



Impact of Typhimurium DT104 Virulence Factors and on the onset, clinical course, colonization patterns and immune response of porcine salmonellosis

Steffi Brumme, Thorsten Arnold, Haukur Sigmarsson, Jörg Lehmann, Holger C. Scholz, Wolf-Dietrich Hardt, Andreas Hensel, Uwe Truyen, Uwe Roesler

► To cite this version:

Steffi Brumme, Thorsten Arnold, Haukur Sigmarsson, Jörg Lehmann, Holger C. Scholz, et al.. Impact of Typhimurium DT104 Virulence Factors and on the onset, clinical course, colonization patterns and immune response of porcine salmonellosis. *Veterinary Microbiology*, 2007, 124 (3-4), pp.274. <10.1016/j.vetmic.2007.04.032>. <hal-00532254>

HAL Id: hal-00532254

<https://hal.science/hal-00532254v1>

Submitted on 4 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Accepted Manuscript

Title: Impact of *Salmonella* Typhimurium DT104 Virulence Factors *invC* and *sseD* on the onset, clinical course, colonization patterns and immune response of porcine salmonellosis

Authors: Steffi Brumme, Thorsten Arnold, Haukur Sigmarsson, Jörg Lehmann, Holger C. Scholz, Wolf-Dietrich Hardt, Andreas Hensel, Uwe Truyen, Uwe Roesler

PII: S0378-1135(07)00214-3
DOI: doi:10.1016/j.vetmic.2007.04.032
Reference: VETMIC 3674

To appear in: *VETMIC*

Received date: 16-3-2007
Revised date: 22-4-2007
Accepted date: 25-4-2007

Please cite this article as: Brumme, S., Arnold, T., Sigmarsson, H., Lehmann, J., Scholz, H.C., Hardt, W.-D., Hensel, A., Truyen, U., Roesler, U., Impact of *Salmonella* Typhimurium DT104 Virulence Factors *invC* and *sseD* on the onset, clinical course, colonization patterns and immune response of porcine salmonellosis, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.04.032

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



1 IMPACT OF *SALMONELLA* TYPHIMURIUM DT104 VIRULENCE
 2 FACTORS *invC* AND *sseD* ON THE ONSET, CLINICAL COURSE,
 3 COLONIZATION PATTERNS AND IMMUNE RESPONSE OF PORCINE
 4 SALMONELLOSIS.

5
 6 Steffi Brumme^{a,b}, Thorsten Arnold^a, Haukur Sigmarsson^a, Jörg Lehmann^c, Holger C.
 7 Scholz^{a,d}, Wolf-Dietrich Hardt^e, Andreas Hensel^{a,f}, Uwe Truyen^a, and Uwe Roesler^{*,a}

8
 9 ^aInstitute of Animal Hygiene and Veterinary Public Health, University Leipzig,
 10 Leipzig, Germany.

11 ^bImpfstoffwerk Dessau-Tornau GmbH, Rodleben, Germany.

12 ^cFraunhofer Institute for Cell Therapy and Immunology, Leipzig, Germany.

13 ^dArmed Forces Institute of Microbiology, Munich, Germany.

14 ^eInstitute of Microbiology, Swiss Federal Institute of Technology, Zürich, Switzerland.

15 ^fFederal Institute for Risk Assessment, Berlin, Germany.

16
 17
 18 **Running title:** Impact of mutations of *Salmonella* *invC* and *sseD* at pigs

19
 20 ***Corresponding author:**

21 Uwe Roesler
 22 Institute of Animal Hygiene and Veterinary Public Health
 23 An den Tierkliniken 1
 24 04103 Leipzig
 25 Germany
 26 Phone: +49 (0) 341 9738153
 27 Fax: +49 (0) 341 9738198
 28 Email: roesler@vetmed.uni-leipzig.de

ABSTRACT

The present study was conducted to study the impact of the virulence factors *invC* and *sseD* of the two type III secretion systems of *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) on the pathogenesis of the porcine *S. Typhimurium* DT104-infection. For this purpose, two *S. Typhimurium* mutant strains with a disrupted *invC* gene of the *Salmonella* pathogenicity island 1 or with a disrupted *sseD*-gene of the *Salmonella* pathogenicity island 2 have been studied in experimental infection of pigs.

Twenty two 7-week-old male hybrid pigs were either infected with one of the mutants or the wild-type *S. Typhimurium* DT104 strain. Each group was examined for clinical signs, *Salmonella* shedding rate and the specific antibody response. Survival and replication were evaluated by qualitative and quantitative determination of the colonisation rate. The humoral and cellular immune responses were examined using isotype-specific ELISA and quantitative real-time PCR of IL-2, IL-4, IL-10, IL-12 and IFN- γ .

The results proved that both mutants had a lower virulence (with marked differences between both mutants) than the wild type and that both virulence factors have importance in porcine salmonellosis. Only pigs infected with the wildtype *S. Typhimurium* DT104 exhibited typical clinical symptoms of salmonellosis like anorexia, vomiting, disturbed demeanour, fever and diarrhoea. Deletion of the *invC* gene resulted in a significantly reduced colonisation rate. Interestingly, the mRNA expression of both type-1 and type-2 cytokines were significantly decreased in pigs infected with either the *invC*-mutant and the *sseD* –mutant strain.

KEYWORDS: *Salmonella Typhimurium*; *invC*; *sseD*; Salmonellosis; pig; swine; immune response; ELISA; Cytokines; Real time PCR

INTRODUCTION

Salmonella enterica serovar Typhimurium (*S. Typhimurium*) is the most common *Salmonella* serotype in pigs and a leading cause of food poisoning in humans. In Europe the prevalence of *S. Typhimurium* phage type DT104 has steadily increased.

Salmonella enterica is a facultative intracellular bacterium capable of surviving within phagosomes of macrophages and dendritic cells (Rathman et al. 1997). Previous studies indicate that survival of *S. Typhimurium* within macrophages correlates with the virulence in mice (Alpuche-Aranda et al. 1995). The most important virulence factors are encoded within different *Salmonella* pathogenicity islands (SPI). SPI-1 is responsible for the membrane ruffling, the invasion of epithelial cells and mediates intestinal pathogenesis (Galan 1999; Barthel et al. 2003). SPI-1 mutants (e.g. sipB mutant) were shown to be significantly impaired to invade the intestinal, but not the tonsillar tissue (Boyen et al. 2006). Furthermore, *in-vitro* studies indicate that SPI-1 is responsible for apoptosis of macrophages, stimulation of chloride secretion and the salmonellae induced Interleukin (IL)-8 production (Lundberg et al. 1999).

SPI-2 is required for survival and replication within the host cells, for systemic spread and for enterocolitis (Bispham et al. 2001; Coombes et al. 2005; Hapfelmeier et al. 2005). Moreover, SPI-2 seems to be responsible for the delayed apoptosis of macrophages within the first 18-24 h of *Salmonella* infection (van der Velden et al. 2000; Monack et al. 2001). Some genes of SPI-1 and SPI-2 encode two different highly conserved type III secretion systems (TTSS) which translocate bacterial effector proteins into the host cell's cytosol to modulate signal transduction pathways within host cells (Galan 1999). Two key components of these TTSS's are *invC* and *sseD*. The *invC* gene, a member of the *invABC* operon encodes a structural component of the TTSS from SPI-1 with a significant ATPase activity. Thus, it is probably an

energy provider for the TTSS (Eichelberg et al. 1994; Akeda & Galan 2004). The *sseD* gene encodes a putative translocon pore subunit of the surface-localized translocon module of the SPI-2 that seems to be important for intracellular survival within macrophages (Nikolaus et al. 2001; Klein & Jones 2001; Zurawski & Stein 2003). In mice and calves *invC* and *sseD* mutants of *S. Typhimurium* were strongly attenuated indicating an important impact of these two virulence factors (Bispham et al. 2001; Coombes et al. 2005; Hapfelmeier et al. 2005).

For efficient control of *salmonellosis* and for better understanding of *Salmonella* infections it is essential to get more detailed information on the virulence factors of salmonellae and on the specific cytokine response during a *Salmonella* infection. As the current knowledge of salmonellosis and of *Salmonella*-induced immunity is predominantly based on experimental infections of mice, further studies should focus on other natural hosts of *S. Typhimurium*, e.g. pigs.

Therefore, the aim of the present study was to analyse the impact of two virulence factors, *invC* and *sseD*, of *S. Typhimurium* DT104 in experimental porcine salmonellosis. Besides analysing different clinical, microbiological and serological parameters, we also established quantitative real-time PCR assays for the determination of interleukin-2, interleukin-4, interleukin-10, interleukin-12, and interferon- γ .

MATERIALS AND METHODS

Bacterial strains and culture conditions. A highly virulent, penta-resistant isolate of *S. Typhimurium* DT 104 originally isolated from a clinically diseased pig was used as wild-type strain. Both mutants, *S. Typhimurium invC::aphT* M810 and the *S. Typhimurium sseD::aphT* M809 have been derived from this wild-type strain.

All *Salmonella* strains were cultured in Luria Bertani (LB) broth to mid-log-arithmetic growth phase in an orbital incubator at 37°C and subsequently pelleted and washed once in PBS by centrifugation (2000 x g for 10 min). Bacteria were then counted and adjusted in ice-cold phosphate buffered saline (PBS; pH 7.2) to a final cell number of 2×10^9 cfu/ml.

