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Intra-peritoneal and intra-rectal immunogenicity induced by rotavirus virus like particles 2/6/7 in mice

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ABSTRACT

We previously developed virus like particles of rotavirus (RV) with VP2, VP6, and VP7 proteins (VLP2/6/7) using stable High-five cell line. To evaluate the immunogenicity of our construct, we assessed the humoral and cytokine responses induced by VLP2/6/7 in BALB/c mice immunized intra-peritoneally and intra-rectally. Enzyme-linked immunosorbent assay (ELISA) and Relative quantitative (RQ) Real-time PCR were used to evaluate the antibody (IgG and IgA) levels in serum and mRNA levels of IL-6, IL-10 and IFN-γ in spleen cells, respectively. Our results showed that VLP2/6/7 is capable of intra-peritoneal (I.P.) and intra-rectal (I.R.) induction of serum IgG and IgA responses. IgA was detected in fecal samples of immunization groups by I.P. and I.R. routes. Interestingly, I.R. route induced higher IgA titer compared with I.P. route which was statistically significant. Moreover, mRNA levels of IL-6 and IFN-γ were significantly elevated in mice immunized intra-peritoneally with VLP2/6/7 compared to control group. As such, the mean change was 7.4 ($P < 0.05$) and 14.8 ($P < 0.001$) for IFN-γ and IL-6, respectively. Likewise, the same pattern was found when mice were immunized intra-rectally. Although elevated, the difference in the mean change for IL-10 was not statistically significant when compared to control group. Our findings indicated that VLPs constructed via a stable insect cell line are able to induce both humoral and cellular responses, a similar pattern as observed after immunization with live RVs.

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1. Introduction

Rotaviruses (RVs), as a genus of the Reoviridae family, are a common cause of severe diarrhea in children <5 years of age worldwide, in addition to noroviruses [1–5]. Efforts to develop a RV vaccine have begun in the late 1970s, and Rotashield was the first RV vaccine licensed but was withdrawn in 1999 by its manufacturer because of its association with intussusception during post-licensure surveillance [6–8]. Recently, RotaTeq™ and Rotarix™, as live attenuated vaccines, were developed and shown to efficiently protect against severe diarrhea [9–13]. However, high degree of genetic exchange and reversion of a live vaccine strain to a more virulent state are main concerns that need to be addressed as they can lead to the development of disease in vaccinated children [14–18]. Moreover, concerns regarding safety and efficacy skewed researchers' focus on alternative vaccine candidates such as non-living RV vaccines among which virus like particle (VLP)-based vaccines have shown the most promising results [15].

Different expression systems such as baculovirus expression vector system in insect cells have been used to produce RV VLPs [19–28]. Administration of RV VLPs by different routes, intra-rectal (I.R.), intra-nasal (I.N.), oral and parenteral has been investigated to evaluate immune responses and it has been shown that VLPs are able to induce antibody responses and protect animal models from RV [29–35]. Moreover, looking at the immunogenicity of RV VLPs or recombinant vaccines, the enhancement of immune response has been found following the administration of different adjuvants [30,34,36–38] although the induction of immunogenicity has been also reported in the absence of adjuvants [30,35,39–41]. Compared to subunit vaccines, a lower dose of VLPs needs to elicit a similar protective response, as they mimic the structure of virus particles [42]. Recently, we have developed VLP2/6/7 using a stable

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High-Five cell system (Shoja et al., submitted for publication). In this study, we investigated the immunogenicity of our construct following intra-peritoneal (I.P.) and I.R. administration in mice, and our results indicated that VLPs are able to induce humeral responses (serum IgG and IgA, fecal IgA) consistent with others [29,34]. Interestingly, efficient IgA response was observed in feces when administrated intra-rectally, indicating the importance of local response because the intestinal tract is the portal for ingestion RV.

Until now, there are few reports about the cytokine profile induced by RV VLPs. Previous studies have shown that VLPs administered to mice can induce a mixed Th1/Th2 profile of cytokines very similar to which induced by live RV administered to suckling mice [32,43]. In addition to humoral responses, we further investigated cytokine profile in immunized mice with VLPs using I.P. and I.R. routes. Increased mRNAs level of cytokines was also found, suggesting the induction of cellular immune response. Our result suggests that I.P. and I.R. immunization with VLPs constructed via a stable insect cell line can elicit both humoral and cellular immune responses, further highlights the role of VLPs as a promising vaccine candidates.

