Protective efficacy of vaccines based on the *Helicobacter suis* urease subunit B and γ–glutamyl transpeptidase

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Abstract

Helicobacter (*H.*) *suis* causes gastric lesions in pigs and humans. This study aimed to evaluate the protective efficacy of immunization with combinations of the *H. suis* urease subunit B (UreB) and γ-glutamyl transpeptidase (GGT), both recombinantly expressed in *Escherichia coli* (rUreB and rGGT, respectively). Mice were intranasally immunized with rUreB, rGGT or a combination of both proteins, administered simultaneously or sequentially. Control groups consisted of non-immunized and non-challenged mice (negative controls), sham-immunized and *H. suis*-challenged mice (sham-immunized controls), and finally, *H. suis* whole-cell lysate-immunized and *H. suis* challenged mice. Cholera toxin was used as mucosal adjuvant. All immunizations induced a significant reduction of gastric *H. suis* colonization, which was least pronounced in the groups immunized with rGGT and rUreB only. Consecutive immunization with rGGT followed by rUreB and immunization with the bivalent vaccine improved the protective efficacy compared to immunization with single proteins, with a complete clearance of infection observed in 50% of the animals. Immunization with whole-cell lysate induced a similar reduction of gastric bacterial colonization compared to rGGT and rUreB in combinations. Gastric lesions, however, were less pronounced in mice immunized with combinations of rUreB and rGGT compared to mice immunized with whole-cell lysate. In conclusion, vaccination with a combination of rGGT and rUreB protected mice against a subsequent *H. suis* infection and was not associated with severe post-vaccination gastric inflammation, indicating that it may be a promising method for control of *H. suis* infections.

34 Keywords: *Helicobacter suis*, vaccination, urease subunit B, γ–glutamyl transpeptidase, mouse
35 model

1. Introduction

Helicobacter (*H.*) *suis* is a worldwide spread bacterium causing chronic gastritis and reduced daily weight gain in pigs [1]. An infection with *H. suis* has also been associated with erosive and ulcerative lesions in the non-glandular part of the porcine stomach [2,3]. Furthermore, this bacterium is the most prevalent non-*H. pylori Helicobacter* species colonizing the stomach of humans suffering from gastric disease [4]. Previous studies in mice have shown that prophylactic intranasal immunization with *H. suis* whole-cell lysate results in significant protection against *H. suis* infections [5,6]. However, production of sufficient *H. suis* whole-cell lysate may be hindered by the laborious *in vitro* cultivation of this bacterium. Also, whole-cell lysates may contain both protective antigens and antigens suppressing protection [7]. To overcome these drawbacks, a subunit vaccine, based on the *H. suis* urease subunit B (UreB) has been developed [6]. Immunization with *H. suis* UreB, recombinantly expressed in *E. coli* (rUreB) only induced a partial protection against *H. suis* challenge in a mouse model and it has been suggested that inclusion of additional antigens might improve the protective efficacy of this subunit vaccine [6].

In addition, immune modulating factors produced by the bacterium may hamper the development of a fully potent immune response against a *H. suis* infection, and thus may influence the effectiveness of certain vaccine formulations. Indeed, *H. suis* γ-glutamyl transpeptidase (GGT) has been shown to modulate the function of lymphocytes *in vitro*, which may result in host immune escape of *H. suis* leading to a chronic infection and lifelong persistence of *H. suis* in the porcine stomach [8]. Inhibition of this *H. suis* virulence factor by vaccination, may lead to an

abrogation of its immune modulatory effect, enabling the development of a fully potent immune response against *H. suis* infection.

The aim of the present study was to evaluate the protective efficacy of simultaneous or consecutive immunization with recombinant *H. suis* GGT (rGGT) and rUreB against *H. suis* infections, and to compare it with that of *H. suis* lysate and univalent vaccination in a standardized mouse model.

2. Materials and methods

2.1. Bacterial strain

H. suis strain 5 (HS5) was used in all experiments. This strain was isolated from the gastric mucosa of a sow, according to the method described by Baele et al. [9].

2.2. Antigens for immunization

Recombinantly expressed GGT (rGGT) was prepared as described previously [10]. Briefly, HS5 DNA was used as template to PCR-amplify the *ggt* gene without predicted signal sequence, cloned into the pENTR™/SD/D-TOPO® vector and transferred into the pDEST™17 destination vector. Chemically competent *E.coli* BL21-AI™ cells were transformed and protein expression was induced with 0.2% L-arabinose. rGGT was purified by $(His)_6$-tag affinity on a Ni-sepharose column (His GraviTrap; GE Healthcare Bio-Sciences AB) following manufacturer's instructions. For further purification, the rGGT was loaded on a Superdex 75 gel filtration column (GE Healthcare Bio-sciences AB). Afterwards, rGGT was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the GGT activity assay [12].

Recombinantly expressed UreB (rUreB) was prepared as described previously [6]. Briefly, a fragment encoding the HS5 UreB sequence was amplified by PCR and cloned into the protein expression vector pET-24d. The rUreB was expressed in *E. coli* strain BL21 (DE3) and *E. coli* cells were lyzed by sonication in a buffer containing 50mM $Na.PO_4$ pH7, 0.5M NaCl, 1M DTT, 1% Triton X-100 and 1mM PMSF. rUreB was purified using Ni-affinity chromatography in buffer consisting of 1 M NaCl, 50 mM PBS, 1% Triton X-100, 250 mM imidazole and 10% glycerol (His GraviTrap; GE Healthcare Bio-Sciences AB) followed by gel filtration on a Superdex™ 200 HR 16/60 column (GE Healthcare Bio-sciences AB). After purification, rUreB was analyzed using SDS-PAGE and Western-blot analysis using anti-hexahistidine-tag mouse monoclonal antibody (Icosagen Cell Factory, Tartu, Estonia). The detergent Triton X-100 was removed from the purified rUreB by using Pierce Detergent Removal Spin columns (Pierce Biotechnology, Rockford, USA) following manufacturer's instructions.