Construction of *invC::aphT* and *sseD::aphT* deletion mutants. The deletion mutants of *invC* and *sseD* were constructed as described by Eichelberg (1994). The SPI-1 and SPI-2 mutants were constructed by P22-transduction of the *invC::aphT* cassette from the *Salmonella* strain SB566 (Eichelberg et al. 1994) or *sseD::aphT* from *Salmonella* strain MvP101 (Medina et al. 1999), respectively.

Experimental design. Twenty-two 7-weeks-old male hybrid piglets (Landrace x Pietrain), tested negative for *Salmonella* by faeces culture and ELISA (SALMOTYPE® Pig STM-WCE, Labor Diagnostik Leipzig, Leipzig, Germany), were divided into three groups. The first group (n=6) was infected with the highly virulent wild-type strain *S. Typhimurium* DT104, originating from a clinical case of salmonellosis, the second group (n=8) and the third group (n=8) received the *invC::aphT* and the *sseD::aphT* mutant, respectively.

All groups were housed in air-conditioned high-security pens. Food and water were provided *ad libitum*. The pigs of the three infection-groups were kept and handled separately. All animal experiments were performed according to the German law of animal welfare (Regierungspräsidium Leipzig; RN 24-9168.11).

After one week of adaptation the pigs were infected orally with the respective *S. Typhimurium* DT104 strain. Feed was withdrawn 20 h before infection. Pigs were sedated with 1 mg/kg i.m. Azaperon and fixed in a hammock in ventral position. Fifty millilitres of bacterial suspension (2×10^9 cfu/ml, 1×10^{11} salmonellae total inoculum per pig) were administered via a gastric tube (Roesler et al. 2004).

1

2 **Clinical signs.** Subsequent to infection, clinical signs including general demeanour,
 3 respiration, coughing, vomiting, ingestion, diarrhoea and rectal body temperatures, were
 4 monitored daily after infection for each pig. Clinical signs were graded into four levels with
 5 0 = no symptoms, 1 = mild symptoms, 2 = medium symptoms and 3 = severe symptoms.
 6 Body weight was measured every third day for each pig.

7

8

9 **Bacteriological examinations.** After infection, the shedding rate of salmonellae in faeces of
 10 each pig was determined daily for one week post infection. Later on, faecal samples were
 11 collected and investigated every third day till day 21.

12 All pigs were euthanized by bleeding under anaesthesia at day 21 post infection (p.i.). Body
 13 cavities were carefully opened to prevent contamination of internal organs by intestinal
 14 content. The tissue samples were taken aseptically, using sterile instruments and bags.
 15 Samples of 5 g from the quadriceps muscle, liver, spleen, lung, tonsils, ileum, jejunum,
 16 caecum, colon, mandibular lymph node, lung (bronchial) lymph node, jejunal lymph node,
 17 ileocolic lymph node, colic lymph node and bile fluid were collected for qualitative and
 18 quantitative bacteriology. Each tissue sample was dipped in ethanol (95 %), flamed and
 19 minced in a sterile bag. Then, peptone water (45 ml) was added and each sample was
 20 homogenized using a stomacher for 1 min at highest speed. The Stomacher bags with contents
 21 were incubated for 24 h at 37 °C, 100 µl of the pre-enrichment suspensions were then added
 22 to 9.9 ml RV-bouillon and incubated for 24 h at 42 °C under aerobic conditions. Finally, the
 23 enrichment broth was streaked in parallel onto XLD-plates and BPLS-plates and incubated for
 24 further 24 h at 37 °C under aerobic conditions. Colonies identified as *Salmonella* spp. were
 25 further characterized by agglutination using polyclonal antisera (Sifin, Berlin Germany).

For quantitative bacteriology, 25 µl of the homogenized tissue suspension was used. A 10-fold serial dilution in PBS was performed from each tissue in microtiter plates, and 25 µl of each dilution was transferred in triplicates on XLD and BPLS agar plates. All plates were incubated for 24 h at 37 °C under aerobic conditions.

Polymerase chain reaction. The *S. Typhimurium* isolates from the faeces and tissue samples were further characterized by a mutant-specific PCR. Primer sets for *invC::aphT* (5'- gcc ggc gaa caa tag act gct t -3' and 3'-ttt ggt atg tga cct ctg cta tct-5') and *sseD::aphT* (5'- caa ccg agg gcg cca gtc aga -3' and 3'- t cag tcc aag taa gcg ccc tat -5') yielded 2.5 and 1.8 kbase pairs. The amplification was performed in a thermocycler „Tpersonal“ (Biometra, Göttingen, Germany). The conditions for the PCR were an initial denaturation at 94 °C for 5 min followed by 35 cycles each of 40 s denaturation at 94 °C, 40 s annealing at 60 °C, and 90 s extension at 72 °C and a final extension step of 5 min at 72 °C. Ten microlitres of each PCR product were separated on a 2 % agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) by electrophoresis. Amplicons were stained with ethidium bromide (0.5 g/ml) visualized with the Bio-Rad Gel Doc 2000 imager system (Bio-Rad, München, Germany).

Serological examinations. The isotype-specific systemic antibody response was evaluated using the “SALMOTYPE® Pig STM-WCE ELISA (Labor Diagnostik Leipzig). All incubations and washing steps were done as described by the supplier. Antibody activities were expressed as ELISA units per ml (EU/ml) using the positive-reference-standard method (Wright et al. 1993). The enzymatic reactions were developed with a TMB-based chromogen and measured by a computer-controlled photometer at 450 nm (Multiscan Ascent®, Thermo

Electron, McLean, USA). The data were analysed using the software Salmosoft™ (Labor Diagnostik Leipzig).

Cytokine quantification. EDTA blood samples were taken at day 0, 7, 14, and 21 after infection. The mononucleated cells were purified by density gradient centrifugation with Bicoll® Separating Solution (Biochrom, Berlin, Germany) and subsequently, RNA was extracted by a silica-based procedure according to the manufacturer's instructions (Qiagen RNeasy Kit™; Qiagen, Hilden, Germany). First strand cDNA was synthesized using the Taq® Reverse Transcription Reagents (Applied Biosystems, Weiterstadt, Germany) with random hexamer oligonucleotides.

For relative quantification of the respective cytokine cDNA we developed duplex real-time PCR assays. To normalize the results of the cytokine quantification the housekeeping gene for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used. Primers and probes (Table 1) for pig IL-2, IL-4, IL-10, IL-12, IFN-γ and GAPDH were provided by biomers Ltd. (biomers.net, Ulm, Germany). The probes were dually labelled with a reporter dye (HEX for the targets or FAM for GAPDH) at the 5' end and the black hole quencher 1 (BHQ 1) at the 3' end. All templates were examined in triplicates and no-template controls (NTC) were additionally included.

The PCRs were performed on a MX 3000P® thermal cycler (Stratagene, West Cedar Creek, USA) by means of TaqMan®-probes. The *ex vivo* real-time amplification for the cytokine quantification of IL-2, IL-4, IL-10, IL-12 and IFN-γ was carried out in a total volume of 25 µl in triplicates. The two-step PCR reactions were cycled 42-times after initial denaturation (95 °C, 15 min) with the following parameters: denaturation at 95 °C for 30 s and annealing (and extension) at 58 °C for 1 min.

The real-time PCR efficiencies of the duplex PCRs (target gene and GAPDH) were determined for each primer/probe set from standard curves generated from serial dilutions of a

cDNAs. The resulting standard curves always revealed linearity and efficiencies of 96.3 % for IFN- γ , 92.4 % for IL-2, 92.3 % for IL-4, 94.2 % for IL-10 and 96.8 % for IL-12.

The results of the PCR were calculated by the comparative C_T method (Pfaffl 2001) for relative quantification (software MxPro-Mx3000TM, Stratagene). The relative expression level is compared with a calibrator, in this study non-treated samples of the pigs taken before experimental infection. The target amount, normalized to the endogenous housekeeping gene (GAPDH) and relative to the calibrator, is then calculated by the formula: $2^{-\Delta\Delta C_T}$.

Statistical analyses. The results of the serological investigation, the bacteriological quantitative examination and the *ex vivo* quantitative real-time PCR were calculated and plotted as notch boxes. The median (internal horizontal line), upper and lower quartiles (the upper and lower horizontal margins of the boxes), the 95 % confidence limits (the oblique margins of the boxes) and the extreme values are shown (McGill et al. 1978). Significant differences were tested by Mann-Whitney U test. A *p* value of ≤ 0.05 was considered significant. The results of the qualitative bacteriological investigation and of the clinical examination were tested by the χ^2 test. *p* values of ≤ 0.05 were considered significant.

RESULTS

Clinical signs. After challenge infection with the respective *S. Typhimurium* DT104 strain only the pigs infected with the wild-type *S. Typhimurium* showed typical clinical symptoms of salmonellosis, such as anorexia, vomiting, disturbed demeanour, fever and diarrhoea. Two pigs of the wild-type group died at day 2 after infection with high-grade signs of salmonellosis (diarrhoea, dehydration, fever). All challenged pigs showed a disturbed demeanour on the first day after infection. The rectal temperature differed significantly

1 ($p \leq 0.05$) between the wild-type group and both mutant groups. The pigs of the wild-type
 2 group showed 1 to 4 days after infection a markedly elevated rectal temperature (39.9 °C -
 3 41.3 °C, mean 40.4 °C on 1st day after infection), whereas the rectal temperatures of both
 4 mutant groups were at normal levels.

