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Enhanced immune responses to viral epitopes by combining macrophage-inducible expression with multimeric display on a Salmonella vector

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Abstract

In this study, the immunogenicity of chimeric 987P fimbriae on a Salmonella vaccine strain was improved by optimizing fimbrial expression. The constitutive tetA promoter and the in vivo activated nirB and pagC promoters were evaluated for their use to express two epitopes of the transmissible gastroenteritis virus (TGEV) spike protein carried by fimbriae which were displayed on a Salmonella vaccine strain. Constructs with the pagC promoter were shown to drive increased expression of chimeric 987P fimbriae in macrophages as well as in Mg²⁺-poor media, mimicking a major environmental signal found in Salmonella-containing endocytic vacuoles of macrophages. Mice immunized orally with a Salmonella vaccine strain which expressed chimeric fimbriae from the pagC promoter elicited significantly higher mucosal and systemic immune responses to both the 987P fimbriae and the TGEV epitopes than mice immunized with the same strain hosting a tetA or nirB promoter-driven expression plasmid. Moreover, only the Salmonella vaccine strains harboring a plasmid with the pagC promoter, with or without an additional tetA promoter in tandem, elicited neutralizing antibodies to TGEV. This indicated that the pagC promoter can be used successfully to improve epitope-display by chimeric fimbriae on Salmonella vaccine strains for the induction of a desired immune response. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Fimbriae; TGEV; Salmonella typhimurium; Vaccine; pagC

1. Introduction

Transmissible gastroenteritis virus (TGEV) is a member of the Coronaviridae family in the Nidovirales order [1]. TGEV replicates both in the villous epithelial cells of the small intestine and in the lung cells of newborn piglets, resulting in a mortality of nearly 100% [2]. Protection of the newborn animals from TGEV infection requires the induction of secretory immunoglobulin A (IgA) in milk, especially in colostrum [2]. Typically, IgA-producing plasma cells in the mammary gland of pregnant sows originate from the gut-associated lymphoid tissues (GALT) where they were activated by different antigenic components. Thus, antigen-specific colostral antibodies can be induced by oral vaccines with enteric tropism [3,4].

Several viral proteins are important for inducing immune responses to coronavirus: the spike protein (S), the nucleocapsid protein (N) and the membrane protein (M) [3,5,6]. Study of the induction of protective immunity to TGEV has focused on the spike protein (S), because it is the major inducer of TGEV neutralizing antibodies [7,8] and mediates binding of TGEV to its cellular receptor aminopeptidase N [9]. The relevant epitopes for neutralization were mapped to the N terminal domain of S protein, and four major antigenic sites (A to D) were identified independently by two groups [7,10]. Sites C and A (using the nomenclature of Delmas et al.) are involved in the induction of neutralization antibodies [7] and stretches of continuous residues in each site were shown to be recognized by neutralizing monoclonal antibodies [7,10].

Various approaches have been used to develop TGEV vaccines. The current commercial vaccines include inactivated or live attenuated TGEV vaccines.
2. Materials and methods

2.1. Mice

Six-week-old female Balb/cByJ mice were obtained from the Jackson Laboratory and housed in filter-top cages in an air-conditioned animal facility. Water and food were provided ad libitum. Mice were adapted for 1 week after arrival before being used for immunization.

2.2. Bacterial strains, media and reagents

E. coli and Salmonella typhimurium strains used in this study are listed in Table 1. Strains χ6212 and χ4550 were grown in LB medium with 50 μg/ml DL-α-aminopimelic acid (DAP, Sigma, St Louis, MO). Medium components were purchased from Difco (Detroit, MI). Restriction and modification enzymes were from New England Biolabs, Inc. (Beverly, MA). Unless specified, reagents were purchased from sigma.

2.3. Plasmid constructs

Standard procedures were used to construct the following plasmids. A DNA fragment comprising the pagC promoter was cloned from S. typhimurium χ4550 by PCR (upper primer 5’CGGGATCCGGTATTAC-CTCTTAATAATAAT, lower primer 5’CGGGATCCCGTGAGCTCCATCCCAGTAC) and was added to the Bam HI site just upstream of fasA in pCS154. This newly generated plasmid, pCS165, carries both the tetA and pagC promoters. Plasmid pCS173, which contains only the pagC promoter, was created by removing the smallest Hind III fragment containing the tetA promoter from pCS165. pCS176, which carries the nirB promoter alone, was constructed by removing the Hind III fragment of pCS155 containing the tetA promoter.