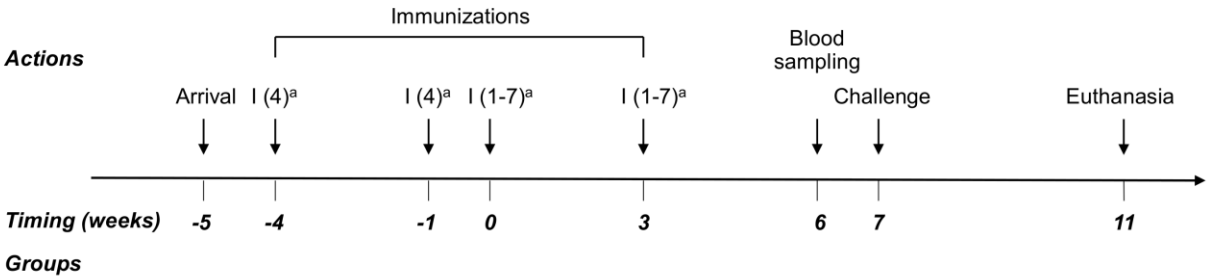
H. suis whole-cell lysate (lysate) was prepared as described by Flahou et al. [5], but without final filtration of the supernatant. The latter was done to prevent potential loss of antigens. Protein concentrations were determined with the *RC DC* protein Assay (Bio-Rad, Hercules, CA, USA).

2.3. Immunization and infection experiments

One week prior to the initiation of the experiments, 70 five-week-old specific-pathogen-free female BALB/c mice were obtained from an authorized breeder (HARLAN, Horst, The Netherlands). The animals were housed on autoclaved wood shavings in filter top cages. They were fed an autoclaved commercial diet (TEKLAD 2018S, HARLAN) and received autoclaved water *ad libitum*. All experiments involving animals were approved by the Animal Care and

Ethics Committee of the Faculty of Veterinary Medicine, Ghent University (EC2011/164). Immunization and infection experiments were performed as presented in Figure 1. Mice were divided over seven groups of 10 animals each. Groups 1, 2, 3, 5, 6 and 7 were intranasally inoculated twice with three weeks interval, with a total volume of 17.5 µl/ dose. In groups 1, 2, 3 and 5, vaccine formulations consisted of Hank's balanced salt solution (HBSS) with 5 µg cholera toxin (CT) (List Biological Laboratories Inc., Madison, NJ, USA), to which 30 µg rUreB, 30 µg rGGT, 30 µg rGGT + 30 µg rUreB, and 100 µg lysate had been added, respectively. Groups 6 (sham-immunized group) and 7 (negative control group) received HBSS only. Mice from group 4 were first immunized twice (with three weeks interval) with a vaccine consisting of HBSS, 5 µg CT and 30 µg rGGT. One week after the second immunization animals were immunized twice (with three weeks interval) with a vaccine consisting of HBSS, 5 µg CT and 30 µg rUreB. Three weeks after the last immunization, blood was collected by tail bleeding from five animals per group and one week later, all animals, except the negative control group, were intragastrically inoculated with 200 µL Brucella broth at pH 5 containing 10^8 viable *H. suis* bacteria [11]. The negative control group was intragastrically inoculated with 200 µL Brucella broth at pH 5. Four weeks after the challenge with *H. suis*, mice were euthanized by cervical dislocation following isoflurane anaesthesia (IsoFlo; Abbott, IL, USA). Blood was collected by sterile cardiac puncture, centrifuged (1000 *g*, 4°C, 10 min) and serum was frozen at -70°C until further use. Stomachs were excised and dissected along the greater curvature. One-half of the stomachs, including antrum and fundus, was immediately placed into 1 mL RNA Later (Ambion, Austin, TE, USA) and stored at -70°C for further RNA- and DNA-extraction. A longitudinal strip of

gastric tissue was cut from the oesophagus to the duodenum along the greater curvature for histopathological examination.



1. rUreB			rUreB	rUreB	*H. suis*
2. rGGT			rGGT	rGGT	*H. suis*
3. rGGT+rUreB			rGGT+rUreB	rGGT+rUreB	*H. suis*
4. rGGT→ rUreB	rGGT	rGGT	rUreB	rUreB	*H.suis*
5. Lysate			Lysate	Lysate	*H.suis*
6. Sham-immunized			HBSS	HBSS	*H. suis*
7. Negative control			HBSS	HBSS	Brucella broth pH5

Figure 1. Experimental design of vaccination study. Per group 10 mice were intranasally immunized twice with 3 weeks interval, each time with 30 µg rUreB + 5 µg cholera toxin (CT), 30 µg rGGT + 5 µg CT, 30 µg rGGT + 30 µg rUreB + 5 µg CT, and 100 µg lysate + 5 µg CT (groups 1, 2, 3 and 5, respectively). Groups 6 (sham-immunized group) and 7 (negative control group) were intranasally immunized with HBSS. Mice from group 4 (rGGT→rUreB) were first intranasally immunized twice with 3 weeks interval, each time with 30 µg rGGT and 5 µg CT. One week after the second immunization animals were immunized twice with 3 weeks interval with 30 µg rUreB + 5 µg CT. Three weeks after the last immunization, blood was collected by tail bleeding from 5 animals per group and one week later, all animals, except the negative control group, were intragastrically inoculated 10^8 viable *H. suis* bacteria. The negative control

group was intragastrically inoculated with 200 µL Brucella broth at pH5. Four weeks after challenge with *H. suis*, mice were euthanized. [a] I (x): Immunization of (number of group).