5 Furthermore, all animals of the wild-type infected group had clearly diminished faeces
 6 consistency. At day 2 post infection all animals of the wild type group showed severe
 7 diarrhoea (score 2-3). Starting at day 7 post infection this diarrhoea became intermittent and
 8 17 days post infection the faecal consistency was back to normal. In summary, pigs infected
 9 with wild-type *S. Typhimurium* DT104 showed a significantly higher mean of the clinical
 10 score ($p \leq 0.05$), which peaked at day 4 post infection (Figure 1).

11

12 **Bacteriological examinations.** *S. Typhimurium* DT104 was not isolated from any
 13 investigated faecal samples before challenge, whereas all infected pigs shed the respective
 14 *S. Typhimurium* strain after challenge infection. The animals of the wild-type group were
 15 continuous shedders (100 %) during the complete observation period. In contrast, all pigs of
 16 the mutant groups were intermittent shedders. The pigs infected with the *invC::aphT* mutant,
 17 shed *S. Typhimurium* DT104 from the 3rd day p.i. till the end of the observation period with
 18 rates between 55.6 and 100 %. The animals of the *sseD::aphT* infection group shed their
 19 challenge strain with faeces from day 3 till day 14 p.i. with a rate of 100 %, but subsequently
 20 they became intermittent shedders (75 %-87.5 %) (Figure 2).

21 The qualitative bacteriological examination of 15 tissue samples of each pig was done with
 22 special regard to organs and tissues, which are directly used in food production (i.e. lung,
 23 liver, spleen and muscles) or which have immunological relevance (tonsil, mandibular lymph
 24 node, lung lymph node, spleen, jejunal lymph node, ileocolic lymph node and colic lymph
 25 node).

Each animal of the wild-type infected group was *Salmonella*-positive in tonsils, caecum, mandibular lymph node, lung lymph node, jejunal lymph node and ileocolic lymph node (Figure 3). However, the wild-type challenge strain could not be isolated from liver and bile.

The qualitative bacteriological examination of the tissue samples of the *invC::aphT* mutant group showed markedly decreased colonization rates (at day 21 *p.i.*) in organs with importance for food production (i.e. lung, liver, spleen and muscles). The *invC::aphT* mutant strain could not be detected in samples derived from liver, spleen, or bile. The majority of *invC::aphT* mutant group had *Salmonella*-positive tonsils, mandibular lymphnodes, and GALT.

In contrast, the examination of the *sseD::aphT* mutant group showed a colonization rate of 100 % in tonsils, ileum, colon, caecum, mandibular lymph node, ileocecal lymph node and colic lymph node. Altogether, the *sseD* mutant infected group showed virtually the same colonization rates as the wild-type infected pigs.

Every positive detection of *Salmonella* could be confirmed with the strain specific (wild-type or both mutants) qualitative PCR.

The quantitative *Salmonella* detection showed markedly decreased *Salmonella* counts in some tissues in the *invC::aphT*, whereas the *sseD* mutant infected group showed similar or even significantly higher (in jejunal lymph nodes and ileocolic lymph nodes) quantities of salmonellas in the organs as the wild-type infected pigs (Figure 4).

Serological examinations. *Salmonella*-specific antibody activities in serum were measured by the reference standard ELISA SALMOTYPE® Pig STM-WCE (Labor Diagnostik Leipzig). Thereby, isotype-specific antibody activities were determined during the observation period of 21 days *p.i.*. Thereby, the attenuation of the strains interfered with the humoral immune responses of the respective groups (Figure 5).

In comparison to the pigs of the mutant groups, the pigs of the wild-type group showed significantly higher antibody activities in serum.

Relative cytokine expression rate of the wild type infection group. All investigated cytokines (IL-2, IL-4, IL-10, IL-12, and IFN- γ) showed similar kinetics (Figures 6 and 7). The *ex-vivo* expression rate of all investigated cytokines decreased in the first days after infection. However, afterwards there was a significant increase (20-fold) of mRNA expression of all investigated cytokines, with exception of IL-10 which only reached the initial value. During the following observation period a further strong increase was seen in case of IL-2 (440-fold), IL-4 (650-fold), IL-12 (460-fold) and IFN γ (83-fold), while only a 2-fold increase of IL-10 mRNA expression was detectable.

Relative cytokine expression rate of the *invC::aphT* mutant group. At the beginning of the observation period there was a suppression of the relative expression rate of IL-2, IL-12 and IFN γ . Later, all measured cytokines were found to be induced, but however, at a significantly lower level in comparison to the wild-type group. The highest mRNA expression rate was found for IFN- γ .

Relative cytokine expression rate of the *sseD::aphT* mutant group. In this group the expression rates of IL-2 and IL-4 were also decreased at the beginning of the experimental *Salmonella* infection. The most significant suppression was found for IL-2 and IL-4 with a decrease of 87 % each. IL-12, IL-10 and IFN γ were only slightly depressed during the first days of infection. Later, the mRNA expression of all cytokines was significantly lower with the sharpest decline for IL-10 (490 fold).

Altogether, the comparative investigation of the cytokine mRNA expression between pigs infected with the wild-type strain of *S. Typhimurium* DT104 and *invC*- or *sseD*-mutants showed, that the cellular immune response against both mutants is in general reduced.

DISCUSSION

Knowledge about pathogenesis (e.g. adhesion, invasion, and survival and replication in hosts) is needed for effective measures to control salmonellosis. The present study demonstrates the impact of the two components *invC* and *sseD* of the two type III secretion systems of *Salmonella* on porcine salmonellosis.

Loss of virulence could be demonstrated with both deletion mutants of *S. Typhimurium* DT104 in the important reservoir pig. As before shown in the mouse model, lower clinical symptoms were shown by the pigs of both mutant groups (Coombes et al. 2005; Hapfelmeier et al. 2005). Only pigs infected with the wild-type strain showed typical clinical signs of salmonellosis. The pigs of both groups infected with one of the mutants shed the pathogen intermittently, whereas the pigs infected with the wild type shed *S. Typhimurium* DT104 bacteria continuously. Thereby, the *invC* mutant displayed the lowest shedding rate. Impaired invasion could explain this phenomenon.

To further analyse the impact of the two components *invC* and *sseD* of the *Salmonella* type III secretion systems the ability for invasion and colonization was also examined. *S. Typhimurium* is an invasive pathogen with the ability of systemic spread via the bloodstream and the lymphatic system. Thus, *Salmonella* are often detected in tonsils, jejunum, ileum, caecum, colon, and mandibular- and ileocolic lymph nodes. Presumably, these organs influence the persistence of *Salmonella* (Wood et al. 1989). However, a link

1 between inoculation route and colonisation rate could not be found (Fedorka-Cray et al.
 2 1995).

3 In this study, significant differences were shown between the *S. Typhimurium* DT104 wild
 4 type strain and both mutants. Previous *in-vitro* studies with an *invC* mutant of *Salmonella*
 5 *enterica* demonstrated a loss of the ability to invade epithelial cells (Gallois et al. 2001). Here,
 6 we could confirm these former findings also in swine, because the colonisation rate of inner
 7 organs was significantly reduced in the *invC* mutant group. In contrast, the *sseD::aphT* mutant
 8 group was colonized in average at 69.2 % and the wild-type group on average at 68.3 % of the
 9 organs examined. *Salmonella* were detectable in mandibular lymph nodes of pigs of all three
 10 groups. We also found high colonization levels of the wild-type and mutant strains in tonsils,
 11 ileum, colic, caecum, jejunal lymph node, ileocolic lymph nodes and colic lymph nodes
 12 indicating the ability of both mutant strains to leave the gut system and to invade the
 13 mesenteric lymph nodes. However, the significantly reduced colonisation rate in organs
 14 outside of the GALT demonstrates the impact of the *invC* gene in the process of invasion.
 15 This is in line with the findings of Boyen (2006) which showed that another SPI-1 mutant,
 16 namely of *sipB*, was also significantly impaired to invade the porcine gut.

17 In contrast, the quantitative bacteriological examination of the tissue samples with
 18 significantly higher counts of salmonellae in jejunic lymph nodes and ileocolic lymph nodes
 19 show a lack of attenuation of the *sseD::aphT* mutant compared to the wild-type bacteria.
 20 These results contradict former results from experimental infections in mice (Coombes et al.
 21 2005; Hapfelmeier et al. 2005).