2.4. Macrophages

Both the murine macrophage-like cell line J774A.1 and murine peritoneal macrophages were used in this study. J774A.1 (ATCC TIB-67; kind gift of Dr Howard Goldfine) was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1.0 mM sodium pyruvate and 10% fetal bovine serum (DMEM-complete) at 37°C under an atmosphere of 5% CO2. Murine peritoneal macrophages were harvested as previously described [27]. Briefly, mice were euthanized by cervical dislocation, and 6–8 ml of Hanks balanced saline buffer (HBSS) was injected into the peritoneal cavity of each mouse. The abdomen of each mouse was massaged vigorously, and peritoneal macrophages were withdrawn using a syringe. Cells were pelleted by cen-
trifugation (250 × g, 10 min) and suspended in DMEM-complete. The cell suspensions were distributed in 24-well plates, and incubated at 37°C for 2 h to allow the macrophage to adhere to the plastic. Nonadherent cells were washed away with HBSS. These enriched populations of macrophages were then cultured in DMEM-complete at 37°C under an atmosphere of 5% CO₂.

2.5. Peptides and fimbriae

The TGEV C and A peptides of the spike protein, corresponding to amino acid residues 379–388 and 521–531, respectively, were both synthesized with a cysteine added to their carboxy termini (SFFSYGEIPC and MKRSGYGQPIAC) at the Protein Chemistry Laboratory of the University of Pennsylvania School of Medicine. Fimbriae expressed on the bacterial surface were prepared by heat extraction, as described previously [28].

2.6. Seroagglutination and antibodies

Slide seroagglutination tests were performed with preadsorbed rabbit anti-987P antiserum [29], with an anti-TGEV-C epitope antiserum [19] and with an anti-TGEV-A epitope antiserum [20].

2.7. SDS-PAGE and Western blotting

Bacterial pellets, isolated fimbriae or macrophage lysates were resuspended in sample buffer, boiled for 5 min, and the proteins were separated by SDS-PAGE. Western blots were probed with rabbit anti-987P fimbriae antibodies, using horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence (ECL) for detection [20]. Relative amounts of chimeric FasA protein of the different constructs were evaluated by densitometry, using the NIH Image software (Division of Computer Research and Technology, National Institutes of Health, Bethesda, MD).

2.8. Phagocytosis assay

To determine the expression of chimeric fimbriae in macrophages, the bacteria were opsonized in 10% fresh mouse serum in phosphate-buffered saline (PBS) for 20 min and added to cell cultures at a multiplicity of infection of 10, as described elsewhere [30]. Internalization were allowed to proceed for 1 h. Nonphagocytosed bacteria were washed away with PBS. The infected macrophages were cultured in DMEM-complete containing 50 µg/ml gentamicin sulfate for 24 h. The macrophages were washed three times with PBS and lysed with 0.1% Triton X-100 in PBS to release the intracellular bacteria. The bacteria were then collected by centrifugation (4000 × g, 5 min), solubilized in SDS-PAGE sample buffer and subjected to Western blotting.

2.9. Immunization and sampling

Mice were immunized and samples were collected and processed essentially as described previously [20,31,32]. For each immunization, a single colony of Salmonella was grown in L broth without any antibiotics at 37°C on a rotary shaker at 150 rev./min overnight. The bacterial cells were gently washed once and resuspended in sterile phosphate-buffered saline (PBS; 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl, pH 7.2) at the concentration of 1–5 × 10¹¹ CFU/ml. Viable counts were performed on all inocula. Before immunization, the mice were deprived of food and water for 4 h. The mice were intubated with feeding needles for intragastrical delivery of 200 µl of bacterial suspensions and fasted for an additional 30 min. The mice were immunized twice at day 0 and 30. For each immunized group of mice, pooled fecal pellets were collected on a weekly basis. Approximately 500 mg feces were added to tubes containing 2 ml of a protease inhibitor solution (PBS with 0.5% BSA and a cocktail of protease inhibitors, Complete™, Boehringer Mannheim, Ger, using the manufacturer’s recommended concentration). The fecal pellets were soaked in ice for 15 min and the tubes were agitated vigorously for 5 min twice on a vortex at maximum speed. The suspensions were centrifuged at 13 000 × g for 15 min and the supernatants were stored at −20°C. To collect serum samples and intestinal secretions, mice were anesthetized with Metofane (methoxyflurane, Mallinckrodt Veterinary Inc., Mundelein, IL) and exsanguinated. Blood was collected by heart puncture. Whole small intestines, from duodenum to the ileo-cecal junction, were excised and luminal contents were carefully collected with the help of 3 ml of protease inhibitor solution introduced into intestinal lumens. Recovered intestinal contents were vortexed vigorously for 5 min. After centrifugation at 13 000 × g for 15 min at 4°C, supernatants were collected and stored at −20°C.

