 2005), the results of Kodama and Matsui (2004) and Adriaensen et al. (2007) also suggest that *Salmonella* flagellin is not a major protective antigen in mice. As stated above, our results confirm and extend these observations on the lack of a major protective role of *S.* Enteritidis flagellin against *Salmonella* infection in chicks. In spite of decreased virulence, the oral vaccination of day-old chicks with this live attenuated (*fliD–,* pSEVΔ) mutant SEΔ155 resulted in pronounced early protective activity against organ invasion and caecal colonisation by, and against shedding of, the highly virulent *S*. Enteritidis 147 challenge strain, as demonstrated during the first four weeks of life. In fact, the level of protection conferred by this *fliD*–, pSEVΔ mutant proved to be very similar to that induced by the wild-type parental strain. By this, we provided data for the first time about a virulence plasmid cured nonflagellated mutant of *S.* Enteritidis to serve as a basis for the development of a negatively markered potential live oral vaccine against virulent *S*. Enteritidis in chicken.

One explanation for this remarkable early protection is presumably the colonisation inhibition or competitive exclusion between bacteria of the same *Salmonella* serovar (Barrow et al., 1987; Nógrády et al., 2003; Methner et al., 2011*a*). Although several commercial and experimental live attenuated *Salmonella* vaccines with certain metabolic mutations have been reported to induce protection against organ invasion and immune response after a single oral application, most of them did not seem to be able to inhibit intestinal colonisation of the challenge *Salmonella* organisms (Van Immerseel et al., 2002; Barrow, 2007). Some of them did exert a modest inhibition of colonisation against homologous challenge (Methner et al., 1997). Live attenuated *Salmonella* vaccines produced by targeted mutagenesis have also been extensively tested in several animal species, and it is known that such strains more readily promote a long-lasting cell-mediated immunity than the killed vaccines (Zhang-Barber et al., 1999; Van Immerseel et al., 2005; Barrow, 2007; Pasquali et al., 2008). Recently a nonflagellated (*ΔguaB, ΔfliC*) mutant produced by Adriaensen et al. (2007), and the nonflagellated (*ΔphoP, ΔfliC)* mutant of *S.* Enteritidis produced by Methner et al. (2011*b*) have been demonstrated to confer sufficient protection against organ in-

vasion by a virulent *S*. Enteritidis challenge strain, but either no reduction or only a weak reduction of colonisation was proven, respectively. Besides, neither of these two papers reported about cloacal shedding of the challenge *S.* Enteritidis strain. Here we found significantly ($P < 0.01$) decreased cloacal shedding compared to the challenge control for the first two weeks and it was completely eliminated for the 3rd and 4th weeks after challenge. In short, in our studies the nonmotile virulence-plasmid cured (*fliD*–, pSEVΔ) mutant did not only provide protection against organ invasion but also effectively reduced caecal colonisation and cloacal shedding of the highly virulent *S*. Enteritidis. In this respect our results were similar to those of Matulova et al. (2013).

In this study we also compared the production of anti-flagellar antibodies in chicks inoculated with the nonmotile-plasmidless (*fliD*–, pSEVΔ) mutant SEΔ155 with that found in chicks inoculated with the motile parental strain SE11. A significant ($P < 0.01$) increase at 4 weeks post inoculation was detected in SE11-inoculated chickens as compared to those inoculated with the nonmotile-plasmidless (*fliD–,* pSEVΔ) mutant SEΔ155. The results indicated that the significant difference in the titre of anti-flagellin antibodies can be used as a negative marker for the differentiation of vaccinated groups from those infected with the wild-type strain, similarly as has been described for the *S.* Enteritidis *fliC*– mutant very recently (Methner et al., 2011*b*; Matulova et al., 2013).

In summary, the negatively markered, nonmotile-plasmidless (*fliD*–, pSEVΔ) mutant of a wild *Salmonella* Enteritidis strain constructed with the further aim of serving as a live, oral chicken vaccine candidate, has been tested for residual virulence in the *in vitr*o (cell culture) and *in vivo* (day-old chick oral infection) models and proved to be sufficiently attenuated without reduced intestinal colonisation capacity. Early protective activity of the nonmotile-plasmidless mutant SEΔ155 against organ invasion and caecal colonisation as well as against long-term shedding of the highly virulent *S.* Enteritidis 147 strain was demonstrable during the first four weeks of life. The serological response of chicks inoculated with the nonmotile-plasmidless mutant SEΔ155 could be differentiated from that of chicks inoculated with the wild-type strain of *S*. Enteritidis. Thus, our studies have shown for the first time that the nonmotile, virulence-plasmid cured (*fliD*–, pSEVΔ) mutant SEΔ155 of *S*. Enteritidis studied here, can be used as a negatively markered live oral vaccine candidate against highly virulent strain of *S*. Enteritidis in chickens.

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