NOTE *Immunology*

Construction of Swine-Specific CpG Motif Enriched Plasmid and the Study of Its Immunostimulatory Effects Both *In Vitro* and *In Vivo*

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ABSTRACT. A swine-specific CpG motif enriched plasmid (pUC18-CpG) was constructed in this study. Its immunostimulant property was tested *in vitro* via lymphocyte transformation assay using swine peripheral blood mononuclear cells (PBMCs). The recombinant plasmid showed higher Stimulation Index (SI) compared to the positive control (LPS). In a following animal experiment, pUC18-CpG was co-administered with a commercial swine FMD killed vaccine. Animals in the pUC18-CpG adjuvanted groups showed much higher antibody titers during the vaccination period.

KEY WORDS: antibody titer, CpG motif, FMD killed vaccine, lymphocyte transformation test, stimulation Index.

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CpG motif was described as a type of Immunostimulatory Sequence (ISS) [8], in which an unmethylated cytosine-phosphate-guanosine (CpG) motif is flanked by two 5'purines and two 3'pyrimidines [13]. Being a pathogen-associated molecular pattern (PAMP), oligodeoxynucleotides (ODN) containing CpG motifs could be recognized by the Toll-like Receptor 9 (TLR9) on certain cell types of the mammalian innate immune system [1, 6]. As a novel immunostimulant, CpG ODN exhibits the characteristic of being species-specific [19], and the sequence for optimal swine PBMCs stimulation was described as GTCGTT [14]. Up to now, CpG ODN with a nuclease-resistant phosphorothioate backbone was used for most of the studies in the relevant research [10, 16, 23], while studies on the adjuvanticity of CpG motif enriched plasmid have been rarely reported.

Foot-and-mouth disease (FMD) is one of the most important OIE notifiable diseases [5]. It is highly contagious to the cloven-footed animals, of great socio-economic consequence and with the potential of rapid international spread [3, 21]. Vaccination is regarded as a powerful strategy for disease control and eradication in FMD endemic areas, as well as in cases of emergency outbreaks in areas normally free from the disease [9]. However, vaccination has failed in some cases to prevent the establishment and transmission of the disease, partly due to the failure of eliciting an effective immune response by the vaccine employed [20], which calls for better FMD vaccine design strategy, including the use of more powerful immunoadjuvant.

In the present study, we have constructed a recombinant plasmid containing multiple swine-specific CpG motifs [14] (pUC18-CpG) and studied its immunostimulatory properties both *in vitro* and *in vivo*.

E.Z.N.A Plasmid Miniprep kit and Gel Extraction Kit were purchased from Omega Bio-Tek (Doraville, GA, U.S.A.); DNA Restriction enzymes and ligation enzyme were from Takara Bio Inc. (Ohtsu, Japan); RPMI Medium 1640 was from Gibco Co., Ltd. (now part of Invitrogen Corporation, Carlsbad, CA, U.S.A.); Fetal calf serum (FCS) was from Sijiqing Biological Engineering Material Co., Ltd. (Hangzhou, China); Lymphocyte separation medium was from Shanghai HuaYi Bio Technoligy Co., Ltd. (Shanghai, China); MTT was purchased from Sigma (St. Louis, MO, U.S.A.); Swine FMD killed vaccine (OS/99 strain) was purchased from China Animal Husbandry Industry Co., Ltd. (Beijing, China); The O type FMDV antibody liquid phase block ELISA (LPB-ELISA) Kit was from Lanzhou Veterinary Institute (Lanzhou, China); 96-well cell culture plates were from Corning Co., Ltd. (Glendale, AZ, U.S.A.).