2.10. ELISA

Individual mouse sera and intestinal secretions, and group-pooled fecal pellet extract were tested for immunoglobulin A (IgA) and IgG antibodies against 987P fimbriae, TGEV C or A peptides by enzyme-linked immunosorbent assay (ELISA) essentially as described [20,29]. Briefly, 96-well ELISA plates (Immulon 4, Dynatech Laboratories, Inc., Chantilly, VA) were coated with isolated wild type 987P fimbriae (0.2 µg in 100 µl 0.1 M carbonate buffer, pH 9.6, per well) overnight at
4°C. TGEV C or A peptid(1.0 µg in 100 µl 0.1 M carbonate buffer, pH 9.6, per well) were coated to the plates using a microwave oven as described [33] and further overnight coating at 4°C. The plates were blocked with 0.5% BSA in PBS at 37°C for 2 h, washed four times with PBS and incubated with serial dilutions of body fluid samples in PBS--0.1% BSA--0.05% Tween-20 for 2 h at 37°C. After the second washing step, plates were incubated with HRP-conjugated anti-mouse IgG, IgG1, IgG2a or IgA antibodies at 37°C for 1 h. Following the last washing step, bound antibodies were detected by using o-phenylenediamine as the chromogenic reagent and reading the absorbance at 450 nm.

2.11. Dot blot assay

Nitrocellulose strips were spotted with TGEV C and TGEV A peptides, the strips were incubated with gut washes, and the blots were developed with HRP-conjugated anti-mouse IgG, IgG1, IgG2a or IgA antibodies at 37°C for 1 h. Following the last washing step, bound antibodies were detected by using o-phenylenediamine as the chromogenic reagent and reading the absorbance at 450 nm.

2.12. Virus seroneutralization test

TGEV neutralization was determined using a limiting dilution microassay with 96-well plates [34]. Briefly, 50 µl of serial twofold dilutions of antisera were mixed with an equal volume of virus suspension containing 100 TCID₅₀ of TGEV Purdue-115 strain (kind gift from Dr Linda J. Saif). After incubation at 37°C for 1 h, 4 × 10⁴ trypsinized swine testis (ST) cells (ATCC CRL-1746) in 100 µl DMEM supplemented with 10% fetal bovine serum and 0.1 mM non-essential amino acids were added to the antiserum–virus mixtures. Neutralization titers were determined 48 h later and calculated as the mean of the highest dilution that neutralized 100% of the cytopathic effect in duplicate experiments. Positive and negative reference sera were included in each experiment.

2.13. Statistical analysis

Groups of log-transformed data were compared by using the unpaired Student’s t-test [35]. Probability (P) values of less than 0.05 indicated that the groups were significantly different.

3. Results

3.1. Construction of the in vivo inducible expression plasmids

To stabilize the antigen-expressing multicopy-number plasmids in live Salmonella vectors in vivo, we used the asd balanced lethal system [36,37]. In plasmid pCS154, expression of the chimeric 987P fimbriae is driven by the promoter for the tetracycline resistance gene tetA [20]. Seroagglutination determined a high and constant level of 987P fimbrial expression of S. typhimurium χ4550/pCS154 in the absence or presence of subminimal inhibitory concentrations of tetracycline (data not shown), suggesting that pCS154 mediated-fimbrial expression was constitutive and not regulated by a TetR repressor [38]. The immunogenicity of chimeric 987P fimbriae expressed by Salmonella crp cya vaccine strains was shown previously to be improved by constructing and using pCS155 which contains a synthetic DNA fragment encoding the nirB promoter inserted between the tetA promoter and the fasA gene of pCS154 [20]. In this study, a similar strategy was used to construct plasmids containing the pagC promoter. A 294-bp fragment of DNA encompassing the pagC promoter of S. typhimurium χ4550 was cloned by standard PCR. The amplified fragment was inserted between the tetA promoter and the fasA gene of pCS154, generating pCS165. Consequently, both pCS155 and pCS165 have two promoters driving the expression of chimeric 987P fimbriae. To construct expression plasmids carrying only the in vivo-inducible promoter nirB or pagC, the tetA promoter-containing Hind III fragments of pCS155 and pCS165 were deleted, resulting in pCS176 and pCS173, respectively.