22 The results of specific serum antibody measurement indicate a significantly decreased
 23 antibody response of both mutant groups. This fact is closely associated with a significantly
 24 lower *ex-vivo* mRNA expression rate of IL-4 in pigs of both mutant groups. IL-4 acts as the
 25 most important differentiation cytokine for the Th2-pathway and thereby, suppresses Th1
 26 activity and additionally it stimulates clonal proliferation of B cells. It is commonly accepted,

1 that antibody response is less important than cell-mediated immune mechanisms during
 2 infection with *Salmonella*. On the other hand, the humoral immune response seems to be
 3 necessary for the protection against a secondary infection with salmonellae (Mittrucker et al.
 4 2000). Furthermore, systemic antibodies eliminate salmonellae from the blood and promote
 5 phagocytosis of salmonellae by opsonization (Mastroeni et al. 2001). It was already shown in
 6 mice, that B cells and their specific antibodies are not essential during a primary or secondary
 7 infection with attenuated salmonellae. However, antibodies accelerate healing. In addition,
 8 there is a correlation between the existence of specific antibodies and vaccine-induced
 9 resistance (McSorley & Jenkins 2000). Moreover, IL-4-dependent immune mechanisms seem
 10 to be indispensable for sterile elimination of attenuated salmonellae as shown in mice
 11 (Lehmann et al. 2006).

12 Salmonellae are facultative intracellular pathogens, which can stimulate both pathways of the
 13 immune system, the cellular and the humoral. The Th1 response starts within few hours post
 14 infection resulting in elimination of salmonellae and protection against re-infection
 15 (Mittrucker et al. 2000).

16 The concerted action of several cytokines including TNF- α , IFN- γ , IL-12, and IL-18 is needed
 17 for the suppression of bacterial growth in the plateau phase of a *Salmonella* infection. IL-12
 18 mediates resistance to several intracellular organisms and it is essentially required for the
 19 establishment of the protective Th1 response (Lehmann et al. 2001). This interleukin induces
 20 IFN- γ in natural killer (NK) cells and presents a co-stimulus required for maximum IFN- γ -
 21 and IL-2-secretion by antigen-activated Th1 cells. The resulting Th1 response promotes
 22 primary cellular immunity, whereas Th2 cells produce IL-4 and IL-10 resulting mainly in
 23 humoral immunity. Our data show an eminently higher IL-12 expression rate in pigs of the
 24 *Salmonella* wild-type group. Additionally, the increase of IFN- γ and IL-2 was also
 25 significantly higher in the wild-type group. IFN- γ stimulates activation of macrophages and
 26 promotes the killing of *Salmonella* within macrophages. The markedly decreased IFN- γ

1 mRNA expression of the *invC::aphT* mutant group probably results from the reduced ability
 2 of invasion and therefore, the lower cell damage. Other studies showed such a correlation
 3 between low level of IFN- γ and cell damage (Trebichavsky et al. 2003). Altogether, the
 4 detected increased expression rates of IL-2, IL-12, and IFN- γ in the *Salmonella* wild-type
 5 group are in accordance with the results of other studies in animals and humans (Bao et al.
 6 2000) and are most probably associated with a protective immune response against
 7 *Salmonella*.

8 IL-10 is one of the most potent anti-inflammatory cytokines. This interleukin inhibits the
 9 antigen presentation to T cells and suppresses phagocytosis and intracellular killing
 10 (Fiorentino et al. 1991). This effect is mediated by the down-regulation of the class II MHC
 11 and co-stimulatory molecules. Furthermore, IL-10 inhibits the production of reactive oxygen
 12 intermediates (ROI) and reactive nitrogen intermediates (RNI) in activated macrophages. On
 13 the other hand, macrophages are a major source of IL-10 production. We could show that both
 14 mutants had the same potency to stimulate IL-10 production as the *S. Typhimurium* DT104
 15 wild-type strain in the first days of infection. There is an interesting involvement of the
 16 factors of SPI-2 in a signal transduction pathway that induces cytokine expression in
 17 *Salmonella* infected macrophages. Thereby, *Salmonella* and some other intracellular
 18 pathogens use IL-10 to survive within macrophages or to inhibit the host defence (Uchiya et
 19 al. 2004). The role of the SPI-2 encoded secretion system is to interfere with intracellular
 20 vesicular trafficking to avoid exposure to toxic agents such as ROI and RNI in infected
 21 macrophages (Chakravorty et al. 2002). Compared to the wild-type group, our data show
 22 high IL-10 expression rates of the *sseD* mutant group (as high as at the wildtype group) in the
 23 first days of infection. We could therefore assume that the loss of *sseD* did not result in a
 24 disability to avoid exposure to toxic agents in the macrophages. This assumption is supported
 25 by the high colonization rates in pigs of the *sseD* mutant group. Therefore, our data indicate
 26 that the loss of the *sseD* gene of SPI-2 did not result in a decreased survival in porcine

macrophages, which is in contrast to the results obtained in mice (Hapfelmeier et al. 2005). Finally, further studies with ROI and RNI measurements at macrophages infected with the used *sseD*-mutant should answer this question.

In general, we noted an immunosuppressive effect in all infection groups at the beginning of the observation period. A possible reason is the high challenge dose to show possible differences in shedding of salmonellae and in clinical symptoms of salmonellosis. Previously, this immunosuppressive effect has been described in chicken (Hassan & Curtiss 1994). The lymphocyte depletion and immunosuppression might play a critical role in the development of the *Salmonella* carrier status. Furthermore, a significant lower expression of IFN- γ was observed in susceptible infected chicken in comparison to resistant animals. The reduced expression rate of IFN- γ can be a valuable indication of immunodeficiency associated with persistence of *Salmonella* in the chicken's digestive tract (Sadeyen et al. 2004).

Altogether, we could show, that the virulent wild-type strain of *S. Typhimurium* DT104 possesses a significantly higher potency to stimulate the cytokine response in porcine immune cells compared to both mutants with deletions in the two *Salmonella* type III secretion systems. Thereby, the mildest reactions were noticed in animals of the *invC::aphT* mutant group; which is in line with the disability of invasion.

In summary, this study shows that the *invC::aphT* and *sseD::aphT* mutants of *S. Typhimurium* DT104 possess a significant reduced virulence in swine. Thereby, the *invC* deletion results in more evident attenuation than the *sseD* deletion, because of a total loss of clinical signs of salmonellosis and significantly reduced colonisation rates. The *sseD* deletion mutant, however, was not able to induce clinical symptoms of porcine salmonellosis and induced lower cellular and humoral immunity, but the colonization rates of the tested tissues were in parts significantly higher compared to the wild-type.

ACKNOWLEDGMENTS

The technical assistance of Eveline Brumme and Nadja Leinecker is gratefully acknowledged.

REFERENCES

- Akeda, Y., Galan, J.E., 2004. Genetic analysis of the *Salmonella enterica* type III secretion-associated ATPase InvC defines discrete functional domains. *J. Bacteriol.*, 186, 2402-2412.
- Alpuche-Aranda, C.M., Berthiaume, E.P., Mock, B., Swanson, J.A., Miller, S.I., 1995. Spacious phagosome formation within mouse macrophages correlates with *Salmonella* serotype pathogenicity and host susceptibility. *Infect. Immun.*, 63, 4456-4462.
- Bao, S., Beagley, K.W., France, M.P., Shen, J., Husband, A.J., 2000. Interferon-gamma plays a critical role in intestinal immunity against *Salmonella* typhimurium infection. *Immunology*, 99, 464-472.
- Barthel, M., Hapfelmeier, S., Quintanilla-Martinez, L., Kremer, M., Rohde, M., Hogardt, M., Pfeffer, K., Russmann, H., Hardt, W.D., 2003. Pretreatment of mice with streptomycin provides a *Salmonella enterica* serovar Typhimurium colitis model that allows analysis of both pathogen and host. *Infect. Immun.*, 71, 2839-2858.
- Bispham, J., Tripathi, B.N., Watson, P.R., Wallis, T.S., 2001. *Salmonella* pathogenicity island 2 influences both systemic salmonellosis and *Salmonella*-induced enteritis in calves. *Infect. Immun.*, 69, 367-377.
- Chakravorty, D., Hansen-Wester, I., Hensel, M., 2002. *Salmonella* pathogenicity island 2 mediates protection of intracellular *Salmonella* from reactive nitrogen intermediates. *J. Exp. Med.*, 195, 1155-1166.
- Coombes, B.K., Coburn, B.A., Potter, A.A., Gomis, S., Mirakhur, K., Li, Y., Finlay, B.B., 2005. Analysis of the contribution of *Salmonella* pathogenicity islands 1 and 2 to enteric disease progression using a novel bovine ileal loop model and a murine model of infectious enterocolitis. *Infect. Immun.*, 73, 7161-7169.
- Eichelberg, K., Ginocchio, C.C., Galan, J.E., 1994. Molecular and functional characterization of the *Salmonella* typhimurium invasion genes *invB* and *invC*: homology of InvC to the F0F1 ATPase family of proteins. *J. Bacteriol.*, 176, 4501-4510.
- Fedorka-Cray, P.J., Kelley, L.C., Stabel, T.J., Gray, J.T., Laufer, J.A., 1995. Alternate routes of invasion may affect pathogenesis of *Salmonella* typhimurium in swine. *Infect. Immun.*, 63, 2658-2664.
- Fiorentino, D.F., Zlotnik, A., Mosmann, T.R., Howard, M., Ogarra, A., 1991. IL-10 inhibits cytokine production by activated macrophages. *J. Immunol.*, 147, 3815-3822.