2. Materials and methods

2.1. VLPs preparation

In our previous study, we have developed VLPs containing VP2, VP6 and VP7 proteins of RV using a stable High-Five cell system (Shoja et al., submitted for publication). High-Five insect cell line stably and constitutively expressing VLP2/6/7 was grown in serum-free SF900 medium (Life Technologies, Carlsbad, CA) in the presence of G418 and hygromycin antibiotics up to two weeks at 28 °C. Cell supernatants were clarified by centrifugation at 2000 × g for 15 min at 4 °C. VLPs were then purified by ultracentrifugation sedimentation through 25% sucrose cushion at 100,000 × g for 75 min. Purified VLPs were finally resuspended in 1 × PBS as previously described [44,45].

2.2. Immunization experiments

To evaluate the induction of immune responses by VLP2/6/7, female BALB/c mice 6–8 weeks of age were used to inject intra-peritoneally and intra-rectally with VLPs. Ten mice (6 mice for I.P. and 4 mice for I.R.) were used for each immunization and control groups and checked regularly for signs of toxicity, and remained healthy up to the end of the immunization protocol. BALB/c mice were injected intra-peritoneally and intra-rectally with 20 μg of purified VLPs resuspended in 100 μl of PBS. For I.R. immunization, mice were first instigated to defecate, and then immunization was gradually performed. Mice were monitored for 5 min to make sure there is no liquid evacuating through the anus during this time. The immunization schedule was based on a 3-dose regimen, where the booster inoculations were administered at 2 and 4 weeks after the primary injection. Blood samples were drawn from the retro-orbital vein 2 days before the priming dose (pre-immunization) and 7 days after each administration. Fecal samples were collected from each mouse on day 42 (7 days after the last immunization). The mice were then sacrificed and spleens were removed for cytokine profile investigation. All experiments were performed according to the Helsinki guidelines.

2.3. Enzyme-linked immunosorbent assay (ELISA)

The presence and the titer of specific anti-VLP antibodies were evaluated in sera collected from immunized mice by ELISA. Nunc-Immuno F8 MaxiSorp™ plates (Thermo Scientific) were coated with 1:80 dilution of purified VLPs resuspended in PBS. Plates were then incubated with dilutions of heat-inactivated mouse sera and mouse feces ranging from 1:250 to 1:64,000 and 1:40 to 1:10,240, respectively. After 4 times washes with PBS-Tween (0.1%) wells were incubated 2 h at 37 °C with Goat anti-mouse IgG-HRP and Goat anti-mouse IgA (Abcam) antibodies (1:4000 dilution). Positive reactions were visualized with TMB solution and stopped with 2 N sulfuric acid. Absorbance was determined at 450 nm and reactions were considered positive when exceeding the mean absorbance + 2 standard deviations (SD) of equal dilutions of pre-immune sera or feces taken from control group.

2.4. RNA extraction from spleen

Total RNA was extracted from the spleen cells of immunized and control mice using Trizol® total RNA isolation reagent (Gibco BRL, Life Technologies, USA) according to the manufacturer's instructions. Extracted RNA was eluted in 80 μl RNase-free water, and to eliminate contaminating genomic DNA, the RNA samples were treated with RNase-Free DNase I (Fermentase) according to the manufacturer's instructions. RNA purity and concentration were determined using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Based on the absorbance ratio at 260/280 nm (range from 1.8 to 2, Mean ± standard error of the mean (SEM) = 1.878 ± 0.03492) and at A_{260}/A_{230} nm (range from 2 to 2.2, Mean ± SEM = 2.271 ± 0.03551), all RNA samples were pure and protein free. Total RNA from 20 samples (immunized and control mice) was then adjusted to a final concentration of 1.5 μg/μl before stored at −80 °C.

2.5. cDNA synthesis

cDNA was synthesized from total RNA (1.5 μg) using RT enzyme mix and 2× RT reaction mix (includes oligo $(dT)_{20}$ and random hexamers) for one cycle in a final volume of 20 μl as follows: 25 °C for 10 min, 50 °C for 30 min and 85 °C for 5 min using SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, California, CA) according to the manufacturer's instructions. The cDNA was then diluted in nuclease free water and stored at −20 °C.

