TITLE

Salmonella Typhimurium LPS mutations for use as DIVA markers in vaccines for pigs

Bregje Leyman*, Filip Boyen, Alexander Van Parys, Elin Verbrugghe, Freddy Haesebrouck and Frank Pasmans

Ghent University, Faculty of Veterinary Medicine, Department of Pathology, Bacteriology and Avian Diseases, Salisburylaan 133, 9820 Merelbeke, Belgium

* Corresponding author: Tel: +32 9 264 73 76

Fax: +32 9 264 74 94

E-mail address: Bregje.leyman@ugent.be

Keywords: pig, lipopolysaccharide, marker strains, *Salmonella* Typhimurium

Abstract

Contaminated pork is a major source of human salmonellosis and the serovar most frequently isolated from pigs is *Salmonella* Typhimurium. Vaccination could contribute greatly to controlling *Salmonella* infections in pigs. However, pigs vaccinated with the current vaccines cannot be discriminated from infected pigs with the LPS-based serological tests used in European *Salmonella* serosurveillance programmes. We therefore examined which LPS encoding genes of *Salmonella* Typhimurium can be deleted to allow differentiation of infected and vaccinated pigs (DIVA), 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*. Primary inoculation of BALB/c mice with the parent strain, Δ*rfaL*, Δ*rfbA* or Δ*rfaJ* strain but not the Δ*rfaG*, Δ*rfaF* or Δ*rfaI* strain 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 clear 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. 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.

1. Introduction

Salmonellosis is one of the most important bacterial zoonotic diseases in humans and *Salmonella* infections are often linked with the consumption of contaminated pork [1] [2]. The serovar most frequently isolated from pigs is *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium), which is also the most prevalent serovar in humans [3]. In order to reduce human *Salmonella* Typhimurium infections, minimization of the *Salmonella* intake into the food chain is very important and efforts to reduce transmission of *Salmonellae* by food should be implemented on a global scale [2]. A combined approach using hygienic measures, the use of feed additives and different protection measures, such as vaccination, has been proposed to reduce the contamination on farms [3] [4] [5]. Vaccination has already proven to be efficient in laying hens, reducing faecal shedding and internal egg contamination of *Salmonella*, resulting in reduction of the number of human salmonellosis cases [6] [7]. Currently, one licensed *Salmonella* Typhimurium live vaccine for pigs is commercially available in Europe [8] and has shown to reduce both shedding and colonization of host tissues [9] and to induce a substantial *Salmonella* antibody response seven days after the second immunization [10]. These antibodies are, however, not distinguishable from those induced after a wild type *Salmonella* Typhimurium infection. An isogenic mutant of the *Salmonella* vaccine strain was developed, which lacks the *ompD* gene. This allowed differentiation of infected and vaccinated animals, using an OmpD based enzyme-linked immunosorbent assay (ELISA) [9]. Unfortunately, this DIVA-vaccine (**D**ifferentiation of **I**nfected and **V**accinated **A**nimals) is not broadly applicable, despite its ability to reduce colonization [9], because European *Salmonella* serosurveillance programmes are mostly based on the detection of antibodies against the lipopolysaccharides (LPS) of *Salmonella* [11]. It was therefore the aim of this study to develop and characterize LPS mutations that might be used as DIVA markers with application in the currently used monitoring programmes in the EU.

Since a marker should not affect the vaccine strain's protective capacity, we first compared the protective capacity of LPS mutants and their parent strain in a standardized mouse virulent assay. Secondly, the LPS deletion mutants were tested for their capability to elicit a DIVA antibody response in pigs.

2. Materials and methods

All *in vivo* experiments were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC 2009/124, EC 2009/131, EC 2010/080 and EC 2010/108).

2.1 Bacterial strains

Salmonella Typhimurium strain 112910a, phage type 120/ad, isolated from a pig stool sample and characterized previously [4], 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*, Δ*rfaF*. These strains were used for immunization of mice and pigs. The bacterial strains and primers used in this study are shown in table 1A/B. The knock-out mutants were constructed as described before [12]. Briefly, the genes of interest were first substituted by a PCR adjusted antibiotic resistance cassette (kanamycin) using the helper plasmid pKD46. This plasmid encodes the phage *λ* Red system, which promotes recombination between the native gene and the PCR adjusted antibiotic resistance cassette. Recombinant clones were selected by plating on Luria-Bertani agar (LB; Sigma Aldrich Chemie Gmbh, Steinheim, Germany) containing 100 µg/ml kanamycin. The substitution was confirmed by PCR. In the last step, the antibiotic resistance cassettes were eliminated using the helper plasmid pcp20. The targeted genes were completely deleted from the start codon through the stop codon, as confirmed by sequencing. *Salmonella* challenge strains comprised, spontaneous mutants resistant to 20 µg/ml nalidixic acid (Nal^{20}) in a NCTC12023 *Salmonella* Typhimurium strain highly virulent in BALB/c mice, in *Salmonella* Enteritidis strain SE147, in *Salmonella* Heidelberg strain 704Sa06 and in

Salmonella Typhimurium strain 112910a. All bacteria were routinely grown in LB broth or on brilliant green agar (BGA) at 37 °C, unless stated otherwise.