3.2. Environmental regulation of chimeric 987P fimbriae on Salmonella

Whether each plasmid construct contained the sequences necessary to drive gene expression after induction by the appropriate environmental signal was determined in vitro. The expression of chimeric 987P fimbriae was studied in S. typhimurium χ4550 carrying plasmids pCS165, pCS173 or pCS176 using different growth conditions. The amounts of fimbriae isolated from the same numbers of bacteria were compared on SDS-polyacrylamide gels. To test activation of the nirB promoter in χ4550/pCS176, the bacteria were grown statically in a capped-flask to reduce the oxygen level in the culture [39]. The aerated culture expressed a low level of fimbriae which increased threefold when the bacteria were cultured statically in a capped-flask (Fig. 1, lanes 8–9). The pagC promoter in pCS165 or pCS173 were repressed by supplementing the bacterial culture medium with MgCl₂. As the concentration of MgCl₂ increased, the level of fimbrial expression decreased (Fig. 1, lanes 2–7). In LB media supplemented with 10 mM MgCl₂, χ4550/pCS173 merely expressed fimbriae, while χ4550/pCS165 still expressed relatively high levels of fimbriae. This result was expected, since pCS165 can also drive fimbrial expression from its tetA promoter (Fig. 1). LB by itself contains approximately 0.23 mM Mg²⁺ (Difco, typical analysis sheets), thus,
fimbrial expression might be even higher with bacteria grown under the Mg\(^{2+}\)-limiting conditions (1 \(\mu\)M) shown to best signal the induction of PhoP-activated gene transcription [40]. Taken together, the data confirmed that fimbriae expression from the constructs with the pagC promoter are regulated by Mg\(^{2+}\) concentrations.

3.3. Expression of chimeric fimbriae by Salmonella in macrophages

To verify that the pagC promoter in plasmids pCS165 and pCS173 can be induced in macrophages, the expression of chimeric fimbriae by *Salmonella* constructs in both J774A.1 macrophage-like cell line and fresh peritoneal macrophages were analyzed by Western blotting. *S. typhimurium* \(\chi 4550\) expressing the fimbriae from the pagC promoter by harboring pCS165 or pCS173, produced high levels of chimeric fimbriae in both the J774A.1 cells (Fig. 2A, lanes 2 and 3) and the peritoneal macrophages (Fig. 2B, lanes 2 and 3). In contrast, *S. typhimurium* \(\chi 4550\) expressing the fimbriae from the tetA promoter of pCS154 (Fig. 2, lane 1) or from the nirB promoter of pCS176 (Fig. 2, lane 4) showed distinctively lower levels of chimeric fimbriae in both kinds of macrophages.

3.4. Systemic humoral immune responses against chimeric fimbriae

The immunogenicity of the chimeric 987P fimbriae expressed by *S. typhimurium* \(\chi 4550\) hosting different plasmids was evaluated after oral immunization of Balb/c mice. 987P-specific serum IgG was detected in all mice immunized with *S. typhimurium* \(\chi 4550\) vaccine strains regardless of which expression plasmid they harbored (Fig. 3A). However, the antibody titers were significantly higher in the mice immunized with \(\chi 4550/pCS165\) or \(\chi 4550/pCS173\) than those in mice immunized with \(\chi 4550/pCS154\) or \(\chi 4550/pCS176\) (Fig. 3A). No other comparison in antibody titers identified significant differences. Thus, the data indicated that use of the plasmids with the pagC promoter significantly enhanced the immunogenicity of the chimeric 987P fimbriae. To better characterize the type of T helper cell response, the serum anti-987P fimbriae IgG1 and IgG2a subclasses of the different mice groups were determined. In mice immunized with strains expressing the fimbriae from the pagC promoter (with or without the tetA promoter), the anti-987P fimbriae IgG response was dominated by the IgG2a subclass (Fig. 3B). In contrast, mice immunized with strains expressing the fimbriae from the tetA or nirB promoter alone had a significantly lower IgG2a response. Statistically significant higher levels of IgG2a were detected \((P < 0.05)\) in the mice immunized with strains expressing the fimbriae from the pagC promoter, whereas statistically significant higher levels of IgG1 were detected \((P < 0.05)\) in the mice immunized with strains expressing the fimbriae from the tetA promoter alone. This suggested that mice from the former immunization groups developed a better type 1 helper T-cell (Th1) response to the 987P fimbriae than mice from the latter group.
3.5. Intestinal humoral immune responses against chimeric fimbriae