2.6. Relative quantitative (RQ) real-time PCR

Target genes (IL-6, IL-10 and IFN-γ) were relatively quantified by real-time PCR with SYBR® GreenER™ qPCRSuperMix Universal (Invitrogen, Carlsbad, California, CA) on the Corbett Research Rotor-Gene™ (Qiagen, USA). Briefly, PCR amplification reactions were performed in 20 μl reaction mixtures containing cDNA (10 fold diluted), 2× Sybr Green supermix and 10 pmol of each primer pair (Table 1). The reactions were incubated at 95 °C for 10 min,

Table 1
Primer sequences of target genes and reference gene used for relative quantitative Real-time RT-PCR.

	Primer sequence	Amplicon size (bp)
Target gene		
IL-6	Forward: 5′-TTCCATCCAGTTGCCTTCTTG-3′	98
	Reverse: 5′-GGGAGTGGTATCCTCTGTGAAGTC-3′	
IL-10	Forward: 5′-GGCAGCCTTGCAGAAAAGAG-3′	85
	Reverse: 5′-GCTGATCCTCATGCCAGTCA-3′	
IFN-γ	Forward: 5′-CCTGCGGCCTAGCTCTGA-3′	90
	Reverse: 5′-TGGCAGTAACAGCCAGAAACA-3′	
Reference gene		
β-actin	Forward: 5′-GCTCTGGCTCCTAGCACCAT-3′	75
	Reverse: 5′-GCCACCGATCCACACAGAGT-3′	

followed by 50 cycles of 95 °C for 15 s, 55 °C for 60 s and 60 °C for 60 s. Distilled water (DW) was used as a negative control. A melting curve analysis was performed to confirm single gene-specific peaks by heating samples from 60 to 99 °C at the end of the amplification cycles.

The linearity and accuracy of Real-time RT-PCR were evaluated using β-actin (Reference gene) standard curve derived from amplifying serially diluted pooled cDNA (a 10-fold dilution series). These diluted pooled cDNA were also used for intra- and inter-assay experiments. A serially diluted cDNA was amplified as triplicates on the same plate to assess the intra-assay accuracy with the standard deviation of the mean C_T value ranged from 0.07 to 0.87, with coefficients of variation < 5%. In addition, three independent assays were used to assess the inter-assay accuracy. The standard deviation of the mean C_T value ranged from 0.17 to 1.6, with coefficients of variation < 5%. The linearity and accuracy were determined by the correlation coefficient ($R^2 = 1$) and by the equation for the standard curve ($y = -3.415X + 34.90$).

IL-6, IL-10 and IFN-γ expression was analyzed according to the $2^{-\Delta\Delta CT}$ method and the levels of each cytokine normalized to the β-actin cDNA amount [46]. The relative values of each cytokine were expressed as the fold-increase of the immunized group over the control group to compare mRNA levels between immunized and control groups.

2.7. Statistical analysis

Statistical analysis was performed with GraphPad Prism software, Version 5. Two-way ANOVA was used to analyze humoral immune responses. Quantification of cytokines mRNA level was assessed using the non-parametric test, one-way ANOVA. P value < 0.05 was considered statistically significant.