pUC18-CpG containing 20 copies of CpG ODN 2006 (sequence: 5'-TCGTCGTTTTGTCGTTTTGTCGTTTT-GTCGTTTTGTCGTT-3') was generated through tandem insertion of 4 copies of CpG ODN 2006 into the multiple cloning sites (MCS) of the pUC18 plasmid vector. Briefly, 4 copies of CpG ODN 2006 were directionally cloned into the MCS of *EcoRI-SacI, KpnI-BamHI, XbaI-SalI* and *PstI- HindIII,* respectively. pUC18-CpG was multiplied in *Escherichia coli* (*E.coli*) by fermentation. Then, the recombinant plasmid was purified by the large scale plasmid purification method [24]. Briefly, after alkali lysis, the

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plasmid was selectively precipitated from the supernatant by CTAB. Further purification was carried out using potassium acetate and Triton X-114 for the removal of host protein and endotoxin, respectively. The purified plasmid was dissolved in endotoxin-free PBS at 10 mg/m*l*, and stored at −20°C until use.

Blood sample was collected from 6-month-old, female SPF Changbai pigs in heparin tubes via venipuncture and was diluted with sterile PBS at the ratio of 1:1. Swine PBMCs were prepared by Ficoll gradient centrifugation procedure based on the method described by Lai Changhua and his colleagues [2]. PBMCs were separated and washed twice with PBS. The viability of the cells was checked by trypan blue staining. Cells were counted and allocated into 96-well cell culture plates at 2×10^5/well in 200μl RPMI-1640 culture medium supplemented with 10% (FCS). Cells were cultured in CO_2 cell culture incubator under standard conditions.

The swine PBMCs were stimulated by pUC18-CpG at different doses (1.25, 2.5, 5.0 and 10.0 μg, respectively). Each dose was performed in triplicates. Lipopolysaccharide (LPS) was added at 10 μg/m*l* and served as a positive control. Negative control cells were cultured without pUC18-CpG or LPS. The pUC18 vector alone was added to additional 3 cell culture wells. MTT assay was carried out as described in an earlier study by Verma *et al.* [22]. Briefly, at 72 hr of cell culture, 20 μl MTT (5 mg/m*l*) was added per well. Cells were incubated for additional 3 hr. Colored crystals of formazan were dissolved with a 100 μl of SDS-isobutanol-HCl. Plates were kept on orbital shaker for 5 min. Optical density (O.D.) was read on a microplate reader (BIO-RAD) at 570 nm. Stimulation index (SI) was calculated as described by Verma *et al.* [22], in a revised manner.

$$SI = \frac{\text{(O.D. of test sample} - \text{O.D. of blank)}}{\text{(O.D. of negative control} - \text{O.D. of blank)}}$$

Twenty 28-day-old Changbai piglets, after being confirmed free of FMDV infection by ELISA, were randomly divided into 4 groups, 5 per group. Group A was immunized with sterile PBS as a control group; Group B was immunized with a full dose FMD killed vaccine; Group C was co-immunized with 250 μg pUC18-CpG and a full dose vaccine; Group D was co-immunized with 500 μg pUC18-CpG and a full dose vaccine. All formulations were delivered in a total volume of 3 m*l* and the injection was administered intramuscularly. All procedures involving animals conformed to the policies of the local animal care committee.

Blood sample collection was performed prior to vaccination, and then at 2-week interval until 4 weeks. Serum was separated by centrifugation and stored at −20°C until use. The O type FMDV antibody liquid phase block ELISA (LPB-ELISA) Kit was commercially available from Lanzhou Veterinary Institute and used to measure the anti-FMDV structural protein antibody level.

Data analysis was performed with Prism 5 software (GraphPad). Comparison was made by using the Student *t*-test. All values are presented as the mean ± SD. A *P*-value <0.05 is considered statistically significant.

pUC18-CpG stimulated PBMCs proliferation was measured by the MTT colorimetric assay method. The SI value of LPS and different doses of the plasmid was shown in Fig.1. pUC18-CpG showed strong stimulation effect at all different doses, compared to LPS positive control. The SI value of pUC18-CpG at each dose was higher than that of LPS. All the pUC18-CpG groups showed higher SI value than that of the positive control. The highest SI was observed from the 5.0 μg dose group, and showed statistical significance of difference when compared to the other groups (P<0.05).