2.2 Characterization of the LPS knock-out mutants of *Salmonella* Typhimurium

Validation of the LPS phenotype occurred by SDS-polyacrylamide gel electrophoresis and fluorescent staining. For this purpose LPS was isolated from *Salmonella* Typhimurium strain 112910a and its isogenic knock-out mutants using a commercially available LPS extraction kit (Intron biotechnology, Gyeonggi-do, Korea). The obtained LPS was quantified using a ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, USA) and was separated by standard SDS-polyacrylamide gel electrophoresis. LPS was stained using a Molecular probes Pro-Q Emerald LPS Gel stain kit (Invitrogen, Oregon, USA), creating a bright green-fluorescent signal, which was visualised with a 300 nm UV-transilluminator. To verify whether LPS mutant strains (Δ*rfbA*, Δ*rfaL*, Δ*rfaJ*, Δ*rfaI*, Δ*rfaG*, Δ*rfaF*) were still expressing O-antigens on their surface, an *in vitro* agglutination test (PRO-LAB O_4 and O_{12} antisera, diagnostics, Austin, Texas) was performed, according to the manufacturer's instructions. The smooth phenotype was also tested by checking sensitivity of *Salmonella* Typhimurium and its isogenic knock-out mutants to bacteriophage P22 as described elsewhere [12]. As a measure of *in vitro* virulence of the wild type strain and its isogenic mutants, invasiveness of all strains was assessed in porcine epithelial cells (IPEC-J2) using a gentamicin protection assay as described previously [13].

2.3 ELISA procedures

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. Coating antigens in this ELISA include LPS of serogroups B, C1 and D (O-antigens 1, 4, 5, 6, 7 and 12) [14]. Besides, an in-house *Salmonella*

Typhimurium strain 112910a whole cell ELISA, to detect porcine anti *Salmonella* Typhimurium antibodies, was prepared as follows. *Salmonella* Typhimurium strain 112910a was cultured overnight at 37 °C in 500 ml LB broth. Inactivation was achieved by adding 0.18% (v/v) formalin overnight at 37 °C. The bacteria were centrifuged three times (5000 X g for 30 min at room temperature) and the resulting pellet was resuspended in a volume of 250 ml Phosphate Buffered Saline (PBS) with 0.18% formalin and incubated overnight at 37 °C. The inactivated culture was centrifuged again (5000 X g for 10 min at 5 °C) and the pellet was resuspended in a final volume of 250 ml coating buffer (1.08 g $Na_2CO_3.10H_2O$, 0.968 g $NaHCO_3$, 0.25 l aqua ad iniectabilia 100 % w/v). F96 maxisorp Nunc-immuno plates (Nunc; Denmark) were coated with 140 µl formalin-inactivated *Salmonella* strains diluted in coating buffer to an optical density of 660 nm, measured using a spectrophotometer (Ultraspec III®), incubated for 24 h at 4 °C and washed three times with 100 µl wash buffer (0.6 g $NaH_2PO_4.2H_2O$, 5.6 g $NaH_2PO_4.12H_2O$, 0.5 ml Tween 20 (Merck, Germany), 12.5 g NaCl). Plates were stored at 4 °C until used. Before starting the assay, the plates were washed with 100 µl destillated water (AD) + 1% milk powder to prevent non-specific binding. A 1/2000 dilution of sera (100 µl) was added to the wells. The cut-off optical density was calculated as the mean obtained from the sera from a bacteriologically and serologically *Salmonella* free pig (the negative control, determined using the HerdChek ELISA) plus two times the standard deviation. All measurements were performed in triplicate.

2.4 Protective capacity of LPS mutant strains against *Salmonella* serovars

The protective capacity of the LPS deletion mutants was compared to that of the wild type strain using a mouse model. Five-week-old specified pathogen-free (SPF) BALB/c mice (Bio services, Janvier, France) were housed in filter-topped cages at 25 °C under natural day-night rhythm with *ad libitum* acces to feed and water and enriched with mouse houses and play tunnels. Bacterial inocula used for oral protection assays were prepared as follows. Strains

were grown overnight on a shaker at 37 °C in 100 ml LB broth. The bacteria were washed twice in PBS at 3500 X g for 15 min at room temperature and adjusted in PBS to the appropriate concentration of 2 x 10^7 colony forming units per ml (CFU/ml). The number of viable bacteria was determined by plating tenfold dilutions on BGA.

In a first experiment, 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 inoculated first via the orogastric route with 2 x 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. A control group of ten mice was sham-inoculated with sterile PBS. Four weeks after primary inoculation, all mice were challenged with a total of 10^8 CFU/ml of the virulent *Salmonella* Typhimurium strain NCTC12023Nal^{20} by the orogastric route.

In a second experiment, we tested whether truncation of the LPS chain in the Δ*rfaJ* strain promotes cross-immunity against other *Salmonella* serovars. Sixty mice were orally inoculated first with 2 x 10^7 CFU/ml of either the Δ*rfaJ* strain (n = 20) or *Salmonella* Typhimurium strain 112910a (n = 20). A control group of 20 mice was sham-inoculated with sterile PBS (n = 20). Sixteen days after primary inoculation, ten mice of each group were challenged with a total of 10^8 CFU/ml of either *Salmonella* Heidelberg strain 704Sa06 Nal^{20} (n = 10) or *Salmonella* Enteritidis strain SE147 Nal^{20} (n = 10).

In both *in vivo* experiments, mice were euthanized nine days post challenge. Tissue samples (spleen, liver and caecum) were examined quantitatively for the presence of the respective *Salmonella* strain. Samples were weighed and 10% (w/v) suspensions were made in buffered

peptone water (BPW; Oxoid, Basingstoke, UK) after which the material was homogenized with a stomacher. The homogenized samples were examined for the presence of *Salmonella* by plating 10-fold dilutions on BGA supplemented with nalidixic acid (BGA^{NAL}). If negative at direct plating, the samples were pre-enriched overnight in BPW at 37 °C, enriched overnight at 37 °C in tetrathionate broth and then plated on BGA^{NAL}. Samples that were negative after direct plating but positive after enrichment were presumed to contain 60 CFU per gram tissue (detection limit for direct plating). Samples that remained negative after enrichment were presumed to contain 0 CFU per gram tissue.