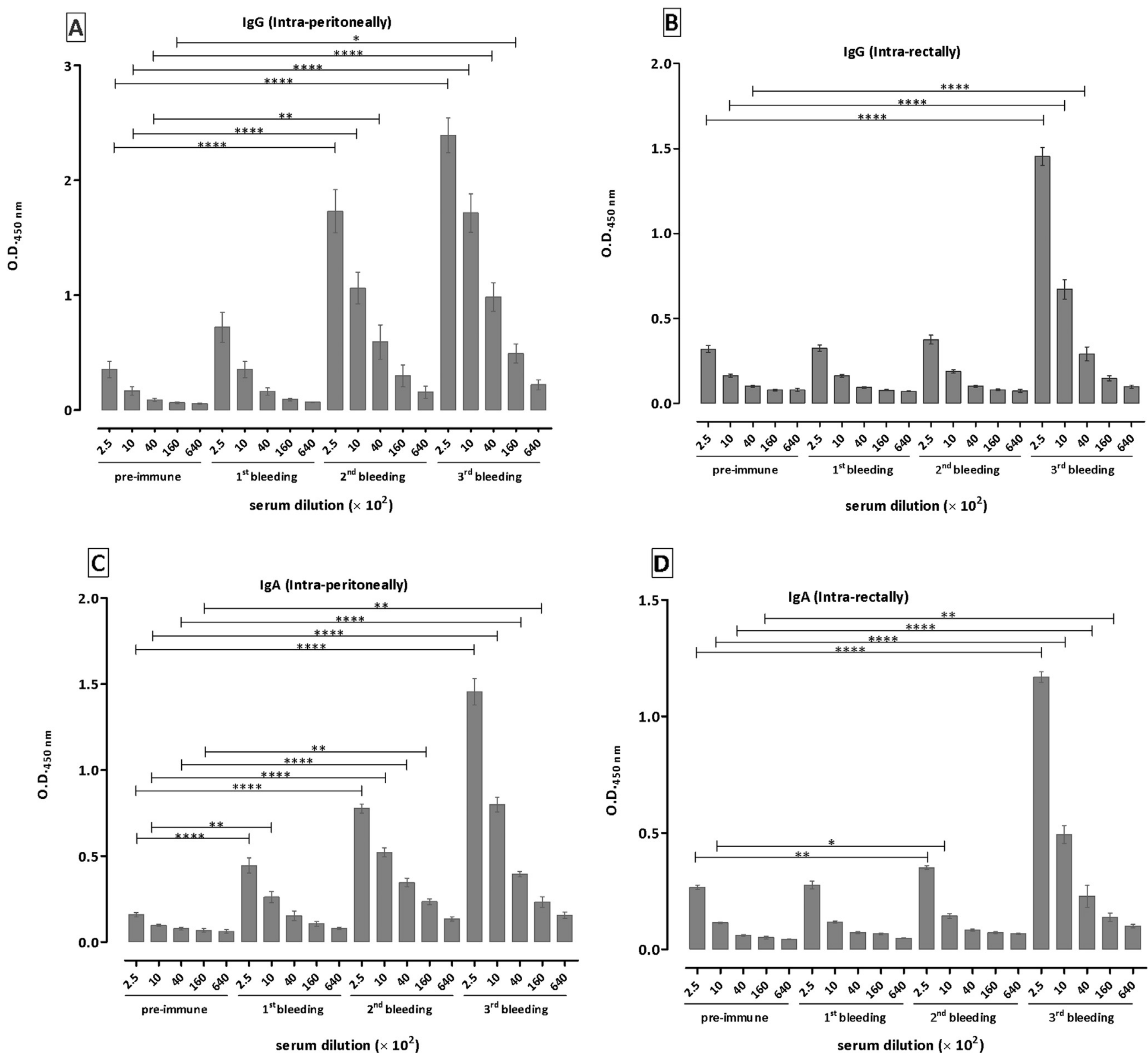


Fig. 1. Anti-VLP2/6/7 IgG and IgA specific responses in serum samples of BALB/c mice immunized intra-peritoneally and intra-rectally. (A and C) O.D.450 values observed per each mice at indicated serum dilutions a week after each intra-peritoneally administration of VLPs (*: P value < 0.05). (B and D) O.D.450 values observed per each mice at indicated serum dilutions a week after each intra-rectally administration of VLPs (*: P value < 0.05). Bars represent the mean ± standard error of the mean (SEM).

3. Results

3.1. In vivo humoral immune response induced by VLP2/6/7 in sera

ELISA tests were performed on Nunc-Immuno F8 MaxiSorp™ plates coated with purified VLPs. As indicated in Fig. 1A, I.P. immunization protocols with VLPs induced anti-VLP IgG titers, reaching from highest dilutions of 1:4000 ($P < 0.01$) to 1:16,000 ($P < 0.05$) for single and double boost immunization, respectively. For anti-VLP IgA titers, the highest dilution for both single and double boost immunization was found 1:16,000 ($P < 0.01$) (Fig. 1C). Moreover, immunizing mice with I.R. induced anti-VLP IgG titers, reaching a highest dilution of 1:4000 ($P < 0.0001$) after double boost immunization (Fig. 1B), whereas the highest dilutions for anti-VLP IgA titers after single and double boost immunization were found 1:1000 ($P < 0.05$) and 1:16,000 ($P < 0.01$), respectively (Fig. 1D). Moreover, as indicated in Fig. 2, IgA was detected in fecal samples collected from mice immunized intra-rectally and intra-peritoneally, reaching from highest dilutions of 1:640 ($P < 0.05$) to 1:160 ($P < 0.05$), respectively. However, low IgA titer was found in feces when the mice were immunized intra-peritoneally compared to intra-rectally.