A positive correlation between the given dose and SI value was observed when the administered dose of the plasmid was below 5.0 μg. When the given dose was increased to 10.0 μg, a drop in the SI value was observed. The blood of two more SPF Changbai pigs was used in separate PBMCs transformation tests, the results of which were consistent with the current study (Data not shown).

The anti-FMDV structural protein antibody titers were tested under the same conditions after the second blood sample collection. The average antibody titers of each group at each time were described in Fig 2. During the whole period of the animal experiment, animals in Group A remained seronegative for FMDV.

On Day 14 after vaccination, animals in Group B showed lower average antibody titers compared to those of Groups C and D. The average antibody levels between Group B and those of Groups C and D showed statistical significance of difference (P<0.05). Meanwhile the average antibody titers between Group C and Group D showed no significant difference (P>0.05).

On day 28 after the vaccination, the average antibody levels in Group B had increased gradually but still maintained a lower level above 5, while the antibody titers in both Groups C and D had increased more rapidly compared to those of Group B, to a higher value at 7 above. At this time point, the average antibody levels between Group B and those of Groups C and D showed statistical significance of difference, separately (P<0.05). Still, no significant difference was observed between the average antibody levels in Groups C and D.

During the past decade, a lot of studies have been carried out in the field of innate immune recognition [4, 11, 15, 17]. In particular, CpG motif as a danger signal could be recognized by TLR9 expressed on certain mammalian cells and cause direct B cell activation, implying its potential use as an immunoadjuvant [7, 12].

In the present experiment, we used a CpG motif enriched plasmid (pUC18-CpG) as an immune stimulator for swine PBMCs lymphocyte transformation test. Our results showed that the Stimulation Index for the optimal dose of pUC18-CpG was more than 2.5 times higher than that of LPS, while overdose administration of the recombinant plasmid could inhibit the stimulation effect. This may be caused by the regulatory mechanisms of the immune system. Our results indicate that pUC18-CpG could be used as a potent immunopotentiator for enhancing humoral immune response.

In a following *in vivo* test, pUC18-CpG was used as vaccine adjuvant and co-administered with a commercial FMD

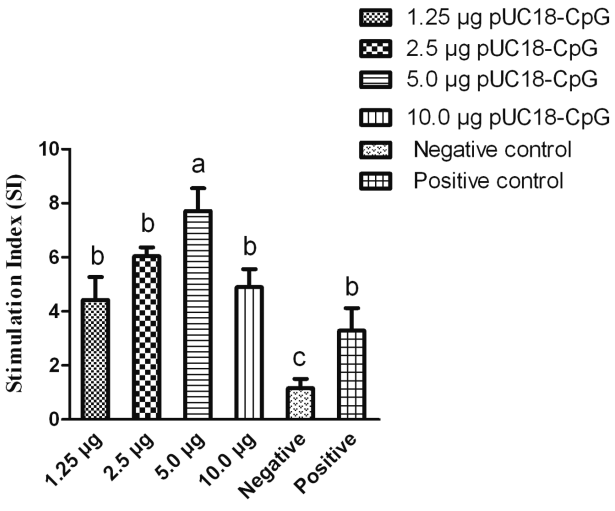

Fig. 1. Stimulation Index (SI) of different doses of pUC18-CpG. For each dose, lymphocyte transformation assay was performed in triplicates. SI was calculated per well and then expressed as mean ± SD (n=3). LPS served as a positive control, pUC18 as a negative control. Different letters mean statistical significance of differences.