- 1 Galan, J.E., 1999. Interaction of *Salmonella* with host cells through the centisome 63 type III
2 secretion system. *Curr. Opin. Microbiol.*, 2, 46-50.
- 3 Gallois, A., Klein, J.R., Allen, L.A., Jones, B.D., Nauseef, W.M., 2001. *Salmonella*
4 pathogenicity island 2-encoded type III secretion system mediates exclusion of NADPH
5 oxidase assembly from the phagosomal membrane. *J. Immunol.*, 166, 5741-5748.
- 6 Hapfelmeier, S., Stecher, B., Barthel, M., Kremer, M., Müller, A.J., Heikenwalder, M.,
7 Stallmach, T., Hensel, M., Pfeffer, K., Akira, S., Hardt, W.D., 2005. The *Salmonella*
8 pathogenicity island (SPI)-2 and SPI-1 type III secretion systems allow *Salmonella* serovar
9 typhimurium to trigger colitis via MyD88-dependent and MyD88-independent mechanisms. *J.*
10 *Immunol.*, 174, 1675-1685.
- 11 Hassan, J.O., Curtiss, R.III, 1994. Virulent *Salmonella* typhimurium-induced lymphocyte
12 depletion and immunosuppression in chickens. *Infect. Immun.*, 62, 2027-2036.
- 13 Klein, J.R., Jones, B.D., 2001. *Salmonella* pathogenicity island 2-encoded proteins SseC and
14 SseD are essential for virulence and are substrates of the type III secretion system. *Infect.*
15 *Immun.*, 69, 737-743.
- 16 Lehmann, J., Bellmann, S., Werner, C., Schröder, R., Schutze, N., Alber, G., 2001. IL-12p40-
17 dependent agonistic effects on the development of protective innate and adaptive immunity
18 against *Salmonella* enteritidis. *J. Immunol.*, 167, 5304-5315.
- 19 Lehmann, J., Springer, S., Werner, C.E., Lindner, T., Bellmann, S., Straubinger, R.K., Selbitz,
20 H.J., Alber, G., 2006. Immunity induced with a *Salmonella enterica* serovar Enteritidis live
21 vaccine is regulated by Th1-cell-dependent cellular and humoral effector mechanisms in
22 susceptible BALB/c mice. *Vaccine*, 24, 4779-4793.
- 23 Lundberg, U., Vinatzer, U., Berdnik, D., von Gabain, A., Baccarini, M., 1999. Growth phase-
24 regulated induction of *Salmonella*-induced macrophage apoptosis correlates with transient
25 expression of SPI-1 genes. *J. Bacteriol.*, 181, 3433-3437.
- 26 Mastroeni, P., Chabalgoity, J.A., Dunstan, S.J., Maskell, D.J., Dougan, G., 2001. *Salmonella*:
27 immune responses and vaccines. *Vet. J.*, 161, 132-164.
- 28 McGill, R.J., Tukey, J.W., Larson, W.A., 1978. Variations of Box Plots. *Am. Statist.*, 32, 12-
29 16.
- 30 McSorley, S.J., Jenkins, M.K., 2000. Antibody is required for protection against virulent but
31 not attenuated *Salmonella enterica* serovar typhimurium. *Infect. Immun.*, 68, 3344-3348.
- 32 Medina, E., Paglia, P., Nikolaus, T., Müller, A., Hensel, M., Guzman, C.A., 1999.
33 Pathogenicity island 2 mutants of *Salmonella* typhimurium are efficient carriers for
34 heterologous antigens and enable modulation of immune responses. *Infect. Immun.*, 67, 1093-
35 1099.
- 36 Mittrucker, H.W., Raupach, B., Kohler, A., Kaufmann, S.H., 2000. Cutting edge: role of B
37 lymphocytes in protective immunity against *Salmonella* typhimurium infection. *J. Immunol.*,
38 164, 1648-1652.

- 1 Monack, D.M., Detweiler, C.S., Falkow, S., 2001. *Salmonella* pathogenicity island 2-
2 dependent macrophage death is mediated in part by the host cysteine protease caspase-1. *Cell*
3 *Microbiol.*, 3, 825-837.
- 4 Nikolaus, T., Deiwick, J., Rappl, C., Freeman, J.A., Schröder, W., Miller, S.I., Hensel, M.,
5 2001. SseBCD proteins are secreted by the type III secretion system of *Salmonella*
6 pathogenicity island 2 and function as a translocon. *J. Bacteriol.*, 183, 6036-6045.
- 7 Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real time RT-
8 PCR. *Nucleic Acids Res.*, 29, e45.
- 9 Rathman, M., Barker, L.P., Falkow, S., 1997. The unique trafficking pattern of *Salmonella*
10 typhimurium-containing phagosomes in murine macrophages is independent of the
11 mechanism of bacterial entry. *Infect. Immun.*, 65, 1475-1485.
- 12 Roesler, U., Marg, H., Schröder, I., Mauer, S., Arnold, T., Lehmann, J., Truyen, U., Hensel,
13 A., 2004. Oral vaccination of pigs with an invasive *gyrA-cpxA-rpoB Salmonella*
14 Typhimurium mutant. *Vaccine*, 23, 595-603.
- 15 Sadeyen, J.R., Trotter, J., Velge, P., Marly, J., Beaumont, C., Barrow, P.A., Bumstead, N.,
16 Lalmanach, A.C., 2004. *Salmonella* carrier state in chicken: comparison of expression of
17 immune response genes between susceptible and resistant animals. *Microbes. Infect.*, 6, 1278-
18 1286.
- 19 Trebichavsky, I., Splichal, I., Splichalova, A., Muneta, Y., Mori, Y., 2003. Systemic and local
20 cytokine response of young piglets to oral infection with *Salmonella enterica* serotype
21 Typhimurium. *Folia Microbiol. (Praha)*, 48, 403-407.
- 22 Uchiya, K., Groisman, E.A., Nikai, T., 2004. Involvement of *Salmonella* pathogenicity island
23 2 in the up-regulation of interleukin-10 expression in macrophages: role of protein kinase A
24 signal pathway. *Infect. Immun.*, 72, 1964-1973.
- 25 van der Velden, A.W., Lindgren, S.W., Worley, M.J., Heffron, F., 2000. *Salmonella*
26 pathogenicity island 1-independent induction of apoptosis in infected macrophages by
27 *Salmonella enterica* serotype typhimurium. *Infect. Immun.*, 68, 5702-5709.
- 28 Wood, R.L., Pospischil, A., Rose, R. 1989. Distribution of persistent *Salmonella* typhimurium
29 infection in internal organs of swine. *Am. J. Vet. Res.*, 50, 1015-1021.
- 30 Wright, P.F., Nilson, E., Van Rooij, E.M.A., Lelenta, M., Jeggo, M.H., 1993. Standardisation
31 and validation of enzyme-linked-immunosorbent assay techniques for the detection of
32 antibody in infectious disease diagnosis. *Rev. sci. tech. Off. int. Epiz.*, 12, 435-450.
- 33 Zurawski, D.V., Stein, M.A. 2003. SseA acts as the chaperone for the SseB component of the
34 *Salmonella* Pathogenicity Island 2 translocon. *Mol. Microbiol.*, 47, 1341-1351.
- 35
- 36

FIGURE LEGENDS

FIG. 1. Mean clinical score (general demeanour, vomiting, anorexia and diarrhoea) of pigs of the *S. Typhimurium* DT104 wild-type group (filled circle), of animals of the *invC::aphT* mutant group (circles), and of pigs of the *sseD::aphT* mutant group (filled triangles). Clinical signs were quantified using a clinical score: 0 = no symptoms, 1 = mild symptoms, 2 = medium symptoms, and 3 = severe symptoms. (#) indicates significant differences ($p < 0.05$) between wild type group and mutant groups.

FIG. 2. Shedding rate of *S. Typhimurium* DT104 (wildtype and two mutants) in faeces of experimentally infected pigs. The black bars represent the pigs infected with the *S. Typhimurium* DT104 wild-type, the white bars the pigs infected with the *invC::aphT* mutant, and the grey bars the animals infected with the *sseD::aphT* mutant.

FIG. 3. Colonization rate of *S. Typhimurium* DT104 (wildtype and two mutants) in tissue samples after qualitative bacterial examination. The black bars represent the pigs infected with the *S. Typhimurium* DT104 wild-type, the white bars the pigs infected with the *invC::aphT* mutant, and the grey bars the animals infected with the *sseD::aphT* mutant. Significant differences ($p \leq 0.05$) between the wild-type group and the mutant groups are indicated by asterisks (*) and between both mutant groups by #. MaLn, mandibular lymphnode; JeLn, jejunal lymphnode; IcLn, ileocolic lymphnode; colic lymphnode.

