**Application of the DIVA principle to *Salmonella* Typhimurium vaccines in pigs avoids interference with serosurveillance programmes**

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**Abstract**

Salmonellosis is one of the most important bacterial zoonotic diseases in humans and *Salmonella* infections are often linked with the consumption of contaminated pork. In order to reduce *Salmonella* Typhimurium infections in humans, minimization of the *Salmonella* intake into the food chain is important. Vaccination has been proposed to control *Salmonella* infections in pigs. However, pigs vaccinated with the current vaccines cannot be discriminated from infected pigs with the lipopolysaccharide (LPS) -based serological tests used in European serosurveillance programmes. We therefore examined which LPS encoding genes of *Salmonella* Typhimurium can be deleted to allow differentiation of infected and vaccinated pigs, without affecting the vaccine strain's protective capacity. For this purpose, deletion mutants in *Salmonella* strain 112910a, used as vaccine strain, were constructed in the LPS encoding genes: Δ*rfbA*, Δ*rfaL*, Δ*rfaJ*, Δ*rfaI*, Δ*rfaG* and Δ*rfaF*. Inoculation of BALB/c mice with the parent strain, Δ*rfaL,* Δ*rfbA* or Δ*rfaJ* strains but not the Δ*rfaG*, Δ*rfaF* or Δ*rfaI* strains protected significantly against subsequent infection with the virulent *Salmonella* Typhimurium strain NCTC12023. Immunization of piglets with the Δ*rfaJ* or Δ*rfaL* mutants resulted in the induction of a serological response lacking detectable antibodies against LPS. This allowed a differentiation between sera from pigs immunized with the Δ*rfaJ* or Δ*rfaL* strains and sera from pigs infected with their isogenic wild type strain.

**Introduction**

*Salmonella* infections in humans are often linked with the consumption of contaminated pork [1] [2]. Vaccination has been proposed to control *Salmonella* infections in pigs [1] [3] [4] and has already proven to be efficient in laying hens, reducing faecal shedding and internal egg contamination [5] [6]. Currently, one licensed *Salmonella* Typhimurium live vaccine for pigs is commercially available in Europe [7]. The use of this vaccine is limited due to interference with European *Salmonella* serosurveillance programmes based on the detection of antibodies against the lipopolysaccharides (LPS) of *Salmonella* [8]. It was therefore the aim of this study to develop a DIVA-vaccine strain (**D**ifferentiation of **I**nfected and **V**accinated **A**nimals), without attenuating the vaccine strain, which would not interfere with current LPS-ELISA based serosurveillance programmes.

**Material and Methods**

*Salmonella* Typhimurium strain 112910a, phage type 120/ad, isolated from a pig stool sample and characterized previously [3], was used as the wild type background to construct several isogenic LPS knock-out mutants: Δ*rfbA*, Δ*rfaL*, Δ*rfaJ*, Δ*rfaI*, Δ*rfaG* and Δ*rfaF*. A commercially available enzyme-linked immunosorbent assay (ELISA) (HerdChek *Salmonella*; IDEXX Laboratories, Schiphol-Rijk, Noord-Holland, The Netherlands) for the detection of porcine antibodies against the LPS of *Salmonella* was used as a reference according to the manufacturer's instructions. Besides, an in-house *Salmonella* Typhimurium strain 112910a whole cell ELISA to detect porcine anti *Salmonella* Typhimurium antibodies, was prepared as described before [9]. In a mouse model, we tested whether the LPS mutants affect the protective capacity of *Salmonella* Typhimurium strain 112910a against a subsequent challenge with a highly virulent strain. For that purpose, seven groups of ten mice were inoc $10^7$ CFU/ml of one of the LPS mutant strains (either:

Δ*rfbA*, Δ*rfaL*, Δ*rfaJ*, Δ*rfaI*, Δ*rfaG* or Δ*rfaF*) or with the wild type *Salmonella* Typhimurium strain 112910a. Four weeks after primary inoculation, all mice were challenged with $10^8$ CFU of the virulent *Salmonella* Typhimurium strain NCTC12023Nal[20] by the orogastric route. In a second *in vivo* study, we examined whether it was possible to discriminate between the serological response induced after immunization of pigs with either *Salmonella* Typhimurium strain 112910a or one of its isogenic strains (Δ*rfaL* Δ*rfaJ*) on the one hand and after infection of pigs with *Salmonella* Typhimurium strain 112910a on the other hand. Therefore, 14 piglets were randomly allocated to three vaccinated groups (n = 12) and one sham-vaccinated control group (n = 2). Vaccinated animals were intramuscularly immunized (2x) with one of the formalin-inactivated *Salmonella* strains (either: *Salmonella* Typhimurium strain 112910a, Δ*rfaJ* or Δ*rfaL*) in Freund's incomplete adjuvant. To obtain sera from *Salmonella* Typhimurium infected piglets, one experimental group (n = 3) was
$10^7$ CFU of *Salmonella* Typhimurium strain 112910aNal[20].

## Results

### Vaccination of mice with Δ*rfbA*, Δ*rfaL* and Δ*rfaJ* but not Δ*rfaI*, Δ*rfaG* and Δ*rfaF* protects mice against a *Salmonella* Typhimurium infection:

Oral immunization of mice with *Salmonella* Typhimurium strain 112910a, Δ*rfbA*, Δ*rfaL* or Δ*rfaJ* induced a significant ($P < 0.05$) protection against subsequent challenge with NCTC12023Nal[20] in both spleen and liver compared to non immunized control animals. Results are shown in figure 1.

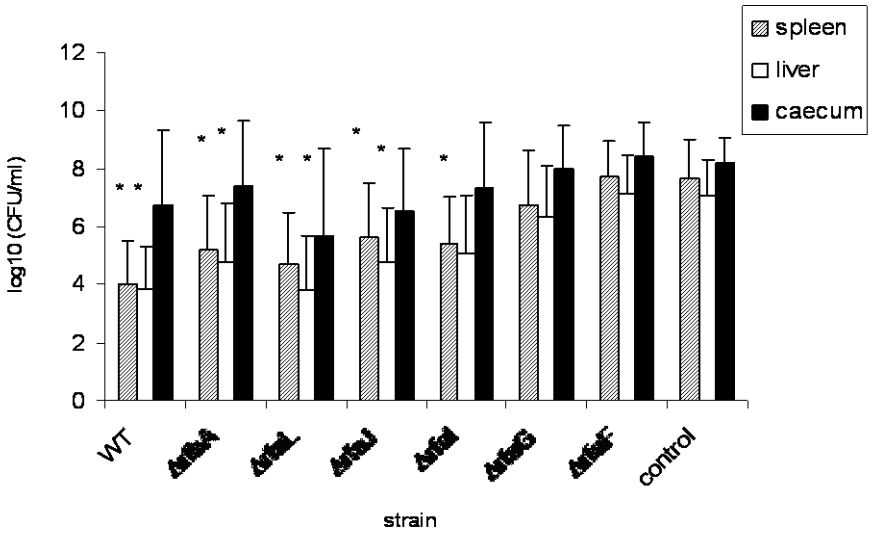


Figure 1: Recovery of *Salmonella* bacteria from various organs of mice immunized with either *Salmonella* Typhimurium, one of its isogenic LPS mutants or non immunized control animals and subsequently challenged with *Salmonella* Typhimurium strain NCTC12023Nal[20]. The $\log_{10}$ value of the ratio of CFU per gram sample and standard deviations are given. An asterisk refers to a significant difference with the control group ($P < 0.05$).