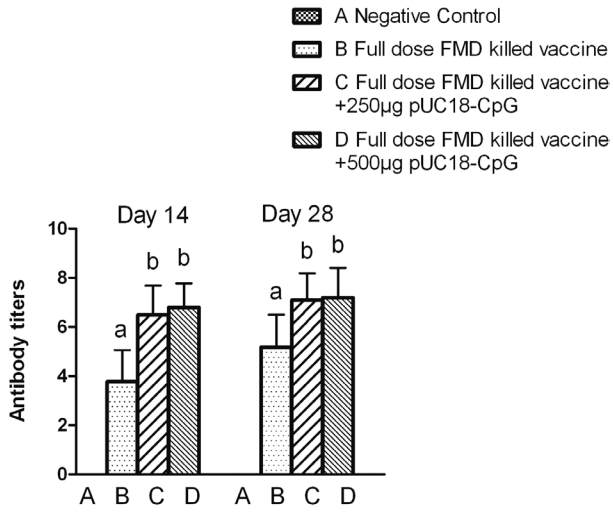

Fig. 2. The average valence of anti-FMDV antibody at different time intervals. Antibody titers were determined by calculating $\log_2$ of the highest serum dilution. For each group, antibody titers were expressed as mean ± SD (n=5). The difference between groups with different letters at each time period is significant (P<0.05).

killed vaccine. On day 14 post vaccination, the average antibody titers in Groups C and D were much higher than those in Group B (P<0.05), implying that co-administration of pUC18-CpG may help bring forward the appearance of anti-structural protein antibodies. It will be of great help when an enforced immunization is necessary [18]. On day 28 prior to challenge, the antibody titers of Groups C and D remained higher than those in Group B (P<0.05). Based on the results from the above study, we conclude that pUC18-CpG is a powerful immunostimulator and shows excellent adjuvanticity when co-administered with the FMD killed vaccine.

REFERENCES

1. Barbalat, R., Ewald, S. E., Mouchess, M. L. and Barton, G. M. 2011. Nucleic acid recognition by the innate immune system. *Annu. Rev. Immunol.* **29**: 185–214. [Medline] [CrossRef]
2. Changhua, L., Jindong, Y., Defa, L., Lidan, Z., Shiyan, Q. and Jianjun, X. 2005. Conjugated linoleic acid attenuates the production and gene expression of proinflammatory cytokines in weaned pigs challenged with lipopolysaccharide. *J. Nutr.* **135**: 239–244. [Medline]
3. Doel, T. R. 2003. FMD vaccines. *Virus Res.* **91**: 81–99. [Medline] [CrossRef]
4. Elinav, E., Strowig, T., Henao-Mejia, J. and Flavell, R. A. 2011. Regulation of the antimicrobial response by NLR proteins. *Immunity* **34**: 665–679. [Medline] [CrossRef]
5. Goris, N., Merkelbach-Peters, P., Diev, V. I., Verloo, D., Zakharov, V. M., Kraft, H. P. and De Clercq, K. 2007. European Pharmacopoeia foot-and-mouth disease vaccine potency testing in cattle: between test variability and its consequences. *Vaccine* **25**: 3373–3379. [Medline] [CrossRef]
6. Gray, R. C., Kuchtey, J. and Harding, C. V. 2007. CpG-B ODNs potently induce low levels of IFN-alphabeta and induce IFN-alphabeta-dependent MHC-I cross-presentation in DCs as effectively as CpG-A and CpG-C ODNs. *J. Leukoc. Biol.* **81**: 1075–1085. [Medline] [CrossRef]
7. Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* **408**: 740–745. [Medline]
8. Herbst, M. M. and Pyles, R. B. 2003. Immunostimulatory CpG treatment for genital HSV-2 infections. *J. Antimicrob. Chemother.* **52**: 887–889. [Medline] [CrossRef]
9. Hutber, A. M., Kitching, R. P., Fishwick, J. C. and Bires, J. 2011. Foot-and-mouth disease: the question of implementing vaccinal control during an epidemic. *Vet. J.* **188**: 18–23. [Medline] [CrossRef]
10. Katsuda, M., Iwahashi, M., Matsuda, K., Miyazawa, M., Nakamori, M., Nakamura, M., Ojima, T., Iida, T., Hayata, K. and Yamaue, H. 2011. Comparison of different classes of CpG-ODN in augmenting the generation of human epitope peptide-specific CTLs. *Int. J. Oncol.* **39** 1295–1302. [Medline]
11. Kawai, T. and Akira, S. 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. I. *Immunity* **34**: 637–650. [Medline] [CrossRef]
12. Klinman, D. M. 2004. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat. Rev. Immunol.* **4**: 249–258. [Medline]
13. Krieg, A. M. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* **20**: 709–760. [Medline] [CrossRef]
14. Linghua, Z., Xingshan, T., Yong, G. and Fengzhen, Z. 2006. Effects of CpG ODN on CD4+ and CD8+ T subpopulations in the immune response to porcine reproductive and respiratory syndrome killed virus vaccine. *Vaccine* **24**: 1874–1879. [Medline] [CrossRef]
15. Loo, Y. M. and Gale, M. Jr. 2011. Immune signaling by RIG-I-like receptors. *Immunity* **34**: 680–692. [Medline] [CrossRef]
16. Mallick, A. I., Parvizi, P., Read, L. R., Nagy, E., Behboudi, S. and Sharif, S. 2011. Enhancement of immunogenicity of a virosome-based avian influenza vaccine in chickens by incorporat-