2.5 Immunization of piglets

In this study, we examined whether it was possible to discriminate between the serological response induced after immunization of pigs with the wild type and its isogenic Δ*rfaL* and Δ*rfaJ* strains. For this purpose, we immunized pigs with adjuvanted bacterins of either the wild type strain, the Δ*rfaL* strain or the Δ*rfaJ* strain to maximize antibody production [15]. Fourteen, 6-week-old, bacteriologically and serologically *Salmonella* negative piglets (commercial closed line based on Landrace) were housed together at 25 °C under natural day-night rhythm with *ad libitum* access to feed and water.

For preparation of antigen suspensions for immunization of pigs, strains were cultured for 9 hours at 37 °C in 400 ml LB broth and were adjusted to 5 x 10^8 CFU/ml. Inactivation was achieved by adding 0.18% (v/v) formalin (VWR international, Fontenay sous bois, France) overnight at 37 °C. The formalin-inactivated *Salmonella* strains were washed twice (5000 X g for 30 min at room temperature) and the resulting pellet was resuspended in 11 ml PBS with 0.18% formalin and incubated overnight. Thereafter, this suspension was mixed with 11 ml marcol oil (Esso Belgium nv, Antwerp, Belgium) containing 3.4% sterilized Tween 80 (Sigma Aldrich Chemie Gmbh, Steinheim, Germany) and 6.4% mannide monooleate (Sigma Aldrich Chemie Gmbh, Steinheim, Germany). To check sterility, all suspensions were

cultured on Columbia agar plates containing 5% sheep blood (COL; Oxoid, Wesel, Germany) and incubated aerobically and anaerobically overnight at 37 °C.

Piglets were randomly allocated to three vaccinated groups (n = 4) and one sham-vaccinated control group (n = 2). One and three weeks after their arrival, pigs were intramuscularly immunized with one of the formalin-inactivated *Salmonella* strains (either: *Salmonella* Typhimurium strain 112910a, Δ*rfaJ* or Δ*rfaL*) with Freund's incomplete adjuvant to elicit an optimal humoral (antibody-mediated/Th2) response [15]. The control group was injected with 1 ml of sterile PBS. Four weeks after the second immunization, the pigs were humanely euthanized and blood samples were taken from the vena jugularis externa, using a Venoject system (Terumo; Roma, Italia). All sera samples were examined for the presence of anti *Salmonella* Typhimurium antibodies using the Herdchek ELISA and the in-house *Salmonella* Typhimurium strain 112910a whole cell ELISA, prepared as described previously.

2.6 Experimental infection of piglets with *Salmonella* Typhimurium

To obtain sera from *Salmonella* Typhimurium infected piglets, an experimental infection was performed with 4 week-old bacteriologically and serologically *Salmonella* negative piglets (commercial closed line based on Landrace). Piglets were randomly allocated in one experimental group (n = 3) and one negative control group (n = 3) and both groups were housed in separate isolation units at 25°C under natural day-night rhythm with ad libitum access to feed and water. One week after their arrival at the facility, three experimental animals were orally inoculated with approximately 2 x 10^7 CFU/ml of a stationary phase culture of *Salmonella* Typhimurium strain 112910aNal^{20} in 2 ml Hank's buffered salt solution (HBSS; Gibco Life Technologies, Paisley, Scotland); the negative control group (n = 3) was sham-inoculated with 2 ml HBSS. The clinical condition of the pigs was monitored daily. Six weeks after oral inoculation, pigs were humanely euthanized and blood samples were taken from the vena jugularis externa, using a Venoject system (Terumo; Roma, Italia). All sera

samples were examined for the presence of anti *Salmonella* Typhimurium antibodies using the Herdchek ELISA and the in-house *Salmonella* Typhimurium strain 112910a whole cell ELISA, prepared as described previously.

2.7 Statistical analysis

In all experiments, statistical analysis was performed using a one-way ANOVA test (in case of homogeneity of variances), with posthoc Bonferroni corrections or a nonparametric Mann-Whitney-U-test (in case of non-homogeneity of variances), using the SPSS Statistics 17.0 software (SPSS Inc., Chicago, USA). ELISA results were analysed by a one-way ANOVA and Bonferroni corrections were applied. A *P-value* of < 0.05 was considered significant.

3. Results

3.1 Characterization of the LPS knock-out mutants of *Salmonella* Typhimurium

A systematic truncation of the LPS chain occurred as a result of defects in genes coding for glycosyl or phosphoryl transferases (or epimerases) and is shown in figure 1. LPS patterns obtained by standard SDS-polyacrylamide gel electrophoresis of *Salmonella* Typhimurium strain 112910a, the O-antigen mutant (Δ*rfbA*), the outer core mutants (Δ*rfaL,* Δ*rfaJ,* Δ*rfaI*) and the inner core mutants (Δ*rfaG*, Δ*rfaF*) are presented in figure 2 and show a visible loss of O-antigens for core mutants (Δ*rfaL,* Δ*rfaJ,* Δ*rfaI* , *rfaG*, Δ*rfaF*) compared to *Salmonella* Typhimurium strain 112910a. Loss of the *rfbA* gene resulted in the presence of a complete core without covalently bound O-antigen ("semirough" LPS), because the rfb locus is responsible for the biosynthesis of O-antigen [16]. The complete lack of O-antigens in core mutants was also confirmed by resistance to bacteriophage P22 and appearance of the "rough" phenotype. *Salmonella* Typhimurium strain 112910a showed the "wild-type" LPS structure and is denoted as "smooth" LPS [16].

A slide agglutination test was used to verify expression of O-antigens on the surface of *Salmonella* Typhimurium 112910a and its isogenic knock-out mutants. While *Salmonella*

Typhimurium strain 112910a showed a distinct agglutination within 60 seconds, little granular clumping was seen with the *rfbA* mutant strain. No agglutination was observed with Δ*rfaL*, Δ*rfaJ*, Δ*rfaI*, Δ*rfaG* and Δ*rfaF* strains, which confirmed a total loss of O_4 and O_{12} antigens.