Our results showed that the I.P. and I.R. administration of VLPs generated by the stable double transfected High-Five cells is able to induce high levels of serum IgG and IgA titer although the difference between two routes (I.P. vs. I.R.) was not statistically significant ($P > 0.05$). In addition, detectable IgA titers in fecal samples suggested that I.R. route can induce efficient IgA response in intestinal tract compared to I.P. route.

3.2. IL-6, IL-10 and IFN-γ relative mRNA level in mice spleen

Prior to assess the relative expression levels of target genes, the linearity and accuracy of the real-time PCR were confirmed (Fig. 3). The mRNA levels of IL-6, IL-10 and IFN-γ genes were normalized and analyzed by the comparative C_T method. Analysis of our results indicated an increase in mRNA levels of cytokine profile. Looking at the mRNA level for IFN-γ and IL-6, significant mean changes in intra-peritoneally immunized mice compared to control group were detected. The mean change observed for IFN-γ and IL-6 was 7.4 ($P < 0.05$) and 14.8 ($P < 0.001$), respectively. Likewise, in VLP-immunized mice the level of IL-10 mRNA increased by a mean of 1.98 fold, however, the difference was not statistically significant ($P > 0.05$) when compared to control group. Additionally, similar pattern was found when mice were immunized intra-rectally with VLPs. In this group, the mean change for IFN-γ and IL-6 was found to be 8.8 ($P < 0.05$) and 14.5 ($P < 0.001$), respectively. For IL-10 the mean change was 4.9 ($P > 0.05$) (Fig. 4). However, comparing immunized mice groups based on the immunization routs (I.P. vs. I.R.) there was no significant difference in the relative mRNA levels of IL-6 and IFN-γ ($P > 0.05$).

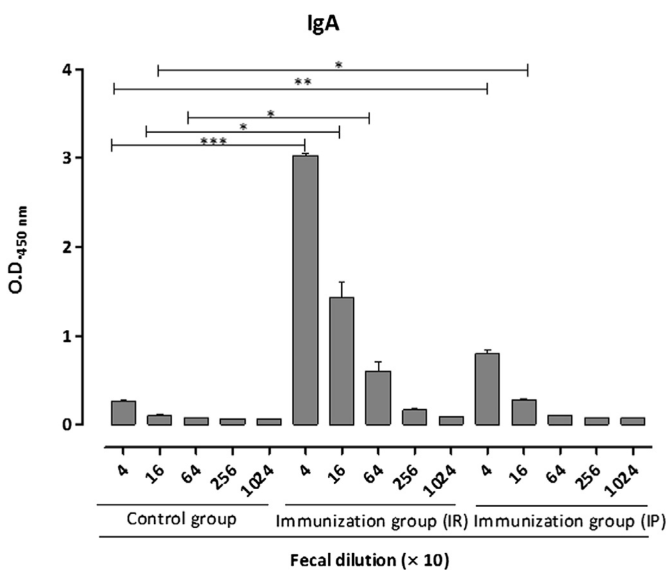


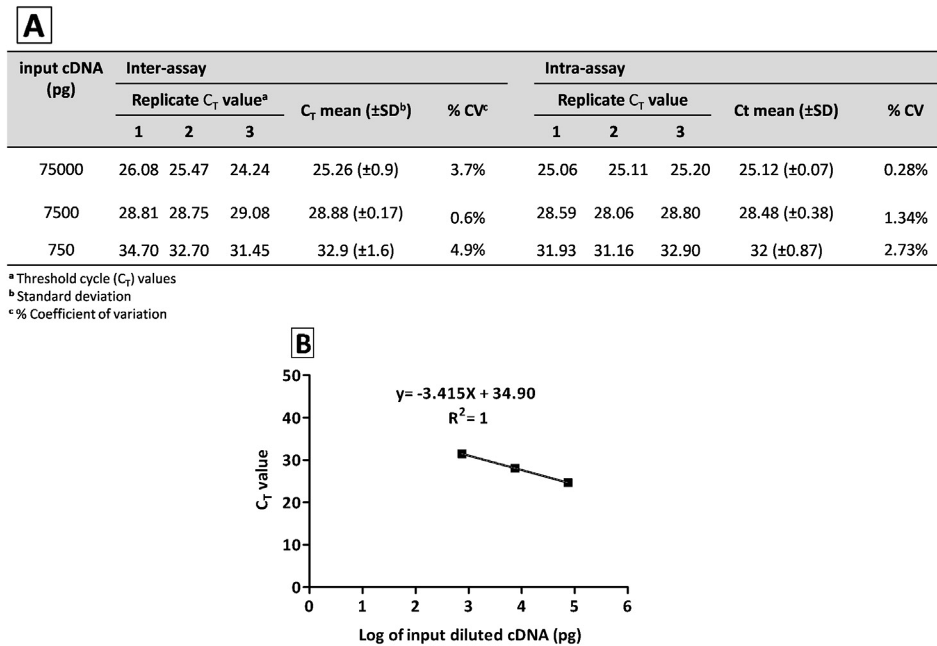
Fig. 2. Anti-VLP2/6/7 IgA specific responses in fecal samples of BALB/c mice immunized intra-peritoneally and intra-rectally. O.D.450 values observed per each mice at indicated fecal dilutions a week after last intra-peritoneally and intra-rectally administration of VLPs (*: P value < 0.05). Bars represent the mean ± SEM.