### Pigs, immunized with the Δ*rfaL* or Δ*rfaJ* mutant, can be serologically differentiated from *Salmonella* infected animals:

Results showed no significant seroconversion ($P > 0.05$) in animals immunized with inactivated Δ*rfaJ* or Δ*rfaL* strains and in sham-vaccinated control animals (non immunized and non infected animals), when using the commercial IDEXX ELISA. Conversely, marked seroconversion occurred in pigs immunized with the inactivated *Salmonella* Typhimurium strain 112910a. Results also illustrate a clear differentiation between sera from piglets immunized with the Δ*rfaJ* strain or Δ*rfaL* strain and sera of pigs infected with their isogenic wild type strain. Anti-*Salmonella*-antibody titers were detected in the serum of all immunized and infected animals, when using the in-house whole cell ELISA. Results are illustrated in figure 2.

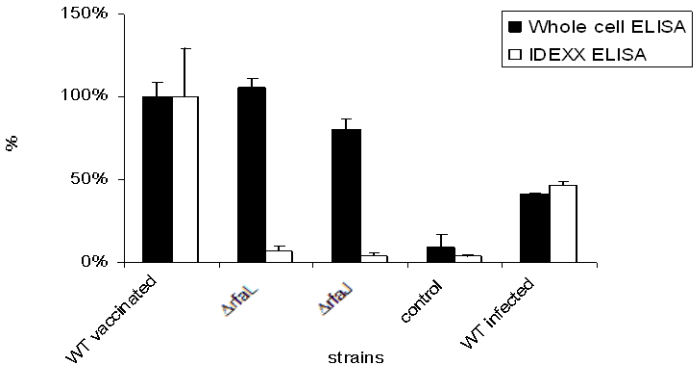


Figure 2: Serological results of pigs immunized with Δ*rfaL*, Δ*rfaJ* or *Salmonella* Typhimurium strain 112910a, control pigs (animals that were not immunized and not infected) and pigs infected with S*almonella* Typhimurium strain 112910a Nal[20]. Values are represented as a percentage compared to the wild type vaccinated group. We emphasize that these results are based on a small sample size.

## Discussion

DIVA vaccines are a recent advance in vaccinology enabling distinction between an animal that is seropositive to a particular infectious agent because it has been vaccinated, and one that is seropositive because it has been infected with virulent field organisms [10]. Because current *Salmonella* serosurveillance programmes are generally based on detection of antibodies against LPS antigens, we selected six LPS genes that might be suitable markers to develop a LPS based DIVA-vaccine. In a mouse *in vivo* experiment we showed that the *rfaG* and *rfaF* mutant strains were not able to protect BALB/c mice against a subsequent infection with *Salmonella* Typhimurium NCT12023Nal[20] and that the Δ*rfaI* strain was only able to significantly reduce bacterial counts in the spleen of mice. Conversely, Δ*rfbA*, Δ*rfaL* and Δ*rfaJ* strains, with less truncated LPS, were able to successfully protect BALB/c mice against a *Salmonella* Typhimurium infection and their protective capacity was not impaired compared to their isogenic wild type strain. These results strongly suggest that a confined truncation of LPS is essential to maintain protection against challenge with the virulent strain *Salmonella* Typhimurium NCTC12023Nal[20] in mice. The ultimate goal of this study was to verify whether LPS mutant strains were able to elicit a DIVA humoral immune response in pigs. Our results illustrate that both the Δ*rfaL* and the Δ*rfaJ* strain gave no seroconversion when using a LPS based ELISA, while a clear-cut seroconversion was observed when using an in-house *Salmonella* Typhimurium strain112910a whole cell ELISA. Besides, immunization of piglets with the Δ*rfaJ* or Δ*rfaL* mutants resulted in the induction of a serological response allowing clear differentiation between sera from piglets immunized with the Δ*rfaJ* or Δ*rfaL* strains and sera of pigs infected with their isogenic wild type strain when using a LPS based ELISA.

## Conclusion

In conclusion, applying deletions in the *rfaJ* or the *rfaL* gene in *Salmonella* Typhimurium strain 112910a allows differentiation of infected and vaccinated pigs in an LPS based ELISA without reducing the strain's protective capacities in mice.

## References

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