Further, invasion of *Salmonella* Typhimurium strain 112910a and its isogenic knock-out strains was compared in an IPEC-J2 cell strain, using a gentamicin protection assay. The Δ*rfbA*, Δ*rfaG* and Δ*rfaF* strains showed a statistically significant decrease ($P < 0.05$) in invasion when compared to the 112910a strain, while the Δ*rfaL*, Δ*rfaJ* and Δ*rfaI* strains were not impaired in invasion. Results are summarized in figure 3.

3.2 Deletion of *rfaI, rfaG, rfaF* genes but not *rfaL* and *rfaJ* severely affects the protective capacity of *Salmonella* Typhimurium strain 112910a in BALB/c mice

Oral immunization of mice with *Salmonella* Typhimurium strain 112910a, Δ*rfbA*, Δ*rfaL* or Δ*rfaJ* strains induced a significant ($P < 0.05$) protection against subsequent challenge with NCTC12023Nal[20] in both spleen and liver compared to non immunized control animals. Bacterial counts (wild type, Δ*rfbA*, Δ*rfaL* and Δ*rfaJ*) in caecum samples showed a non significant ($P > 0.05$) reduction of the numbers of *Salmonella* Typhimurium compared with with control animals. Deletion of *rfaI, rfaG* and *rfaF* genes but not *rfbA*, *rfaL* and *rfaJ* genes thus significantly ($P < 0.05$) reduced protection against challenge with *Salmonella* Typhimurium strain NCTC12023Nal[20]. None of the animals immunized with Δ*rfbA*, Δ*rfaG* or Δ*rfaF* died as a result of vaccination, whereas eight mice vaccinated with either strain 112910a (n = 3), Δ*rfaL* (n = 2), Δ*rfaJ* (n = 2) or Δ*rfaI* (n = 1) died as a consequence of vaccination. After challenge, > 60% of the unvaccinated animals or mice vaccinated with either Δ*rfaG* or Δ*rfaF* died opposed to < 40% of mice vaccinated with wild type, Δ*rfaL* or Δ*rfaJ*. Results are illustrated in figure 4.

3.3 Immunization with the Δ*rfaJ* strain does not confer enhanced cross-protection against subsequent challenge with serovars Heidelberg and Enteritidis in BALB/c mice

In this experiment we determined to which extent the Δ*rfaJ* strain and *Salmonella* Typhimurium strain 112910a were able to confer cross-protection against *Salmonella* Heidelberg or *Salmonella* Enteritidis. Both strains were equally able to induce a significant ($P < 0.05$) reduction of *Salmonella* Heidelberg in the spleen compared to control animals. In liver and caecum both strains induced a noticeable, but non significant ($P > 0.05$) reduction of *Salmonella* Heidelberg compared to control animals.

Recovery of *Salmonella* Enteritidis was not significantly different ($P < 0.05$) in spleen, liver and caecum between animals immunized with *Salmonella* Typhimurium strain 112910a and mice immunized with Δ*rfaJ*. Results are shown in figure 5.

3.4 Immunological responses in pigs

IDEXX ELISA No significant seroconversion ($P > 0.05$) was noticed in pigs immunized with inactivated Δ*rfaJ* or Δ*rfaL* strains and in control animals (non immunized and non infected animals). Conversely, marked seroconversion occurred in animals immunized or orally infected with the inactivated *Salmonella* Typhimurium strain 112910a. Statistical analysis showed a significant difference ($P < 0.05$) between the antibody response against *Salmonella* Typhimurium LPS in pigs infected with *Salmonella* Typhimurium 112910a and control animals. Results are shown in figure 6. 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.

Whole-cell ELISA Significant anti-*Salmonella*-antibody titers were detected in the serum of all immunized and infected animals. No significant distinction ($P > 0.05$) regarding *Salmonella*-specific antibody responses could be made between animals that were immunized

with the inactivated 112910a strain and those immunized with the inactivated Δ*rfaJ* and Δ*rfaL* strains. Results are shown in figure 6.

4. Discussion

Marker 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 [17]. 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. Deletion of LPS genes, however, has some consequences: LPS represent the main surface antigens of Gram-negative bacteria (O-antigens) and harbour binding-sites for antibodies [18]. Therefore, LPS are important in the recognition and the elimination of bacteria by the host's immune system [19]. Truncation of LPS may lead to over-attenuated strains that are not able to fully colonize their host and therefore no longer elicit a sufficient protective immune response [20]. Possibly smooth LPS are indispensable for the early steps of the infection process [21] and contribute to invasiveness [22]. Data on LPS and invasion are often unclear and sometimes contradictory [22]. Our results on invasiveness of *Salmonella* Typhimurium strain 112910a and its isogenic LPS mutants, illustrate that the Δ*rfbA*, Δ*rfaG* and Δ*rfaF* strains were less able to invade IPEC-J2 cells, which might indicate that these strain are less able to colonize their host and therefore are no longer able to elicit a protective immune response. In a mouse *in vivo* experiment we showed that the *rfaG* and *rfaF* mutant strains were indeed 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.

Cross-protection against other enterobacterial pathogens induced by 'rough' mutants is sometimes explained by better accessibility of less immune-potent molecules, such as lipid A and core antigens [21] [23] [24]. Hence, truncation of LPS might confer enhanced cross protection to other serovars. Therefore, we used the Δ*rfaJ* strain to conduct a cross-protection study. The finding that smooth strains are less capable of inducing a cross-protection against other *Salmonella* serovars could not be confirmed in this study. The wild type and the Δ*rfaJ* deficient strains were equally able to provoke cross-protection against *Salmonella* Heidelberg, whereas only the wild type strain was able to protect against a challenge with *Salmonella* Enteritidis.

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.