4. Discussion

Diarrhea, as the most common illnesses in children, results from various pathogenic agents particularly enteric viruses. RVs, as a major cause of severe acute gastroenteritis, are responsible for around 6% of diarrheal episodes and 20% of diarrheal related deaths in children <5 years of age in developing countries [47,48]. Data obtained from epidemiological and hospital-based studies supports the necessity of further investigation to prevent RV infection and vaccine development [49,50]. Enormous efforts have been made to develop a safe and effective vaccine. So far, non-living vaccines particularly VLPs-based vaccines have provided the most promising results. In line with this, a wide range of homologous or heterologous VLPs have been developed.

We have previously constructed VLP2/6/7 using stable High-Five cell system (Shoja et al., submitted for publication). In this study we evaluated the immunogenicity of VLP2/6/7 in BALB/c mice administered by I.P. and I.R. routes, and investigated the mRNA levels of IL-6, IL-10 and IFN-γ using relative quantitative real-time PCR.

Our results showed that specific serum anti-VLP IgG was induced following VLP administration regardless of I.P. and I.R. routes although I.P. immunization group induced a 1-dilution rise in the specific serum anti-VLP titers compared to I.R. immunized group. A progressive increase of specific serum anti-VLP IgA effectively elicited in both the I.P. and I.R. immunization groups. Our findings showed that I.P. route is able to induce a significant IgG and IgA responses, whereas, I.R. route induced a stronger IgA than IgG response. Moreover, IgA was detected in fecal samples of immunization groups by I.P. and I.R. routes. Interestingly, I.R. route was found to induce significantly higher IgA titer compared to I.P. route. In previous studies, parenterally administered live RV or VLPs have been shown high levels of serum and fecal antibody in animal models [30,33,35,51–53]. In 1996 and 2003, Conner et al., and Bertolotti-Ciarlet et al., documented the immunogenicity and protective efficacy of VLPs administered intra-muscularly to rabbits and mice, respectively [30,52]. Parenteral administration of RV vaccine has been demonstrated to be protective in several animal models. Moreover, mucosally (oral and I.N. routes) administered VLPs have been indicated to elicit serum and intestinal antibodies [30,35,54,55] although I.N. route offered high level of antibodies and protection against virus challenge [30,54]. In 2006, Parez et al., first documented the induction of high levels of mucosal and systemic antibodies via rectal administration of VLPs [34]. Similar findings were also found following mice immunization via I.N. and I.R. routes although I.R. route displayed more efficient response in intestinal tract [29]. Our findings were in agreement with previous studies suggesting VLPs can induce IgG and IgA responses through both the I.P. and I.R. routes, providing further evidence that VLPs are immunogenic. We also demonstrate that I.R. route induces higher IgA response than I.P. route in the intestinal tract, similar to



A

input cDNA (pg)	Inter-assay					Intra-assay				
	Replicate C_T value[a]			C_T mean (±SD[b])	% CV[c]	Replicate C_T value			Ct mean (±SD)	% CV
	1	2	3			1	2	3		
75000	26.08	25.47	24.24	25.26 (±0.9)	3.7%	25.06	25.11	25.20	25.12 (±0.07)	0.28%
7500	28.81	28.75	29.08	28.88 (±0.17)	0.6%	28.59	28.06	28.80	28.48 (±0.38)	1.34%
750	34.70	32.70	31.45	32.9 (±1.6)	4.9%	31.93	31.16	32.90	32 (±0.87)	2.73%