2.4. Determination of the number of H. suis *in the stomach*

After thawing, stomach tissues were homogenized (MagNAlyser, Roche, Mannheim, Germany) in 1 mL Tri Reagent® RT (MRC, Brunschwig Chemie, Amsterdam, The Netherlands) and DNA was extracted from the inter- and organic phase according to Tri Reagent® RT manufacturer's instructions. The bacterial load in the stomach was determined using a previously described *H. suis* specific quantitative real-time PCR (qPCR) [13].

2.5. Stomach cytokine responses

The mRNA expression levels of IFN-γ, TNF-α, IL-4, IL-10 and IL-17 were assessed by RT-qPCR using cDNA synthesized from stomach tissue as described previously [14]. The threshold cycle (Ct) values were normalized to the geometric mean of the Ct-values from the reference genes after which normalized mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method [15].

2.6. Serum antibody responses

Anti-rUreB, -rGGT and -lysate serum immunoglobulin G (IgG) responses were assessed by using the Protein Detector™ enzyme-linked immunosorbent assay (ELISA) Kit (KPL, Gaithersburg MD, USA). Measurement of anti-rUreB, anti- rGGT and -lysate specific serum IgG was performed as previously described [6]. In brief, 96 well flat bottom plates (Nunc MaxiSorp, Nalge Nunc Int., Rochester, NY, USA) were coated with 1 µg/well of purified rUreB, 2 µg/well

of purified rGGT, or 1 µg/well of *H. suis* whole cell proteins diluted in 100 µL coating buffer. After blocking with 1% bovine serum albumin in PBS, 100 µL of 1/400 diluted serum was added to each well. After further washing, 100 µL of HRP-labeled anti-mouse IgG (H+L) in a final concentration of 50 ng per well was added. Absorbance was read at 405nm (OD_{405nm}).

2.7. Histopathological examination

Longitudinal strips of gastric tissue were fixed in 4% phosphate buffered formaldehyde, processed by standard procedures and embedded in paraffin. For evaluation of gastritis, haematoxylin - eosin (HE) stained sections of 5 µm were blindly scored based on the degree of infiltrating lymphocytes, plasma cells and neutrophils using a visual analog scale similar to the Updated Sydney System (on a scale of 0-3) [16] with additional specifications for each score. The inflammation scores used in the grading system were as follows: 0, no infiltration with mononuclear and/or polymorphonuclear cells; 1, mild diffuse infiltration with mononuclear and/or polymorphonuclear cells; 2, moderate diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of one or two inflammatory aggregates; 3, marked diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of at least three inflammatory aggregates.

2.8. Statistical analysis

Significant differences in *H. suis* colonization and mRNA cytokine expression among groups were assessed by performing one-way ANOVA analysis. Bonferroni's multiple comparison test was used as post-hoc when equal variances were assessed. Dunnett's T3 post-hoc test was used when no equal variances were assessed. OD_{405nm} levels from ELISA were compared by Kruskall-

Wallis analysis, followed by a–Dunn's multiple comparison test. Histological inflammation scores were compared using the Mann-Whitney *U* test. For correlations between different variables, Spearman's rho coefficient (ρ) was calculated. GraphPad Prism5 software (GraphPad Software Inc., San Diego, CA) was used for all analyses. Statistically significant differences between groups were considered at $p < 0.05$.

3. Results

3.1. Protective effect of immunizations against H. suis *challenge*

All immunizations induced a significant reduction of gastric bacterial load compared to sham-immunized infected mice ($p < 0.05$), albeit significantly less pronounced in the group solely immunized with rGGT compared to all other immunizations ($p < 0.01$) (Figure 2). Highest levels of protection were seen in animals immunized with the combination of rGGT+rUreB or with lysate, with a 10 000-fold and 1000-fold reduction, respectively, of *H. suis* numbers (expressed as median) in the stomachs, compared to non-immunized infected controls ($p < 0.001$). Although not significant ($p > 0.05$), an enhanced protective effect was observed in mice immunized with combinations of rGGT and rUreB compared to rUreB alone. Immunization with rUreB alone, lysate, rGGT+rUreB and the subsequent immunization of rGGT followed by rUreB resulted in 33%, 50%, 57% and 44% of mice negative for *H. suis* DNA, respectively. Immunization with rGGT alone did not result in mice negative for *H. suis* DNA in the stomach. During the study 14 animals died, and the mortality rate per group is shown in Supplementary file 1.

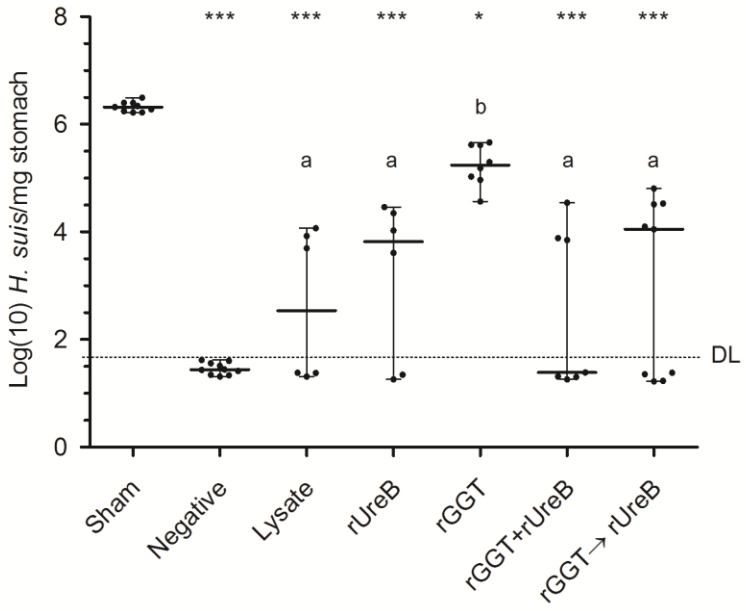


Figure 2. Protection against *H. suis* challenge after prophylactic immunization. Bacterial load per mg stomach tissue was determined for individual mice in each group by qPCR and is illustrated as dots with indication of median (horizontal lines) and range (vertical lines). The dotted line (DL) designates the detection limit of 41.8 copies/mg stomach tissue.* $p < 0.05$, *** $p < 0.001$ compared to non-immunized (sham) *H. suis*-challenged mice. Immunized groups which differed significantly ($p < 0.01$) are marked with different letters. rGGT→rUreB: group of mice which were sequentially immunized with rGGT and rUreB.