ing CpG-ODN. *Vaccine* **29**: 1657–1665. [Medline] [CrossRef]
17. Osorio, F. and Reis e Sousa, C. 2011. Myeloid C-type lectin receptors in pathogen recognition and host defense. *Immunity* **34**: 651–664. [Medline] [CrossRef]
18. Patil, P. K., Bayry, J., Nair, S. P., Gopalakrishna, S., Sajjanar, C. M., Misra, L. D. and Natarajan, C. 2002. Early antibody responses of cattle for foot-and-mouth disease quadrivalent double oil emulsion vaccine. *Vet. Microbiol.* **87**: 103–109. [Medline] [CrossRef]
19. Rankin, R., Pontarollo, R., Ioannou, X., Krieg, A. M., Hecker, R., Babiuk, L. A. and van Drunen Littel-van den Hurk, S. 2001. CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved. *Antisense Nucleic Acid Drug Dev.* **11**: 333–340. [Medline] [CrossRef]
20. Sakwiwatkul, K., Li, R. L., Song, X. M. and Hu, S. H. 2010. Effect of the extract made from Cochinchina momordica seeds on the humoral immune responses of mice to a commercial foot-and-mouth disease vaccine (serotypes O and Asia 1). *Microbiol. Immunol.* **54**: 31–37. [Medline] [CrossRef]
21. Sumption, K., Rweyemamu, M. and Wint, W. 2008. Incidence and distribution of foot-and-mouth disease in Asia, Africa and South America; combining expert opinion, official disease information and livestock populations to assist risk assessment. *Transbound. Emerg. Dis.* **55**: 5–13. [Medline] [CrossRef]
22. Verma, A., Prasad, K. N., Singh, A. K., Nyati, K. K., Gupta, R. K. and Paliwal, V. K. 2010. Evaluation of the MTT lymphocyte proliferation assay for the diagnosis of neurocysticercosis. *J. Microbiol. Methods* **81**: 175–178. [Medline] [CrossRef]
23. Vollmer, J. and Krieg, A. M. 2009. Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists. *Adv. Drug Deliv. Rev.* **61**: 195–204. [Medline] [CrossRef]
24. Zhang, Q., Yu, K., Yuan, W., Xue, F., Sun, H. and Zhu, H. 2008. [Purification of large-scale plasmid DNAs by selective precipitation with cetyltrimethylammonium bromide]. *Sheng Wu Gong Cheng Xue Bao* **24**: 2117–2121 (in Chinese with English Abstract). [Medline]