In conclusion, we proved that immunization with *Salmonella* Typhimurium strain 112910a and its isogenic mutant strains: Δ*rfaL* and Δ*rfaJ*, is equally able to provoke protection against a virulent *Salmonella* Typhimurium strain. In addition, deletion of the *rfaL* or the *rfaJ* genes can be used as DIVA markers in current *Salmonella* serosurveillance programmes based on the detection of antibodies against LPS of *Salmonella*.

Acknowledgements

The technical assistance of Nathalie Van Rysselberghe and Rosalie Devloo is greatly appreciated. This work was supported by the Federal Public Service for Health, Food chain safety and Environment (FOD), Brussels, Belgium: project code RT/ 09/5 SALMOSU and the Bijzonder Onderzoeksfonds (BOF): starTT project IOF 09/StarTT/020.

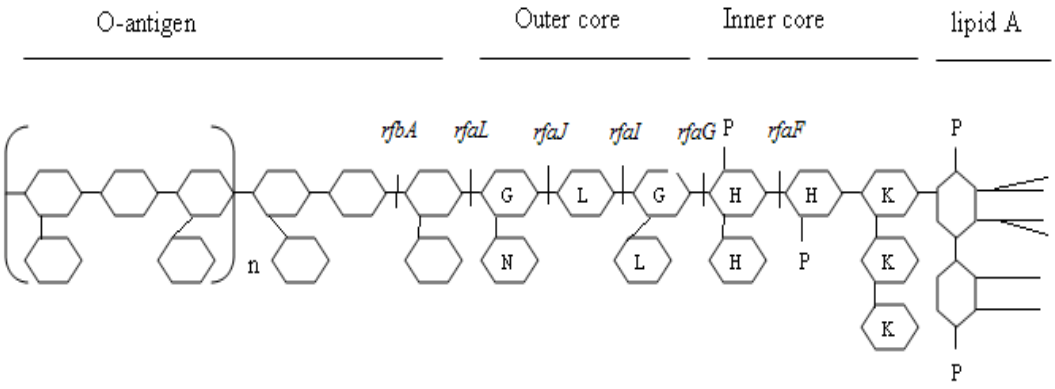
Figures

Strain	**Genotype and O-antigens**	**Product of the deleted gene**	**Source or reference**
WT	*Salmonella* Typhimurium 112910a (O: 1, 4, 12)	no deletions	[4]
NCTC12023Nal^{20}	*Salmonella* Typhimurium NCTC 12023 Nal^{20} (O: 1. 4, 12)	no deletions	[25]
Δ*rfaL*	*Salmonella* Typhimurium 112910a Δ*rfaL*	*Salmonella* Typhimurium O-antigen ligase	This study
Δ*rfaJ*	*Salmonella* Typhimurium 112910a Δ*rfaJ*	LPS 1,2-glucosyltransferase	This study
Δ*rfaI*	*Salmonella* Typhimurium 112910a Δ*rfaI*	LPS 1,3-galactosyltransferase	This study
Δ*rfaG*	*Salmonella* Typhimurium 112910a Δ*rfaG*	LPS core biosynthesis protein	This study
Δ*rfaF*	*Salmonella* Typhimurium 112910a Δ*rfaF*	LPS heptosyltransferase II	This study
Salmonella Enteritidis	*Salmonella* Enteritidis Nal^{20} (O: 1, 9, 12)	no deletions	This study
Salmonella Heidelberg	*Salmonella* Heidelberg Nal^{20} (O: 1, 4, 5, 12)	no deletions	This study
Δ*rfbA*	*Salmonella* Typhimurium 112910a Δ*rfbA*	glucose-1-phosphate thymidylyltransferase	This study
WT Nal^{20}	*Salmonella* Typhimurium 112910a Nal^{20} (O: 1, 4, 12)	no deletions	[4]

Table 1A: Strains used in this study

Primers	Sequences
rfbA forward	5'- TAATAAATTTAAATGCCCATCAGGGCATTTTCTATGAATGAGAAATGGAATGTGTAGGCTGGAGCTGCTTC - 3'
rfbA reverse	5'- GGCTCTAAGATCAAGACATCTGGTATTGCTGTTTTAATCACAATCACCATATGAATATCCTCCTTAG -3'
rfaL forward	5'- CATTAAAGAGACTCTGTCTCATCCCAAACCTATTGTGGAGAAAAGTGTGTAGGCTGGAGCTGCTTC - 3'
rfaL reverse	5' - TTGAGTCCTGATGATGGAAAACGCGCTGATACCGTCATATGAATATCCTCCTTAG - 3'
rfaJ forward	5' - ATAGCCTACTTTAAACGTAAACTTCTTGAATAAAACCCATAGGTGATGTATGTGTAGGCTGGAGCTGCTTC - 3'
rfaJ reverse	5' - AGTTTTTAATCTTTTTTTCAATAATCATAATGGAGATTTAGGGAGGGGAACATATGAATATCCTCCTTAG -3'
rfaI forward	5' - TTTAAAAATTTTAATAATGCAATATTCTCGAAATTACAAAAGTGATCACTTGTGTAGGCTGGAGCTGCTTC - 3'
rfaI reverse	5' - TTCAGCTATTTCTATCTCAGGAAATGAATCCATTACATCACCTATGGGTTCATATGAATATCCTCCTTAG - 3'
rfaG forward	5'- GAAAAAATGCTGCCGCATGAGGCACGCACCATAGATTTGGACAGCCTGCTTGTGTAGGCTGGAGCTGCTTC - 3'
rfaG reverse	5' - CCTCAAAAGCATCTTTACCGCGCCATAGTGTGGTTAACGGCGCTTTCAGCCATATGAATATCCTCCTTAG -3'
rfaF forward	5' - GCCGAAGGCGTCACGGAGTATATGGCCTGGCTGAACCGCGACGCGTAAGTTGTGTAGGCTGGAGCTGCTTC - 3'
rfaF reverse	5' – GGTATGTAATACGTCGCCCATCGATGATGTTTTAACGATCAAAACCCGCACATATGAATATCCTCCTTAG – 3'

359 Table 1B: Primers used in this study



360

361 Figure 1: Schematic representation of the structure of lipopolysaccharide (LPS). Truncation of the LPS chain as
362 a consequence of Δ*rfbA*, Δ*rfaL,* Δ*rfaJ,* Δ*rfaI,* Δ*rfaG* and Δ*rfaF* deletions is shown (G: glucose, L: galactose, H:
363 heptose, K: 2-keto 3-deoxy-octulosonate (KDO), N: N-acetylglucosamine).