3.2. Stomach cytokine responses

mRNA cytokine expression levels (IFN-γ, TNF-α, IL-4, IL-10 and IL-17) in gastric tissue are presented in Figure 3. Significantly higher levels of IL-17 and INF-γ were observed in animals

from all immunized groups, except in the group immunized with rGGT only, compared to sham-immunized mice ($p < 0.05$). Increased levels of IL-17 and IFN-γ were significantly correlated with a decrease in bacterial load ($p < 0.05$, $\rho = -0.513$ and $\rho = -0.2955$, respectively). For IL-4, IL-10 and TNF-α no significant differences in mRNA expression levels were seen between groups after infection. However, mRNA expression levels of TNF- α were increased in all immunized groups, except in the group immunized with rGGT only, compared to non-immunized mice. In addition, a mild negative correlation was observed between gastric bacterial load and TNF-α expression levels ($p < 0.05$, $\rho = -0.349$). Lower levels of IL-10 were observed in immunized animals, compared to sham-immunized mice ($p > 0.05$) and a mild positive correlation was observed between gastric bacterial load and IL-10 expression levels ($p < 0.05$, $\rho = 0.356$).

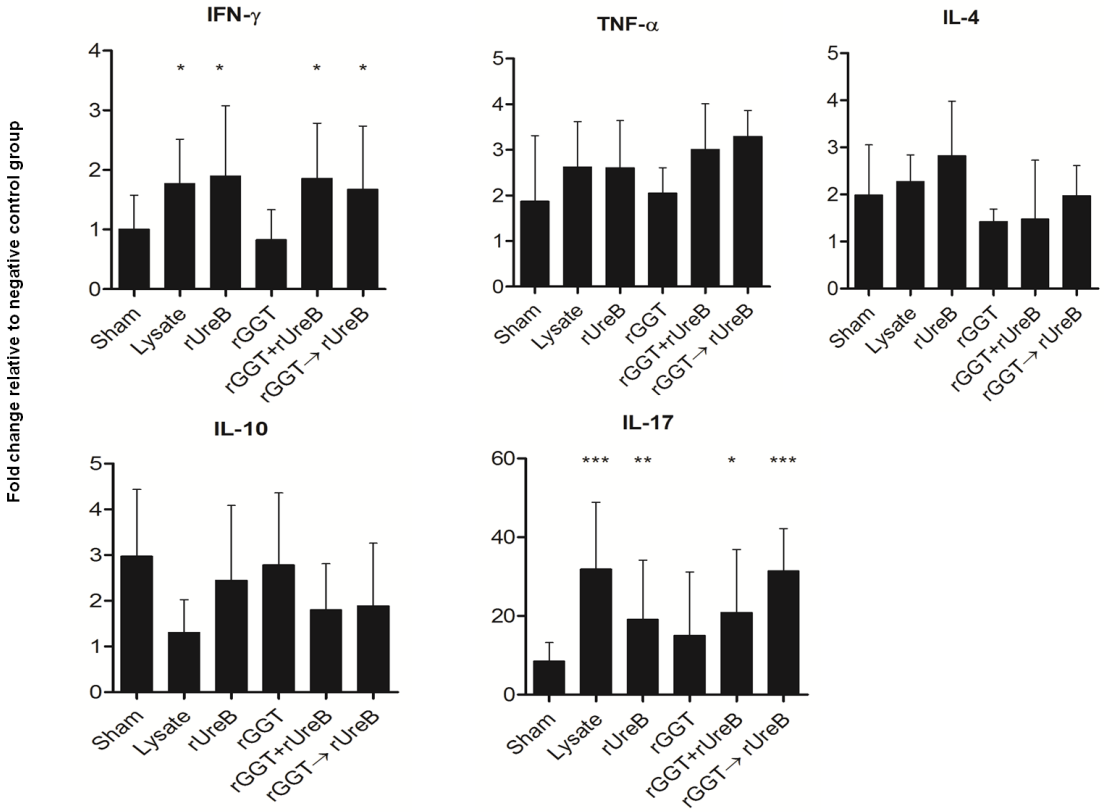


Figure 3. Fold change in cytokine gene expression levels in stomach tissue relative to negative control animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to non-immunized (sham) *H. suis*-challenged group. rGGT→rUreB: group of mice which were sequentially immunized with rGGT and rUreB.

3.3. Humoral immune responses

Three weeks after the last immunization, 1 week prior to challenge, specific serum anti-rUreB, anti-rGGT and anti-lysate IgG antibodies, were significantly increased in animals immunized with respective antigens compared to negative control mice (Supplementary file 2). Serum

antibody responses against *H. suis* lysate, rUreB and rGGT at euthanasia are shown in Figure 4. Negative controls and sham-immunized mice showed significantly lower anti-lysate, -rUreB and -rGGT serum IgG antibodies at euthanasia compared to groups vaccinated with lysate, -rUreB and/or -rGGT, respectively. A weak, but significant ($p < 0.05$, $\rho = -0.235$) correlation was observed between decreased bacterial load and increased specific serum IgG.