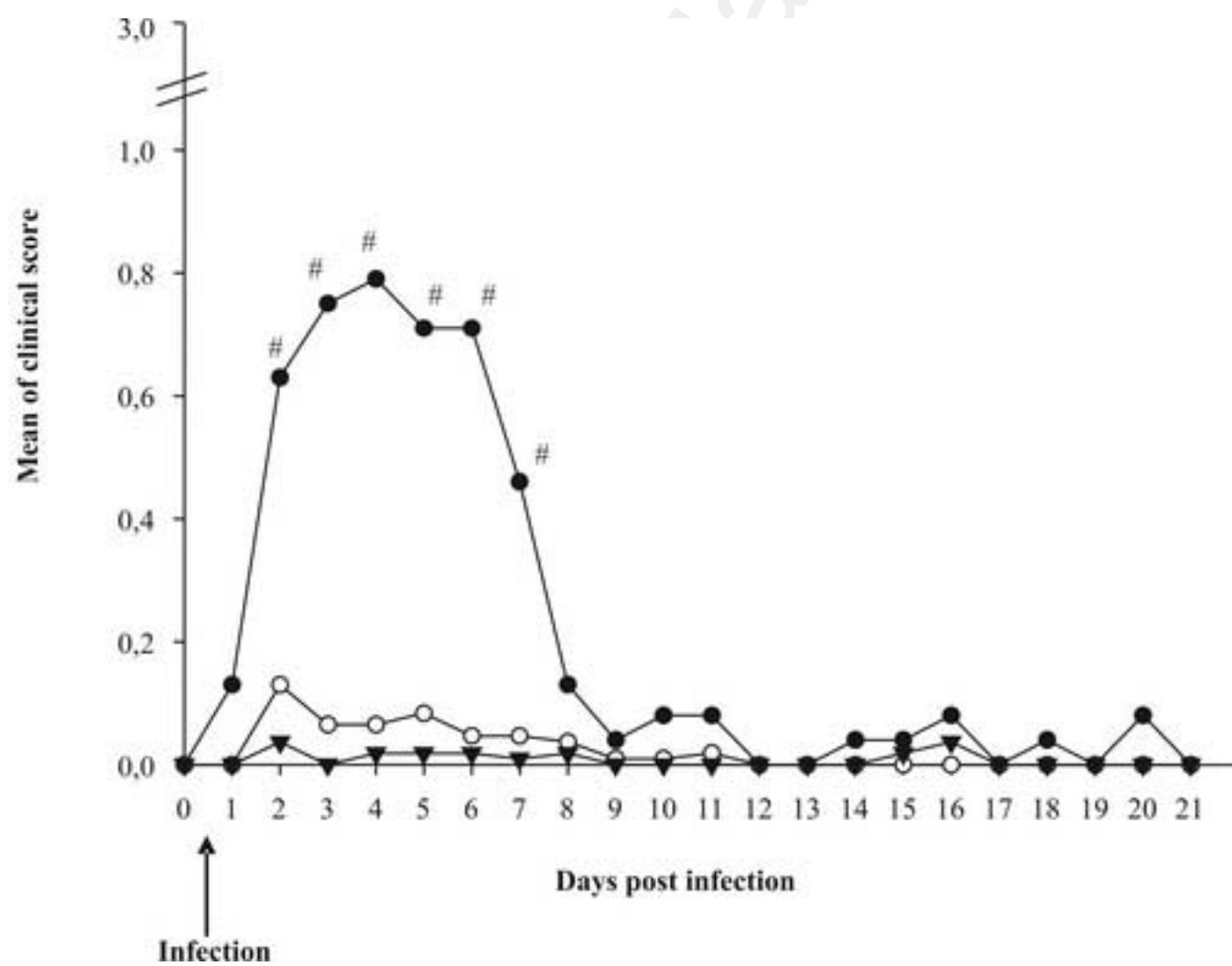
FIG. 4. Colonization rate of *S. Typhimurium* DT104 in tissue samples after quantitative bacterial examination. The black notch boxes represent the pigs infected with the *S. Typhimurium* DT104 wild-type, the white notch boxes the pigs infected with the *invC::aphT* mutant, and the grey notch boxes the animals infected with the *sseD::aphT*

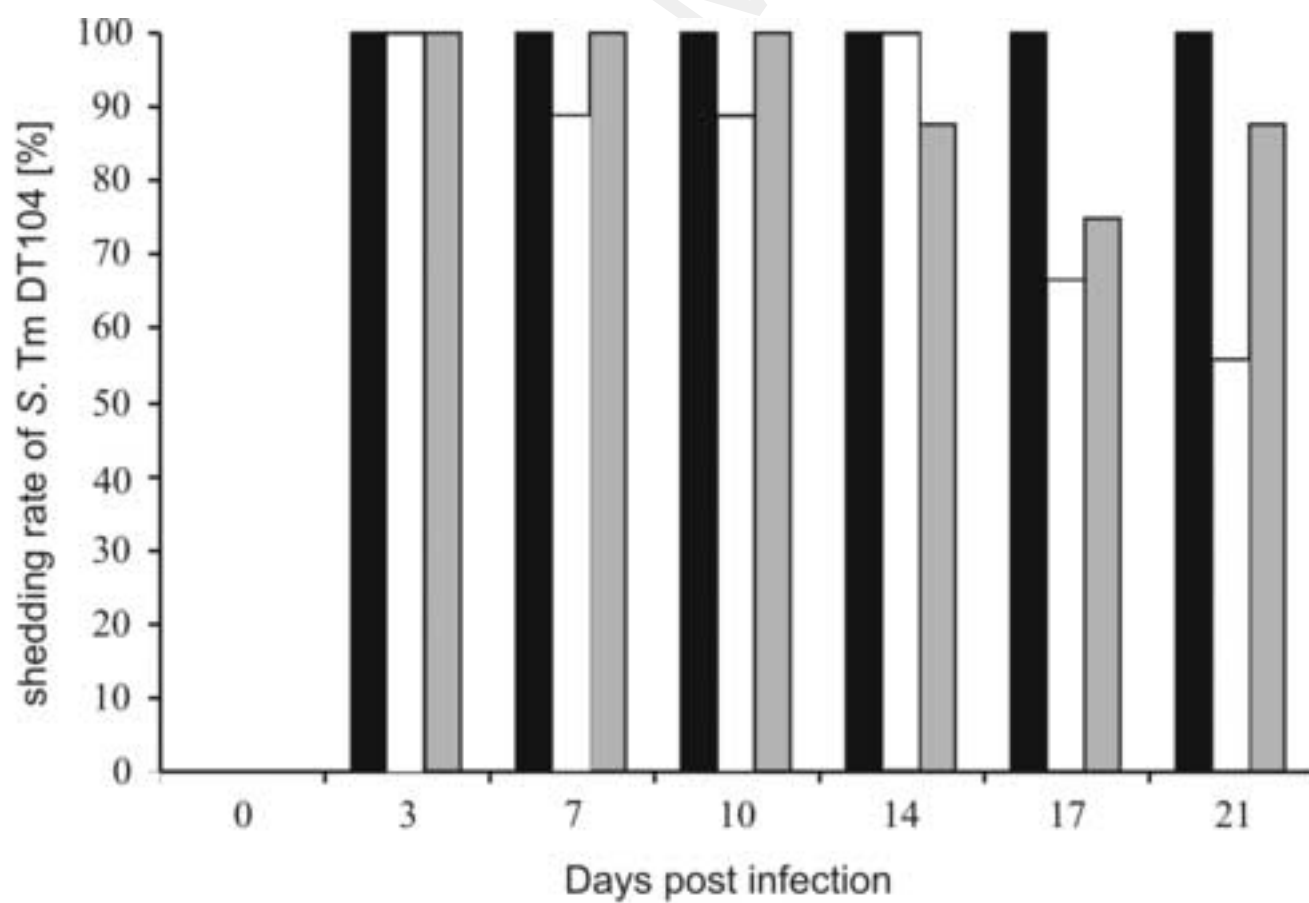
mutant. Significant differences ($p \leq 0.05$) between the wild-type group and the mutant groups are indicated by asterisks (*) and between both mutant groups by the # sign. MaLn, mandibular lymphnode; JeLn, jejunal lymphnode; IcLn, ileocolic lymphnode; colic lymphnode.

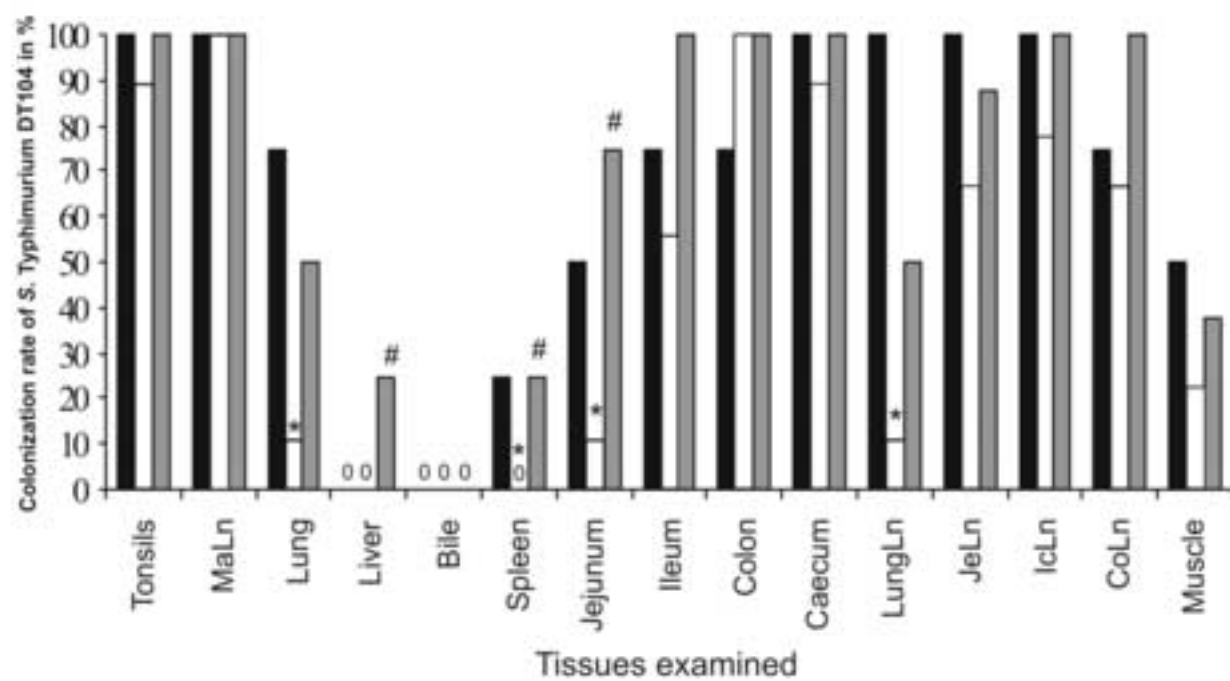
FIG. 5. *Salmonella* specific IgM antibody activities in serum of pigs experimentally infected with the *S. Typhimurium* DT104 wild type strain (black bars), *invC::aphT* mutant strain (white bars) and *sseD::aphT* mutant strain (grey bars). Significant differences ($p \leq 0.05$) between the wild-type group and the mutant groups are indicated by asterisks (*) and significant differences between both mutant groups by the # sign.