364
365

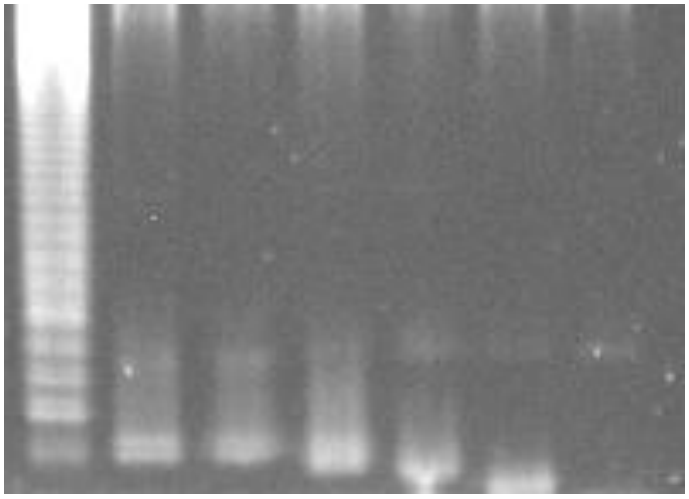


Figure 2: SDS-polyacrylamide gel electrophoresis patterns of LPS of *Salmonella* Typhimurium 112910a (lane 1) and Δ*rfbA* (lane 2), Δ*rfaL* (lane 3), Δ*rfaJ* (lane 4), Δ*rfaI* (lane 5), Δ*rfaG* (lane 6) and Δ*rfaF* (lane 7) mutants is shown. Apart from *Salmonella* Typhimurium strain 112910a (lane 1) all strains show a classical 'rough' type ladder pattern. Staining occurred with fluorescent staining and a ten-fold dilution of 25µg/ml LPS of each strain was loaded.

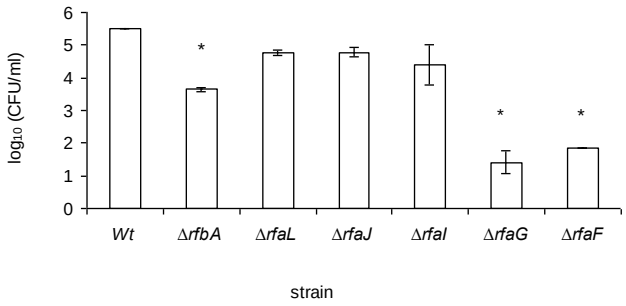


Figure 3: The invasiveness of *Salmonella* Typhimurium and its isogenic knock-out mutants in IPEC-J2 cells. The log values of the number of gentamicin protected bacteria are shown. The results represent the means of three independent experiments conducted in triplicate and standard deviations are given. An asterisk refers to a significantly lower invasion compared to the wild type strain ($P < 0.05$).

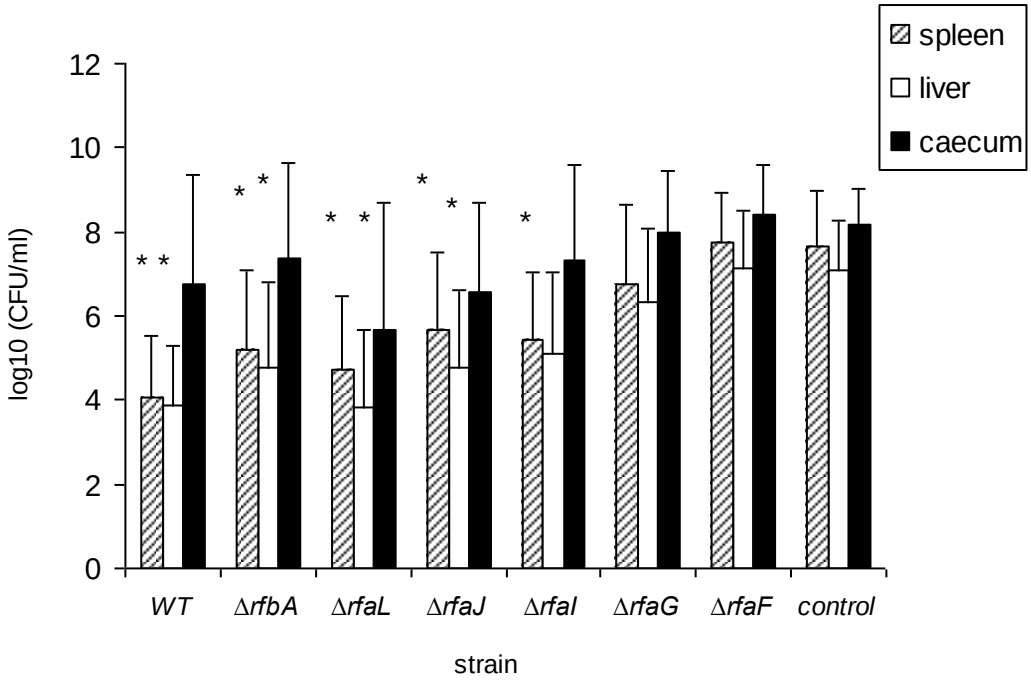


Figure 4: Recovery of *Salmonella* bacteria from various organs of mice immunized with either *Salmonella* Typhimurium, one of its isogenic LPS mutants or non immunized control animals and subsequently challenged with *Salmonella* Typhimurium strain NCTC12023Nal[20]. The $\log_{10}$ value of the ratio of CFU per gram sample and standard deviations are given. An asterisk refers to a significant difference with the control group ($P < 0.05$).