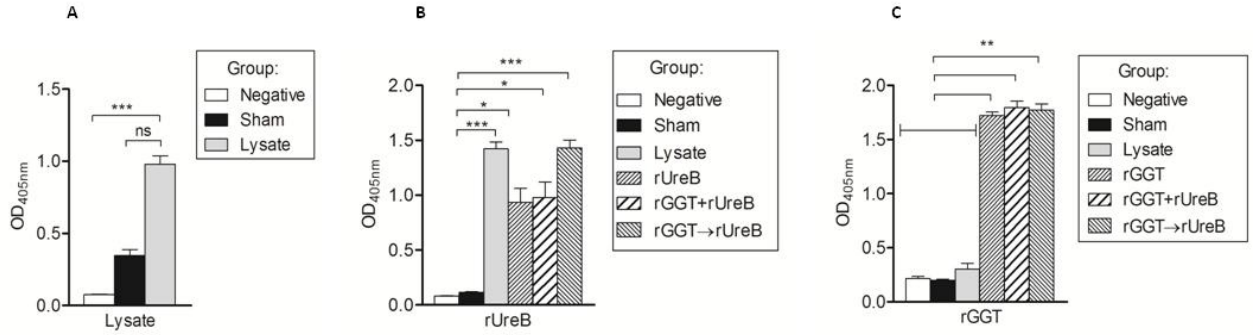


Figure 4. Serum antibody responses against *H. suis* lysate (A), rUreB (B) and rGGT (C) at euthanasia. Different groups are indicated by the bars with levels of specific IgG shown as the mean OD_{405nm} + SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: not significant. rGGT→rUreB: group of mice which were sequentially immunized with rGGT and rUreB.

3.4. Histopathology

Figure 5 provides the results of histopathological examination of the stomachs. Higher inflammation scores were observed in the fundus compared to the antrum (Fig 5A and B). All negative controls had a normal gastric histomorphology (score 0) and sham-immunized, infected mice showed a weak gastric infiltration of mononuclear and/or polymorphonuclear cells (Fig 5C,

median score: 0.25). Most pronounced inflammation was observed in mice immunized with *H. suis* lysate (Fig 5C, median score: 1.125), followed by animals immunized with rUreB alone (Fig 5C, median score: 1.00) and mice immunized with the bivalent vaccine of rGGT and rUreB (Fig 5C, median score: 0.75). Immunization with rGGT alone and consecutive immunization of rGGT and rUreB resulted in less gastric infiltration of mononuclear and/or polymorphonuclear cells compared to all other immunizations, with a median score of 0.50 (Fig 5C). Inflammation in the fundic region and the average inflammation score of animals sequentially immunized with rGGT and rUreB were significantly lower compared to lysate-immunized mice ($p = 0.0033$ and $p = 0.0062$, respectively). Lysate-immunized mice also showed significantly higher overall gastric inflammation and more severe inflammation in the fundic region compared to sham-immunized mice ($p = 0.016$ and $p = 0.013$, respectively). Average gastritis scores of *H. suis*-challenged groups immunized with lysate, rUreB, rGGT and rGGT+rUreB (simultaneously administered) differed significantly from that of non-infected negative control mice ($p < 0.05$).

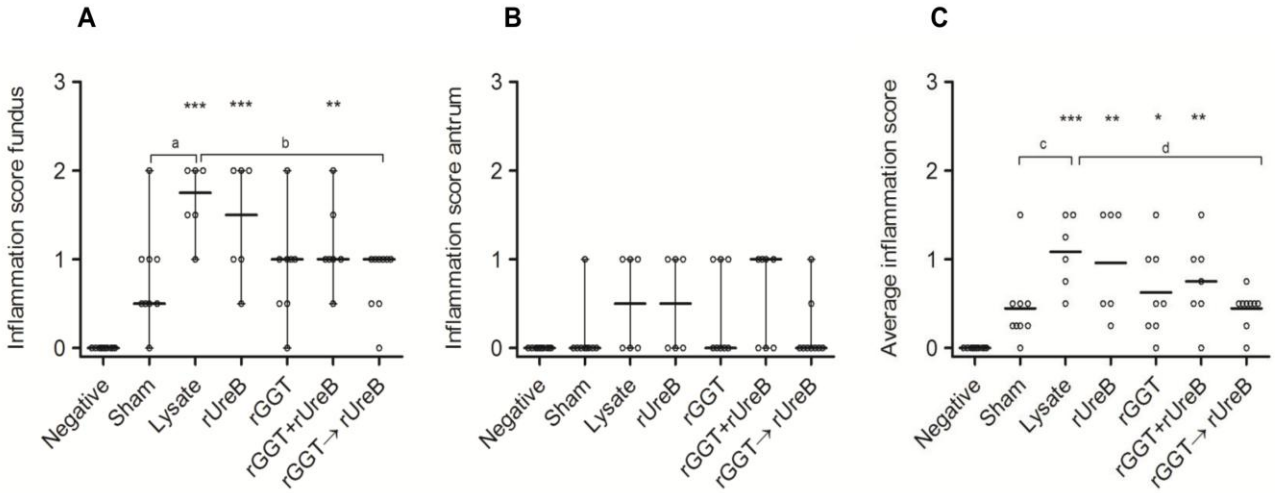


Figure 5. (see legend on next page)

Figure 5. Gastric inflammation scores per group. Scores in negative controls, immunized and non-immunized (sham) mice 4 weeks after challenge were determined in fundus (A) and antrum (B) using haematoxylin-eosin-stained gastric sections. Average of inflammation score of fundus and antrum (average inflammation score) were calculated for each animal per group (C). 0, no infiltration with mononuclear and/or polymorphonuclear cells; 1, mild diffuse infiltration with mononuclear and/or polymorphonuclear cells; 2, moderate diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of one or two inflammatory aggregates; 3, marked diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of at least three inflammatory aggregates. Gastric scores of individual mice per group are illustrated as dots with indication of median (horizontal lines) and range (vertical lines). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to non-infected negative control group. a: significant difference in inflammation in fundic region between sham-immunized and lysate-immunized mice, $p = 0.013$. b: significant difference in inflammation in fundic region between lysate-immunized mice and mice sequentially immunized with rGGT and rUreB (rGGT→rUreB), $p = 0.0033$. c: significant difference in average inflammation score between sham-immunized and lysate-immunized mice, $p = 0.016$. d: significant difference in average inflammation score between lysate-immunized mice and rGGT→rUreB immunized group, $p = 0.0062$.