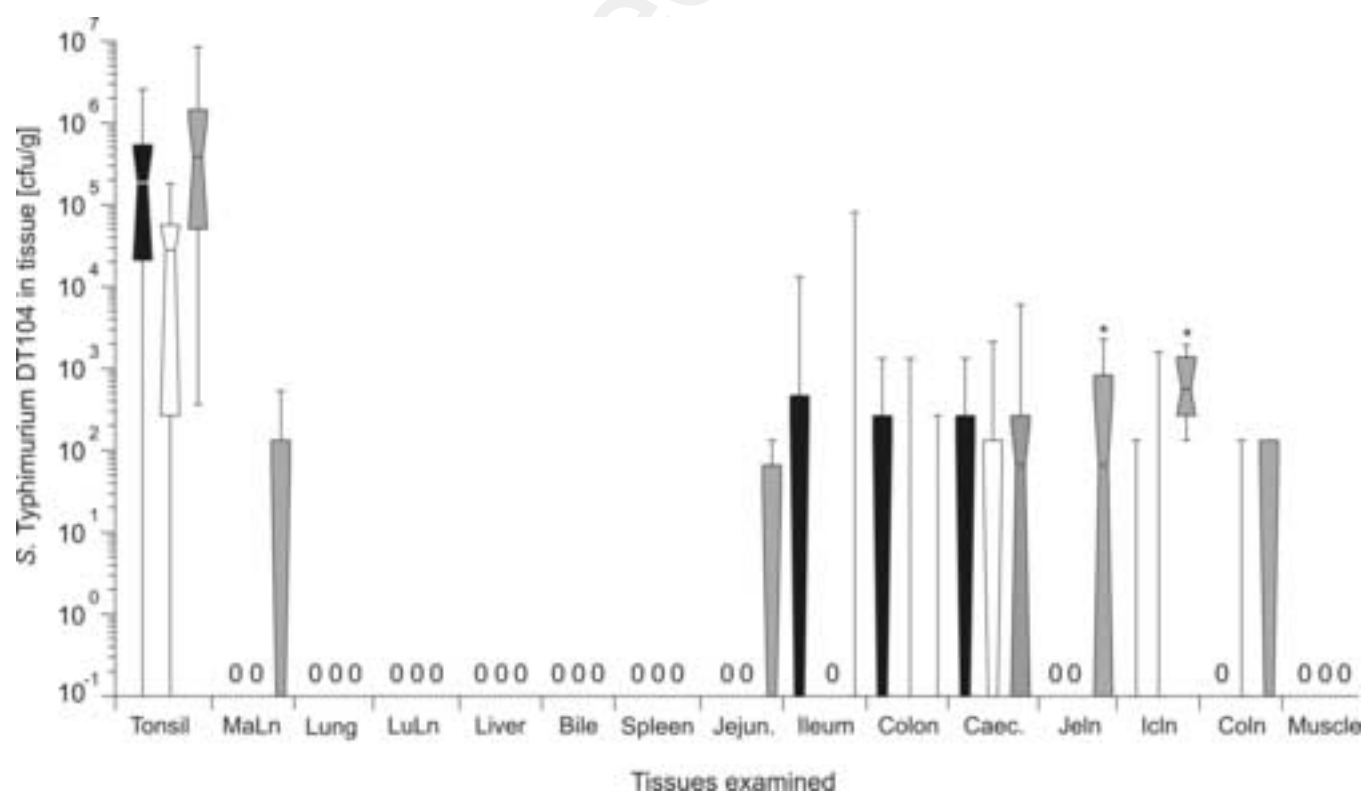
FIG. 6. Relative *ex-vivo* mRNA expression rates of IL-2 (A), IL-4 (B) and IL-10 (C) after experimental infection with *S. Typhimurium* DT 104 in animals of the wild-type group (black notch boxes), the *invC::aphT* mutant group (white notch boxes), and the *sseD::aphT* mutant group (grey notch boxes). Asterisks (*) indicate significant differences ($p < 0.05$) between wild-type group and the respective mutant group.

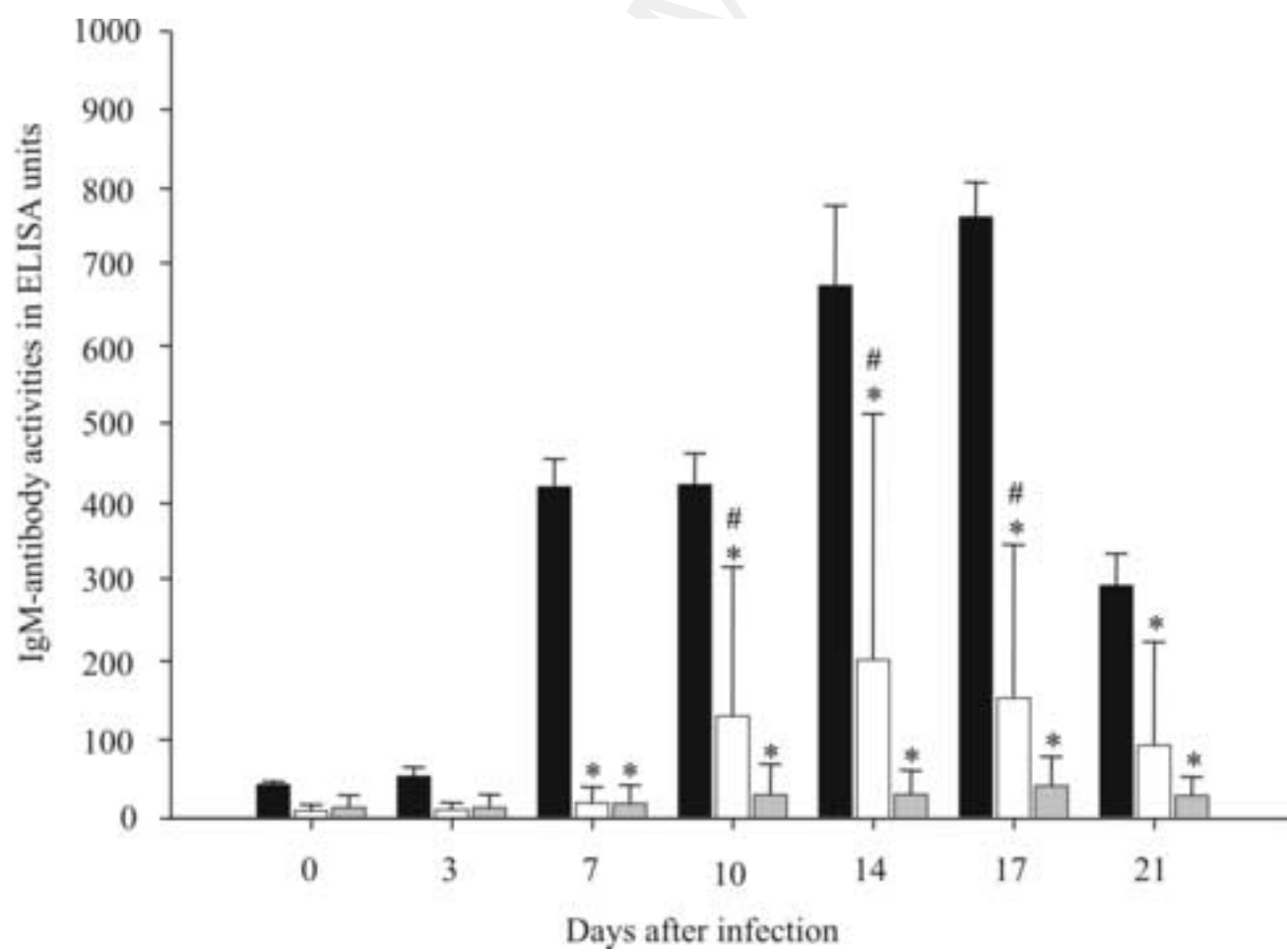
FIG. 7. Relative *ex-vivo* mRNA expression rates of IL-12 (A) and Gamma-Interferon (B) after experimental infection with *S. Typhimurium* DT 104 in animals of the wild-type group (black notch boxes), the *invC::aphT* mutant group (white notch boxes), and the *sseD::aphT* mutant group (grey notch boxes). Asterisks (*) indicate significant differences ($p < 0.05$) between wild-type group and the respective mutant group.