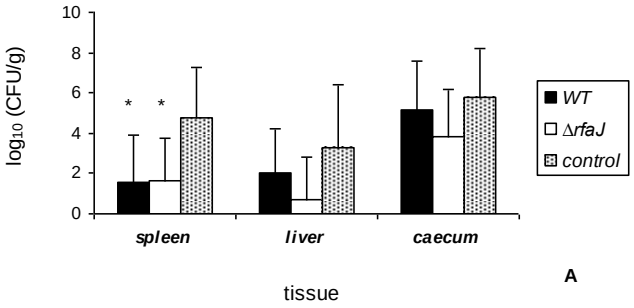


Figure 5A: Recovery of *Salmonella* Heidelberg bacteria from various organs of BALB/c mice immunized with *Salmonella* Typhimurium, one of its isogenic LPS mutants or uninfected animals subsequently challenged with *Salmonella* Heidelberg. The $\log_{10}$ average value of the number of CFU per gram sample is given with its standard deviation. An asterisk refers to a significant difference with the control group ($P < 0.05$).

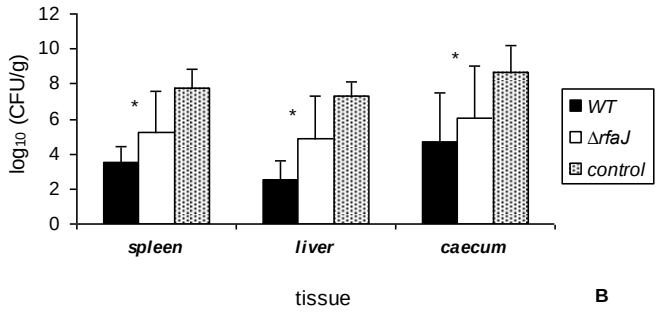


Figure 5B: Recovery of *Salmonella* Enteritidis bacteria from various organs of BALB/c mice immunized with *Salmonella* Typhimurium, one of its isogenic LPS mutants or uninfected animals subsequently challenged with *Salmonella* Enteritidis. The $\log_{10}$ average value of the number of CFU per gram sample is given with its standard deviation. An asterisk refers to a significant difference with the control group ($P < 0.05$).

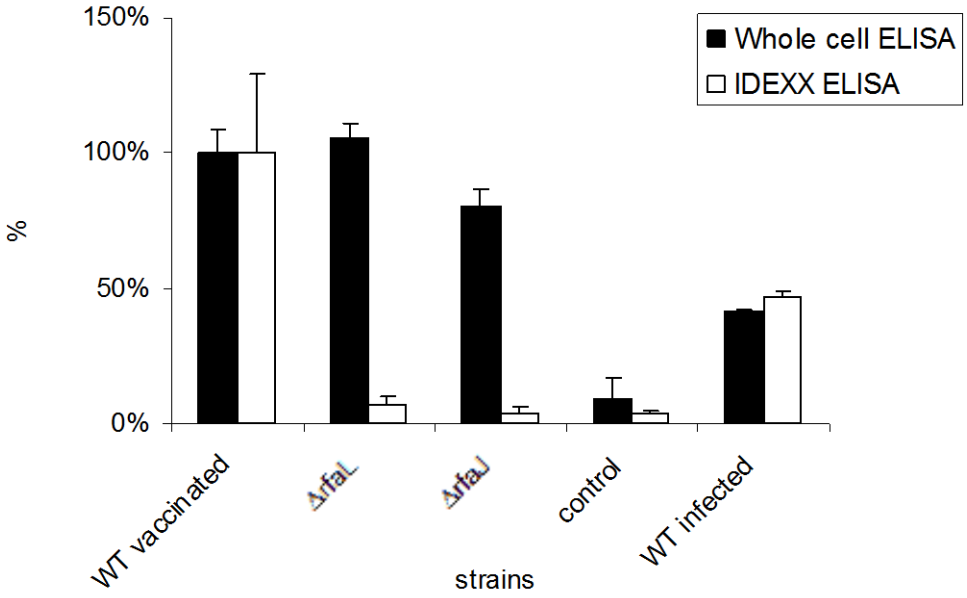


Figure 6: Serological results of pigs immunized with Δ*rfaL*, Δ*rfaJ* or *Salmonella* Typhimurium strain 112910a, control pigs (animals that were not immunized and not infected) and pigs infected with S*almonella* Typhimurium strain 112910a Nal^{20}. Values are represented as a percentage compared to the wild type immunized group.

References

[1] Boyen F, Pasmans F, Van Immerseel F, Morgan E, Botteldoorn N, Heyndrickx M, et al. A limited role for SsrA/B in persistent *Salmonella* Typhimurium infections in pigs. Vet Microbiol 2008 Apr 30;128(3-4):364-73.

[2] Majowicz S.E., Musto J., Scallan E., Angulo F.J., Kirk M., O'Brien S.J., Jones T.F., Fazil A., Hoekstra R.M., The global burden of nontyphoidal *Salmonella* gastroenteritis, Clin. Infect. Dis. (2010) 50:882-889.

[3] Boyen F, Haesebrouck F, Maes D, Van Immerseel F, Ducatelle R, Pasmans F. Non-typhoidal *Salmonella* infections in pigs: A closer look at epidemiology, pathogenesis and control. Vet Microbiol 130 (2008) 1-19.