Discussion

In a recent study, we demonstrated that intranasal vaccination with rUreB alone resulted in a significant reduction of gastric *H. suis* colonization, although complete protection was not achieved [6]. Therefore, the present study aimed to evaluate whether a combination of rUreB and rGGT could increase the vaccine efficacy. *H. suis* GGT is a secreted virulence factor that acts in a similar way as the *H. pylori* GGT. The enzyme causes a glutathione degradation-dependent epithelial cell death [10,17]. In addition, it inhibits the proliferation of T-cells and thus may prevent the generation of an effective host immune response [8,18].

Although immunization with rUreB or rGGT alone induced a significant reduction of gastric *H. suis* colonization, consecutive or simultaneous immunization with both antigens was more effective. The improved protective effect, of vaccination with combinations of rGGT and rUreB compared to immunization with rUreB only, may be related to the consistent anti-GGT response, which might overcome immune evasion induced by this enzyme, enhancing clearance of the bacteria after challenge. Vaccination with *H. suis* rGGT alone seems, however, to be less effective than vaccination with rUreB alone.

H. suis colonization in mice generally induces a predominant Th17 response, in combination with a less pronounced Th2 response [14]. Despite this clear immune response, *H. suis* persists in infected mice. In contrast to *H. pylori* infection, *H. suis* infection does not result in increased levels of IFN-γ, a signature Th1 cytokine [14]. In the present study, vaccinated and protected mice showed significantly increased IFN-γ mRNA levels after challenge compared to sham-immunized mice and the degree of protection was correlated with increased levels of IFN-γ.

Increased expression levels of the pro-inflammatory cytokine, TNF-α were also correlated with decreased bacterial gastric colonization. This indicates that a Th1 response may be involved in protective immunity against *H. suis* infection in mice. Indeed, we previously suggested that a combination of local Th17 and Th1 responses, complemented by antibody responses are involved in the protective immunity against *H. suis* infections [6]. Also in this study the degree of protection was correlated with increased levels of IL-17, a marker of Th17 response, and specific serum IgG responses.

A decreased expression level of IL-10 was correlated with a reduction in gastric *H. suis* colonization. IL-10 is an anti-inflammatory cytokine, which is known to down-regulate immunity to infection and in this way may help gastric *Helicobacter* spp. to persist in their host [6,19,20]. In addition to IL-10, combined vaccination of rGGT and rUreB depicts reduced levels of IL-4. Although increased levels of pro-inflammatory cytokines (IFN-γ, TNF-α and IL-17) and a downregulation of IL-10 and IL-4 mRNA were observed in mice immunized with combinations of rGGT and rUreB, rather a decrease of the microscopic gastric lesions were observed. The reason for this seemingly contradictory result remains to be investigated.

Ideally, an efficacious vaccine should induce protection whilst limiting side-effects. In prophylactic *H. pylori* mouse vaccination experiments, a more pronounced gastritis is often observed after challenging of immunized mice. This post-immunization gastritis is an important issue in the development of vaccines against *H. pylori*, especially when using whole-cell lysates [21-23]. In the present study, the severity of gastric inflammation was higher in mice immunized with whole-cell lysate compared to other immunizations and sham-immunized, infected mice.

315 Animals immunized first with rGGT followed by rUreB showed remarkably lower gastric
316 inflammation compared to other immunized groups. The reason for this reduced inflammation is
317 unclear and requires further studies. For example, the role of gastric and systemic cellular
318 immune responses in induction and evolution of post-immunization gastritis may be determined
319 by using $CD4^+$-, B cell- or neutrophil deficient mice [24]. In addition, it has been shown that
320 post-immunization gastritis disappears over time, indicating that it is a transient event [22,25]. A
321 long term study could therefore be interesting to examine the evolution of the inflammatory
322 response in all immunized, *H. suis*-challenged mice.

323 The results obtained in our mouse model may also be relevant for pigs, which act as the natural
324 host of *H. suis*. Further studies are, however, necessary to confirm this. From an anatomical
325 point-of-view, the nasopharynx-associated lymphoid tissue (NALT) of pigs is organized as
326 tonsils, and forms the basis of the Waldeyer's ring [26], while NALT in rodents are presented as
327 paired lymphoid aggregates in the floor of the nasal cavity at the entrance to the pharyngeal duct
328 [27]. In rodents, lymphocytes from the nose preferentially home back to NALT, as well as
329 cervical and mesenteric lymph nodes, but not to Peyer's patches [28]. It is not clear whether this
330 is also true for lymphocytes of the porcine NALT. Nevertheless, intranasal vaccination of pigs
331 could be a promising route of vaccination for inducing protection not only at the local mucosa,
332 but also at distant mucosal surfaces, as has been described for immunization against enteric
333 colibacillosis [29].