[a] Threshold cycle (C_T) values
[b] Standard deviation
[c] % Coefficient of variation

Fig. 3. Real-time RT-PCR was performed with primer pair of β-actin as housekeeping gene. (A) Intra-assay accuracy and Inter-assay reproducibility of the β-actin cDNA amounts by Real-time RT-PCR. (B) Linearity and accuracy of β-actin Real-time RT-PCR. 10-fold serial dilutions of the total RNA standard starting from 10 to 1000 ng were amplified in triplicate by Real-time RT-PCR. The linearity and accuracy were determined by the correlation coefficient ($R^2 = 1$) and by the equation for the standard curve ($y = -3.415X + 34.90$).

that reported for the nasal route, which, to date, is considered the most efficient [54,56,57].

In addition to antibody responses, cellular immune responses have also been investigated in few studies. Considering the immunogenicity of our VLP construct, we further evaluated the mRNA levels of IFN-γ a representative of Th1 cytokines, IL-10 a representative of Th2 cytokines, and IL-6 a representative of pro-inflammatory cytokines. Significantly higher IL-6 and IFN-γ mRNA levels were found in immunized groups by both I.P. and I.R. routes. Although not significant, an increased fold of IL-10 mRNA level was observed in immunized group compared to control groups. In 2001, Fromantin et al., have shown for first time that I.N. immunization of mice with VLPs induces high IFN-γ and IL-10 production in spleen and cervical lymph nodes (CLN) [32]. In addition, in the study of Agnello et al. [29], they also reported that I.N. and I.R. immunizations with VLPs induced IFN-γ production in spleen and Peyer's patch cells. Similar results have also been reported for the cellular immune responses following I.N. immunization of mice with VLPs where IL-10, IL-17, IL-2 and IL-4 were induced in spleen and CLN, whereas I.R. immunization only induced IL-2 and IL-17, but not IL-10 and IL-4, in spleen, lumbar lymphoid nodes (LLN) and mesenteric lymphoid nodes (MLN) [43]. A similar cellular immune response was also observed by homologous and heterologous strains of live RV administered orally to suckling mice [58]. Our results showed that VLPs increased IFN-γ and IL-10 mRNAs level in mice immunized intra-peritoneally and intra-rectally which are agreement with the previous reports although the increased IL-10 level was not statistically significant. We also reported increased IL-6 mRNA level in mice immunized intra-peritoneally and intra-rectally which was not reported in previous studies using mice models, suggesting that VLPs may induce high inflammatory stimulus in immunization group. Moreover, cytokine patterns of our study were similar to previous reports described in human study [43,59–61].

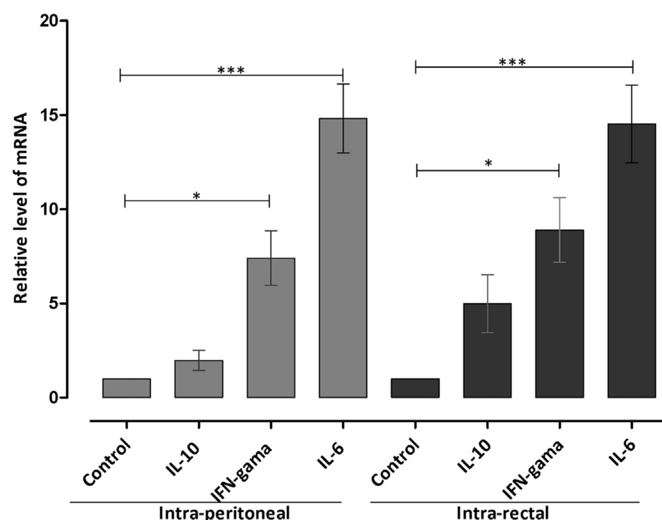


Fig. 4. mRNA levels of IL-6, IL-10 and IFN-γ quantified by relative quantitative (RQ) Real-time RT-PCR of spleen cells RNA extracts of BALB/c mice immunized with VLP2/6/7 intra-peritoneally and intra-rectally. Bars represent the mean ± SEM.

In summary, our findings indicated that VLPs constructed via a stable insect cell line not only induce humoral immune responses but also elicit cellular immune responses in mice administered intra-peritoneally and intra-rectally. Interestingly, the immune response resulted from VLPs suggests a similar pattern observed from studies carried out by live RVs, further highlighting the importance of VLPs for vaccine development.

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