[4] Boyen F, Pasmans F, Van Immerseel F, Donne E, Morgan E, Ducatelle R, et al. Porcine *in vitro* and *in vivo* models to assess the virulence of *Salmonella enterica* serovar Typhimurium for pigs. Lab Anim 2009 Jan; 43(1):46-52.

[5] Van Immerseel F, Boyen F, Gantois I, Timbermont L, Bohez L, Pasmans F, Haesebrouck F, Ducatelle R. Supplementation of coated butyric acid in the feed reduces colonization and shedding of *Salmonella* in poultry. Poultry Science Association, Inc. June 9, 2005.

[6] Collard JM, Bertrand S, Dierick K, Godard C, Wildemauwe C, Vermeersch K, et al. Drastic decrease of *Salmonella* Enteritidis isolated from humans in Belgium in 2005, shift in phage types and influence on foodborne outbreaks. Epidemiol Infect 2008 Jun;136(6):771-81.

[7] Gantois I, Ducatelle R, Timbermont L, Boyen F, Bohez L, Haesebrouck F, et al. Oral immunisation of laying hens with the live vaccine strains of TAD *Salmonella* vac E and TAD *Salmonella* vac T reduces internal egg contamination with *Salmonella* Enteritidis. Vaccine 2006 Sep 11;24(37-39):6250-5.

[8] Lindner T, Springer S, Selbitz HJ. The use of a *Salmonella* Typhimurium live vaccine to control *Salmonella* Typhimurium in fattening pigs in field and effects on serological surveillance. Safepork 2007 – Verona (Italy).

[9] Selke M, Meens J, Springer S, Frank R, Gerlach GF. Immunization of pigs to prevent disease in humans: Construction and protective efficacy of a *Salmonella enterica* serovar Typhimurium live negative-marker vaccine. Infection and immunity 2007 May; 2476-2483.

[10] Eddicks M, Palzer A, Hormansdorfer S, Ritzmann M, Heinritzi K. Examination of the compatibility of a *Salmonella* Typhimurium-live vaccine *Salmoporc*® for three day old suckling piglets. Dtsch Tierärztl Wochenschr 116, 249-254 (2009).

[11] Cortinas Abrahantes J, Bollaerts K, Aerts M, Ogunsanya V, Van der Stede Y. *Salmonella* serosurveillance: different statistical methods to categorise pig herds based on serological data. Prev Vet Med 2009 May 1; 89 (1-2): 59-66.

[12] Boyen F, Pasmans F, Donne E, Van Immerseel F, Adriaensen C, Hernalsteens JP, *et al.* Role of SPI-1 in the interactions of *Salmonella* Typhimurium with porcine macrophages. Vet Microbiol 2006 Mar 10;113(1-2):35-44.

[13] Boyen F, Pasmans F, Van Immerseel F, Morgan E, Adriaensen C, Hernalsteens JP, *et al.* *Salmonella* Typhimurium SPI-1 genes promote intestinal but not tonsillar colonization in pigs. Microbes Infect. 2006 Nov-Dec; 8(14-15):2899-907.

[14] Farzan A, Friendship RM, Dewey CE. Evaluation of enzyme-linked immunosorbent assay (ELISA) tests and culture for determining *Salmonella* status of a pig herd. Epidemiol Infect 2007 Feb;135(2):238-44.

[15] Nichols EF, Madera L, Hancock REW. Immunomodulators as adjuvants for vaccines and antimicrobial therapy. Ann.N.Y.Acad.Sci. (2010) 1-16

[16] Hitchcock PJ, Leive L, Makela PH, Rietschel ET, Strittmatter W, Morrison DC. Lipopolysaccharide nomenclature: past, present, and future. J Bacteriol 1986 Jun;166(3):699-705.

[17] Michael JD. Clinical Immunology of the Dog and the Cat. Second edition. Copyright 2008 Manson publishing/The Veterinary press. ISBN: 978-1-84076-098-9.

[18] Van Amersfoort E.S., Van Berkel T.J.C., Kuiper J. Receptors, mediators and mechanisms involved in bacterial sepsis and spetic shock. Clinical microbiology reviews, July 2003, p. 379-414.

[19] Morrison DC, Ryan JL. Bacterial endotoxic lipopolysaccharides. Volume I: Molecular Biochemistry and Cellular Biology. CrC press.

[20] Karasova D, Sebkova A, Vrbas V, Havlickova H, Sisak F, Rychlik I. Comparative analysis of *Salmonella enterica* serovar Enteritidis mutants with a vaccine potential. Vaccine 2009.

[21] Nagy G, Pal T. Lipopolysaccharide: a tool and target in enterobacterial vaccine development. Biol Chem 2008 Mar 6.

[22] Martin G, Chart H, Threlfall E, Morgan E, Lodge J, Brown N, Stephen J. Invasiveness of *Salmonella* serotypes Typhimurium and Enteritidis of human gastro-enteritic origin for rabbit ileum: role of LPS, plasmids and host factors. J.Med.Microbiol. – Vol. 49 (200), 1011-1021.

[23] Nagy G, Palkovics T, Otto A, Kusch H,Kocsis B, Dobrindt U, Engelmann S, Hecker M, Emödy L, Pál T, Hacker J. Gently rough: the vaccine potential of a *Salmonella* enterica regulatory lipopolysaccharide mutant. J Infect Dis 2008 Dec 1; 198 (11): 1699-706.

[24] Nagy G, Danino V, Dobrindt U, Pallen M, Chaudhuri R, Emödy L, Hinton J C, Hacker J. Down-regulation of key virulence factors makes the *Salmonella enterica* serovar Typhimurium *rfaH* mutant a promising live-attenuated vaccine candidate. Infection and immunity 2006 Oct. p. 5914-5925.

[25] Hensel M, Shea JE, Gleeson C, Jones MD, Dalton E, Holden DW. Simultaneous identification of bacterial virulence genes by negative selection. Science 1995 Jul 21;269(5222):400-3.