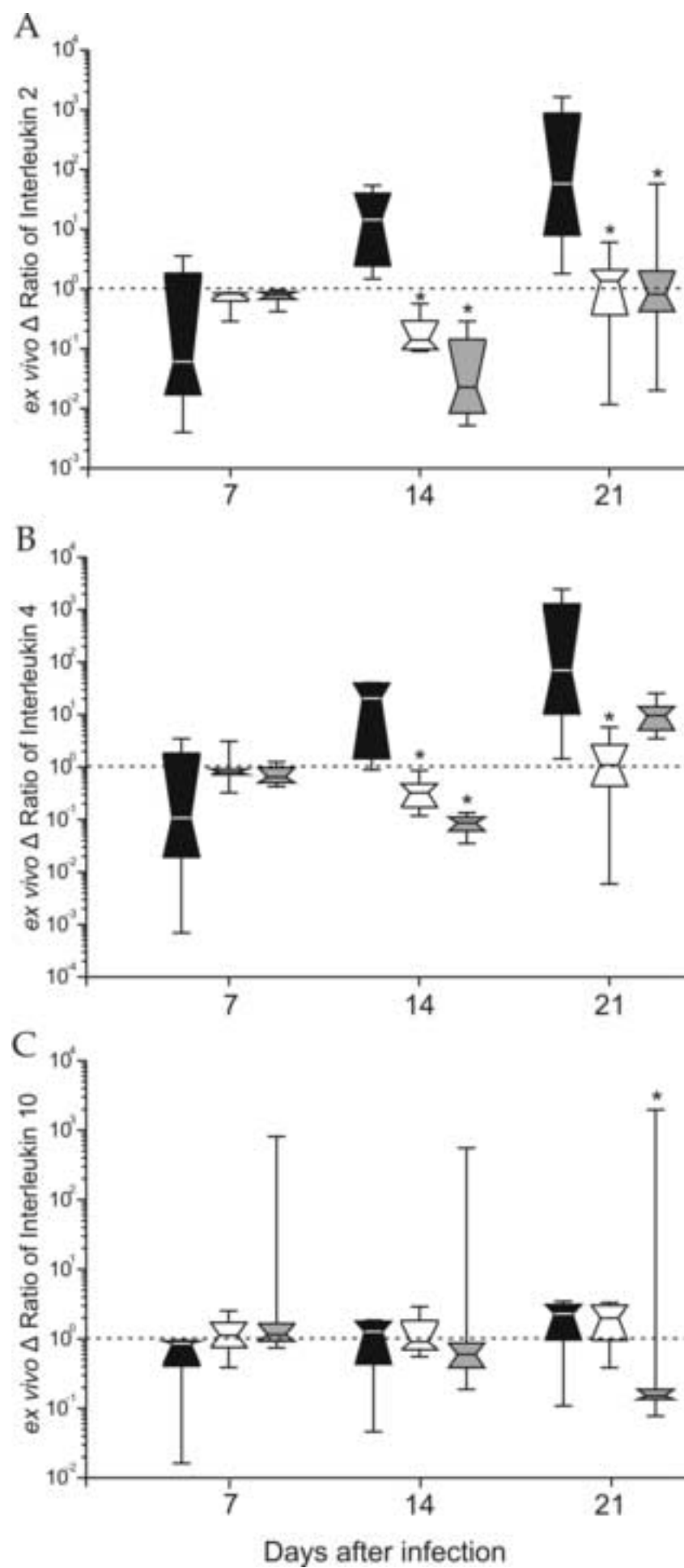


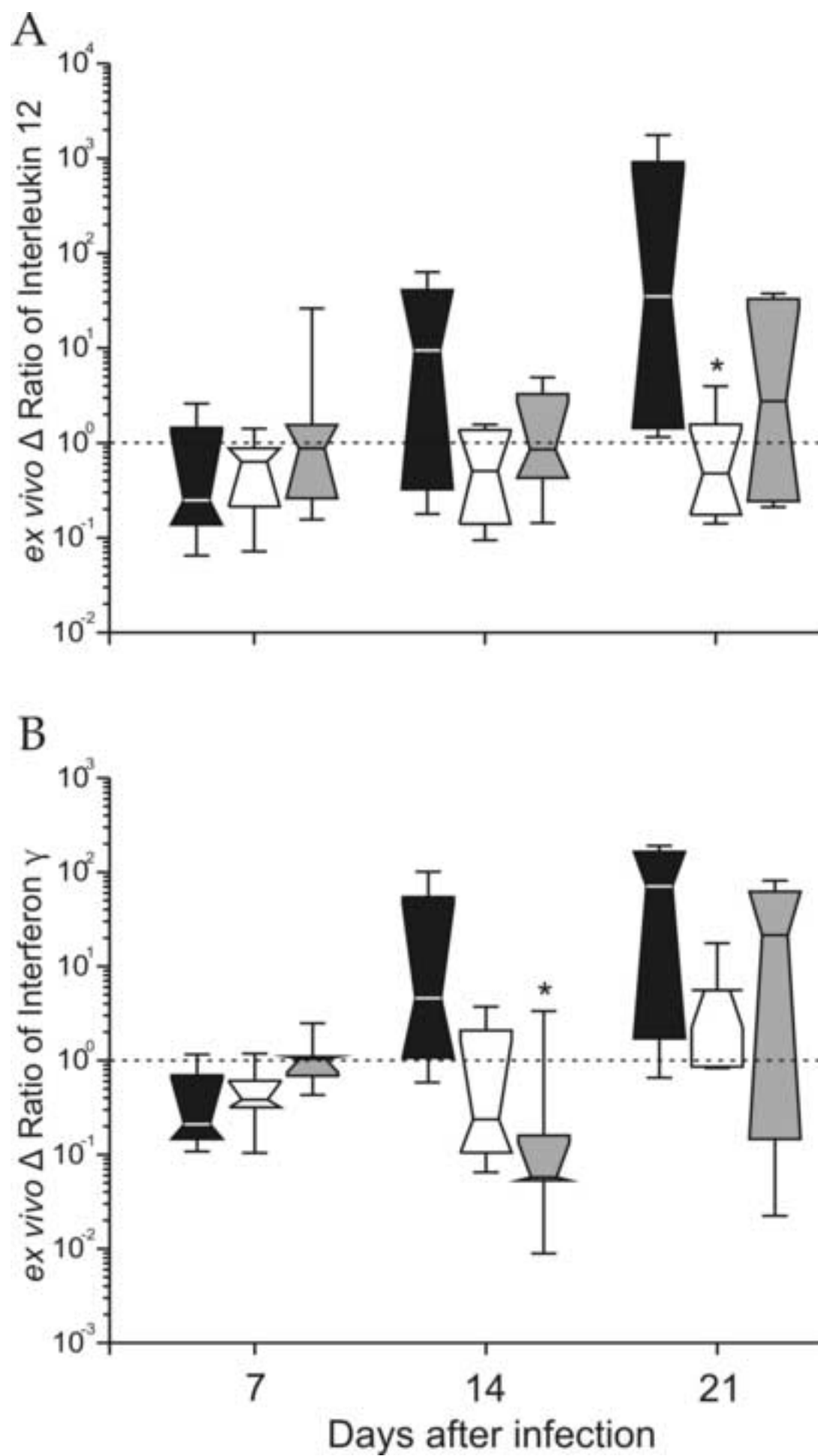












Target	Forward primer	Reverse primer	Probe
GAPDH	GAf (5'-gggctgccagaacatca-3')	GAr (5'-tgtcatcatatttgcaggtttctc-3')	GA (5'-ccggcgctgccaaaggctgt-3')
IL-2	IL-2f (5'-cagctctgtgttgcatgca-3')	IL-2r (5'-gcagcaatggctccagttgt-3')	IL-2 (5'-taaccctgcactcatggcaaacgg-3')
IL-4	IL-4f (5'-agaacacgacggagaaggaaa c-3')	IL-4r (5'-ttgcatgctgctcaggtt-3')	IL-4 (5'-cttcggcacacacacg-3')
IL-10	IL-10f (5'-cggcgctgtcatcaatttct- 3')	IL-10r (5'-gagcttgctaaaggcactttca-3')	IL-10 (5'-tgtgaaaacaagagcaaggccgtgg- 3')
IL-12	IL-12f (5'-cacagcagg cccaggaat-3')	IL-12r (5'-tggtgctgacggccttca-3')	IL-12 (5'-tcaaccactcccaaat- 3')
Gamma-Interferon	IFN- γ f (5'-gcgcaaagccatcagtga-3')	IFN- γ r (5'-tgcaggcaggatgacaattatt-3')	IFN- γ (5'-cagactatgtgcatca-3')