334 In this study, an unexpected high mortality was observed in immunized groups that were
335 experimentally infected with *H. suis*, within days after challenge. This was not observed in sham-

immunized and negative control groups, indicating that this most likely relates to the combination of immunization and subsequent challenge. The exact cause of death, however, was unclear. An extensive local immune response after immunization and subsequent challenge with *H. suis* might be a possible cause of death. Based on autopsy results of some animals, a pronounced local immune response related to the administration route (intranasal) and the adjuvant (CT) after immunization and subsequent challenge, may lead to excessive swelling of the nasal cavity mucosae, resulting in oxygen deficiency. In future *H. suis* mouse vaccination experiments, it should therefore be evaluated whether other mucosal administration routes, such as sublingual or oral immunization, could lead to a similar degree of protection without increased mortality.

In conclusion, immunization of mice with the combination of rGGT and rUreB, protected mice against a *H. suis* infection and induced less severe gastric lesions after *H. suis* challenge than immunization with a whole-cell vaccine. Both proteins are potential candidates for inclusion in subunit vaccines for control of *H. suis* infections. However, additional studies are needed to confirm the present results.

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References

[1] De Bruyne E, Flahou B, Chiers K, Meyns T, Kumar S, Vermoote M, et al. An experimental *Helicobacter suis* infection causes gastritis and reduced daily weight gain in pigs. Vet Microbiol 2012;160:449-54.

[2] Barbosa AJA, Silvia JCP, Nogueira AMMF, Paulino E, Miranda CR. Higher incidence of *Gastrospirillum* sp. In swine with gastric ulcer of the pars oesophagea. Vet Pathol 1995;32:134-9.

[3] Roosendaal R, Vos JH, Roumen T, Van Vugt R, Cattoli G, Bart A, et al. Slaughter pigs are commonly infected with closely related but distinct gastric ulcerative lesion-inducing gastrospirilla. J Clin Microbiol 2000;38:2661-4.

[4] Haesebrouck F, Pasmans F, Flahou B, Chiers K, Baele M, Meyns T, Decostere A, Ducatelle R. Gastric helicobacters in domestic animals and nonhuman primates and their significance for human health. Clin Microbiol 2009;22:202-23.

[5] Flahou B, Hellemans A, Meyns T, Duchateau L, Chiers K, Baele M, et al. Protective immunization with homologous and heterologous antigens against *Helicobacter suis* challenge in a mouse model. Vaccine 2009;27:1416-21.

[6] Vermoote M, Van Steendam K, Flahou B, Pasmans F, Glibert P, Ducatelle R, et al. Immunization with the immunodominant *Helicobacter suis* urease subunit B induces partial protection against *H. suis* infection in a mouse model. Vet Res 2012;43:72.

374 [7] Haesebrouck F, Pasmans F, Chiers K, Maes D, Ducatelle R, Decostere A. Efficacy of
375 vaccines against bacterial diseases in swine: what can we expect? Vet Microbiol 2004;100:255-
376 68.

377 [8] Zhang G, Ducatelle R, Pasmans F, Haesebrouck F, Flahou B. Immune modulating effects
378 of *Helicobacter suis* γ-glutamyl transpeptidase and its substrates, glutamine and glutathione, on
379 lymphocytes. Plos One, provisionally accepted.

380 [9] Baele M, Decostere A, Vandamme P, Ceelen L, Hellemans A, Chiers K, et al. Isolation
381 and characterization of *Helicobacter suis* sp. nov. from pig stomachs. Int J Syst Evol Microbiol
382 2008;58:1350-8.

383 [10] Flahou B, Haesebrouck F, Chiers K, Van Deun K, De Smet L, Devreese B, et al. Gastric
384 epithelial cell death caused by *Helicobacter suis* and *Helicobacter pylori* γ-glutamyl
385 transpeptidase is mainly glutathione degradation-dependent. Cell Microbiol 2011;13:1933-55.

386 [11] Flahou B, De Baere T, Chiers K, Pasmans F, Haesebrouck F, Ducatelle R. Gastric
387 infection with *Kazachstania heterogenica* influences the outcome of a *Helicobacter suis* infection
388 in Mongolian gerbils. Helicobacter 2010;15:67-75.

389 [12] Orlowski M, Meister A. γ-Glutamyl-p-nitroanilide: a new convenient substrate for
390 determination and study of L- and D- γ-glutamyltranspeptidase activities. Biochim Biophys Acta
391 1963;73:679-81.

392 [13] Vermoote M, Pasmans F, Flahou B, Van Deun K, Ducatelle R, Haesebrouck F.
393 Antimicrobial susceptibility pattern of *Helicobacter suis* strains. Vet Microbiol 2011;153:339-42.

[14] Flahou B, Van Deun K, Pasmans F, Volf J, Rychlik I, Ducatelle R, et al. The immune response of mice after *Helicobacter suis* infection: strain differences and distinction with *Helicobacter pylori*. Vet Res 2012;43:75.

[15] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 2001;25:402-8.

[16] Dixon MF, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis: the updated Sydney system. Am J Surg Pathol 1996;20:1161-81.

[17] Shibayama K, Kamachi K, Nagata N, Yagi T, Nada T, Doi Y, et al. A novel apoptosis-inducing protein from *Helicobacter pylori*. Mol Microbiol 2003;47:443-51.

[18] Schmees C, Prinz C, Treptau T, Rad R, Hengst L, Voland P, et al. Inhibition of T-cell proliferation by *Helicobacter pylori* γ-glutamyl transpeptidase. Gastroenterology 2007;132:1820-33.

[19] Matsumoto Y, Blanchard TG, Drakes ML, Basu M, Redline RW, Levine AD, et al. Eradication of *Helicobacter pylori* and resolution of gastritis in the gastric mucosa of IL-10-deficient mice. Helicobacter 2005;10:407-15.

[20] Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity of infection. J Immunol 2008;180:5771-7.