The local gut anti-987P fimbriae IgA and IgG responses were measured by analyzing gut washes of immunized mice. Similar to the systemic humoral response described above, the mucosal anti-987P IgA (Fig. 4A) and IgG (Fig. 4B) titers were much higher in the mice immunized with strain χ4550/pcS165 or χ4550/pcS173 than in the mice immunized with strain χ4550/pcS154 or χ4550/pcS176. There were no significant differences between the χ4550/pcS165- and χ4550/pcS173-immunized groups or between the χ4550/pcS154- and χ4550/pcS176-immunized mice. All the mucosal IgA titers correlated significantly with the serum IgG titers (correlation coefficient \( r = 0.66; P < 0.001 \)). The kinetics of the mucosal antibody response were evaluated by analyzing extracts of fecal pellets. As expected, the responses were quite variable. After the first vaccination, the mice immunized with strain χ4550/pcS165 or χ4550/pcS173 developed higher mucosal IgA (Fig. 5A) and IgG (Fig. 5B) responses for 2–3 weeks. After 3 weeks, mucosal antibody titers in most groups declined; however, both the mucosal IgA and IgG titers increased dramatically after the mice were boosted orally at 4 weeks. In all immunized groups, mucosal antibody titers reached a peak at 6 weeks and did not decrease significantly for at least two more weeks when the mice were sacrificed.
4. Discussion

In this paper, we evaluated the use of combining the display of multimeric foreign epitopes on the surface of *S. typhimurium* with an in vivo-inducible promoter as a delivery system for vaccine development. The *pagC* promoter was particularly attractive, since it is activated in the environment of phagocytic vacuoles, after *S. typhimurium* is taken up by macrophages [41]. By genetically engineering *asd*<sup>+</sup>-stabilized plasmids expressing chimeric 987P fimbriae on *S. typhimurium* under the control of the *pagC* promoter, the systemic and mucosal immune responses to the chimeric fimbriae were improved significantly, in comparison to a construct directing antigen expression from a constitutive *tetA* promoter or from the *nirB* promoter. Most importantly, and in addition to the finding that the *pagC* promoter was the most effective one for inducing anti-fimbriae antibodies, TGEV-neutralizing antibodies were induced only with *S. typhimurium* constructs carrying this promoter for antigen expression. We had shown previously that chimeric fimbriae on *S. typhimurium* induced a better immune response when the fimbriae were expressed from a tandem promoter, where the in vivo-inducible *nirB* promoter was added to the constitutively-expressed *tetA* promoter (x<sub>4550</sub>:pCS155) [20]. In this study, the new vaccine construct with the *pagC* promoter alone induces higher immune responses than the vaccine using the *nirB* promoter together with the *tetA* promoter [20]. Moreover, the immune response was improved by using a construct combining the *pagC* and *tetA* (x<sub>4550</sub>/pCS165) promoters. Curiously, the consistent slight improvement in immune responses we observed with the *pagC* and *tetA* construct were statistically significant only for the TGEV C epitope, but not for the fimbrial antigen itself. Consistent with previous studies, the TGEV A epitope was not an effective immunogen for Balb/c mice [18,20].

Among the various in vivo-inducible promoters, including the *nirB*, *htrA*, *groE*, *osmC*, *katG*, *spa*, *dps* and *pagC* promoters, which have been investigated to improve the immunogenicity of heterologous antigens expressed by *Salmonella* vectors in the last decade [31,32,42–44], the *pagC* promoter appears to be the most efficient one. This promoter is regulated through the PhoP/PhoQ two-component signal transduction system of *Salmonella* that senses environmental Mg<sup>2+</sup> and Ca<sup>2+</sup> concentrations [45,46]. The *pagC* promoter is activated at relatively low concentrations of Mg<sup>2+</sup>, a condition which is found within *Salmonella*-containing vacuoles in macrophages [47–49]. As shown previously for single heterologous protein antigens [31,45], it was demonstrated here that the *pagC* promoter can also drive 987P fimbrial expression in response to a low Mg<sup>2+</sup> concentration, and thus activates 987P fimbriation in macrophages. In *Salmonella*, the *pagC* gene encodes an outer membrane protein which is essential