411 [21] Goto T, Nishizono A, Fujioka T, Ikewaki J, Mifune K, Nasu M. Local secretory
412 immunoglobulin A and postimmunization gastritis correlate with protection against *Helicobacter*
413 *pylori* infection after oral vaccination of mice. Infect Immun 1999;67:2531-9.

414 [22] Garhart CA, Redline RW, Nedrud JG, Czinn SJ. Clearance of *Helicobacter pylori*
415 infection and resolution of postimmunization gastritis in a kinetic study of prophylactically
416 immunized mice. Infect Immun 2002;70:3529-38.

417 [23] Morihara F, Fujii R, Hifumi E, Nishizono A, Uda T. Effects of vaccination by a
418 recombinant antigen ureB138 (a segment of the β-subunit of urease) against *Helicobacter pylori*
419 infection. J Med Microbiol 2007;56:847-53.

420 [24] Becher D, Deutscher ME, Simpfendorfer KR, Wijburg OL, Pederson JS, Lew AM, et al.
421 Local recall responses in the stomach involving reduced regulation and expanded help mediate
422 vaccine-induced protection against *Helicobacter pylori* in mice. Eur J Immunol 2010;40:2778-90.

423 [25] Sutton P, Danon SJ, Walker M, Thompson LJ, Wilson J, Kosaka T, et al. Post-
424 immunization gastritis and *Helicobacter* infection in the mouse: a long term study. Gut 2001;
425 49:467-73.

426 [26] Liebler-Tenorio E, Pabst R. MALT structure and function in farm animals. Vet Res
427 2006;37:257-80.

428 [27] Kuper CF, Koornstra PJ, Hameleers DMH, Biewenga J, Spit BJ, Duijvestijn AM, et al.
429 The role of nasopharyngeal lymphoid tissue. Immunol Today 1992;13:219-24.

[28] Koornstra PJ, Duijvestijn AM, Vlek LF, Marres EH, van Breda Vriesman PJ. Tonsillar (Waldeyer's ring equivalent) lymphoid tissue in the rat: lymphocyte subset binding to high endothelial venules (HEV) and in situ distribution. Reg Immunol 1992;4:401-8.

[29] Lin J, Mateo KS, Zhao M, Erickson AK, Garcia N, He D, et al. Protection of piglets against enteric colibacillosis by intranasal immunization with K88ac (F4ac) fimbriae and heat labile enterotoxin of *Escherichia coli*. Vet Microbiol 2013;162:731-9.

Supplementary files

Supplementary file 1. Mortality rate in different groups during the study.

Group	Mortality rate (%)
1. rUreB	40%[a]
2. rGGT	20%[a]
3. rGGT+rUreB	30%[a]
4. rGGT→rUreB	10%[a]
5. Lysate	40%[b]
6. Sham-immunized	10%[c]
7. Negative control	0%

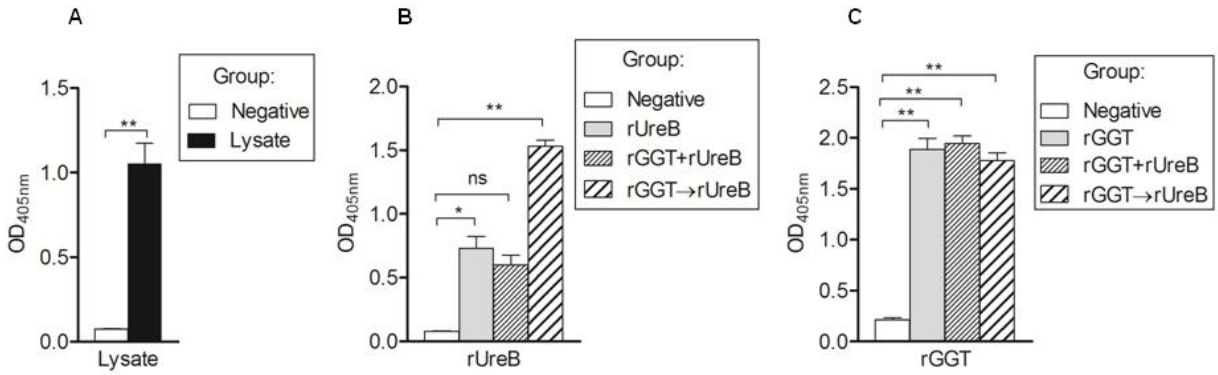
Mice of groups 1, 2, 3 and 5 were intranasally immunized twice with rUreB, rGGT, rGGT+rUreB or lysate, respecitively. Groups 6 (sham-immunized group) and 7 (negative control group) were intranasally inoculated with HBSS. Mice from group 4 (rGGT→rUreB) were first intranasally immunized twice with 30 µg rGGT and 5 µg CT. One week after the second immunization animals were intranasally immunized twice with 30 µg rUreB and 5 µg CT. Four weeks after the last immunization, all animals, except the negative control group, were intragastrically inoculated 10^8 viable *H. suis* bacteria. The negative control group was intragastrically inoculated with 200 µL Brucella broth at pH5. Four weeks after challenge with *H. suis*, mice were euthanized.

[a]Mice died 2 to 5 days after intragastric challenge with *H. suis*.

[b]Two mice died before challenge: one animal 5 days after the first immunization and one animal 4 days after the second immunization. Two animals died 2 and 3 days after intragastric challenge with *H. suis*.

[c]One mouse was euthanized because of a reason unrelated to the study.

Supplementary file 2. Serum antibody responses against *H. suis* lysate (A), rUreB (B) and rGGT (C) at three weeks after last immunization.



Different groups are indicated by the bars with levels of specific IgG shown as the mean OD_{405nm} + SD. * $p < 0.05$, ** $p < 0.01$, ns: not significant. rGGT→rUreB: group of mice which were sequentially immunized with rGGT and rUreB.