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**Fig. 4.** 987P fimbriae-specific mucosal IgA (A) and IgG (B) titers. Balb/c mice were immunized orally with two doses (30-day interval) of *S. typhimurium* x<sub>4550</sub> containing pCS165, pCS173, pCS154 or pCS176. Gut washes were collected before immunization, to be used as negative controls, and at the end of the immunization experiments. The samples were measured individually and error bars represent the S.D. of the values for 10 mice. Mucosal IgA and IgG titers elicited by *S. typhimurium* x<sub>4550</sub>/pCS173 or x<sub>4550</sub>/pCS165 were significantly higher than the titers induced by *S. typhimurium* x<sub>4550</sub>/pCS154 or x<sub>4550</sub>/pCS176 (*P < 0.05; **P < 0.01).
for full bacterial virulence in Balb/c mice and is highly expressed in vivo. Thus, the observed induction of the neutralizing anti-TGEV immune response can most probably be attributed to the expression of large amounts of TGEV peptide antigen on the Salmonella and to the intracellular location of the bacteria in professional antigen-presenting cells.

It has been shown recently that fimbriae expressed by Salmonella vaccine strains stimulate a mixed T helper cell response [20,50,51]. Interestingly, a comparison of the anti-987P IgG1/IgG2a ratios of the animals immunized with strains χ4550/pCS165 or χ4550/pCS173 with the ratios of the animals immunized with strains χ4550/pCS154 or χ4550/pCS176 suggests that the former groups developed a biased Th1 response, while the latter groups developed a predominant Th2 response [50–53]. Moreover, stronger mucosal antibody responses were developed in the former groups. Taken together, the data suggest that the pagC promoter is more appropriate than the nirB or tetA promoter for expressing antigens when a polarized Th1 response or a strong mucosal response is required for immunity, although more in-depth studies, including cytokine profiling, will be required for confirmation.

Compared to other expression methods for heterologous antigen display on Salmonella vaccines, the 987P fimbrial display system has several advantages that favor its use. First, fimbriae are polymeric proteins which allow inserted foreign epitopes to be presented hundreds of times along fimbrial threads, which themselves are numbered in the hundreds on the bacterial surface. Second, because enteroadhesion of 987P fimbriae is essentially mediated by its minor subunit FasG [28,54], the major subunit FasA can be genetically modified as a carrier molecule without affecting the enteroadhesive property of the fimbriae [19]. Having demonstrated previously that the FasA subunit can be used as a carrier of protective epitopes of TGEV [19] and that Salmonella vaccine strains can be genetically manipulated to express the chimeric fimbriae [20], we combined these approaches with the beneficial aspects of in vivo expression by the pagC promoter as a novel strategy for designing better vaccines. The developed system was found to be efficient, since anti-TGEV neutralizing antibodies were detected for the first time after oral administration of a Salmonella vector delivering a TGEV immunogen. Moreover, the observed high titers of mucosal and systemic anti-987P antibodies, as well as the high titers of anti-Salmonella antibodies (data not shown), demonstrated the benefits of our combined approach to develop a multivalent vaccine targeting simultaneously major enteropathogens. It will be of interest to test whether the system can be developed further to display protective epitopes derived from other swine pathogens, such as rotavirus and foot-and-mouth disease virus. Most importantly, future studies will have to be undertaken in the pig to confirm our findings in the relevant host.
Fig. 6. TGEV C epitope-specific serum IgG titers. Balb/c mice were immunized orally with two doses (30-day interval) of S. typhimurium χ4550 containing pCS165, pCS173, pCS154 or pCS176. Sera collected before immunization were used as negative controls. The sera were measured individually and error bars represent the S.D. of the values for 10 mice. IgG titers elicited by S. typhimurium χ4550/pCS165 were significantly higher than the titers induced by any of the three other strains, and IgG titers elicited by S. typhimurium χ4550/pCS173 were significantly higher than the titers induced by χ4550/pCS154 or χ4550/pCS176; (*P < 0.05; **P < 0.01; ***P < 0.001).

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