UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

Departamento de Sanidad Animal

THE STUDY OF THE ADJUVANTICITY OF PLASMID CONTAINING SWINE-SPECIFIC CPG MOTIF ON TWO SWINE BACINES. ESTUDIO DE LA CAPACIDAD ADYUVANTE DEL PLÁSMIDO QUE CONTIENE LA SECUENCIA CPG ESPECÍFICA DE CERDOS EN DOS VACUNAS PORCINAS

MEMORIA PARA OPTAR AL GRADO DE DOCTOR
PRESENTADA POR

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Bajo la dirección del doctor

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MADRID, 2013

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

CENTRO VISAVET

DEPARTAMENTO DE SANIDAD ANIMAL

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DOCTORAL THESIS

Directed by Prof. Dr. José Manuel Sanchez-Vizcaíno Rodríguez

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Madrid, Spain

ACKNOWLEDGMENTS

Some people said that the process of doing a PhD was like running a marathon, but I consider it more complicated than that. No one could complete his or her PhD without the help of countless people, and that is what has got me in the first place when I start writing this part. Over the past 5 years, there are so many people that I am indebted to for their aid and support during my work as a PhD student.

First of all, I am deeply grateful to my advisors Prof. Dr. José Manuel Sánchez-Vizcaíno Rodríguez and Prof. Dr. ZHU Hongfei. Learning abroad had been a dream for me and you both helped to make that dream come true. To work with you has always been a great pleasure. During my Ph.D. process, you have guided and supported me with your extensive professional knowledge, and have always been patient and encouraging in times of difficulties. All the guidance and support make me feel more about two old friends aside from the professor—student relationship, and I highly appreciate that.

During my stay in Madrid, I have been very privileged to get to know and work with many great people who become friends as time goes on. Many thanks to Raquel, who helps me with the registration and other paperwork over the years; to Debora, who offers me a lot of help and advice in my thesis writing; to Belen Ri and Rocío, who help me a lot in my experiments and the Spanish language learning; to Ana, the very first person that received me at the airport and drove me to the city; to Marta, for the language learning and showing me around the city; to Bea, for the help with my computer work and the very nice trip to the mountain near Madrid; to Belen Ro, who gives a lot of professional advice in my work. I am also indebted to Marisa, Fernando, Lina, Victor,

Chelo, Almudena, Marina, and Mar, for the kind help in many ways during my last trip for the DEA. My thanks also go to the members of Prof. Bruno's group!

Furthermore, I am very grateful to my working colleagues in China. Prof. ZHANG Quan has given me a lot of help during the animal experiments and paper preparation. Moreover, I would like to express my gratitude to my workmates Dr. HOU Shaohua, Dr. JIA Hong, Dr. YUAN Weifeng, Dr. XIN Ting and many other students that working on their Ph.D or Master projects in the lab. I am also highly grateful to the animal health workers from Xinjiang Tiankang Animal Science Bio-Technology Co. Ltd and the College of Veterinary Medicine in Yangzhou University, for their work on animal breeding and blood sample collection.

Very special thanks go out to my dear aunts: aunt Maria Jesus and aunt Isabel Minguez Tudela. They have given me a lot of help during my study abroad, both in my work and in my daily life. If not with their care and encouragement, I may not have completed this long process.

Last but not least, I would like to thank my family for the support they have provided through my entire life. In particular, I must acknowledge my parents and my wife, without whose love, encouragement and assistance, I would not have finished this thesis.

This study was supported by grants from a Key Project in the National Science & Technology Pillar Program during the Eleventh Five-year Plan Period of China (No. 2006BAD31B03), partly by Special Funds for Scientific Research on Public Causes (No. 200903027) and by Central Level Special Funds for Institute Fundamental Scientific Research on Public Causes (No. 2010js-1).

DECLARATION

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree or diploma of the university or other institutes of higher learning, except where due acknowledgment has been made in the text.

Place: Madrid, Spain **Signature：**

Date： 16／05／2013 **Name:** GUO Xiaoyu

CERTIFICATE

This is to certify that the thesis entitled: **THE STUDY OF THE ADJUVANTICITY OF PLASMID CONTAINING SWINE-SPECIFIC CpG MOTIF ON TWO SWINE VACCINES,** submitted by Mr. GUO Xiaoyu to the Facultad de Veterinaria de la Universidad Complutense de Madrid towards the fulfillment of the requirements for the award of the Degree of Doctor, is a bona fide record of the work carried out by him under our supervision and guidance.

At Madrid, on Day of of 2013.

Prof. Dr. José Manuel Sánchez-Vizcaíno Rodríguez **Prof. Dr. ZHU Hongfei**

INDEX:

List of abbreviations：

ADE: antibody dependent enhancement

APCs: antigen presenting cells

BEI: binary ethylenimine

cDCs: conventional dendritic cells

CMI response: cell-mediated immune response

CpG: cytosine-phosphate-guanosine

DIVA: differentiating infected from vaccinated animals

DPI: day post infection

dsRNA: double-stranded RNA

FA： fiebre aftosa

FMDV: Foot-and-mouth disease virus

IFN: interferon

IKK: the I kappa B kinase

IRAK: interleukin-1 receptor-associated kinase

ISS: Immunostimulatory Sequence

KV vaccine: killed virus vaccine

LPB-ELISA: Liquid phase block ELISA

LPS: lipopolysaccharides

MCS: multiple cloning sites

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NSP: non-structural protein

ODN: oligodeoxynucleotide

OIE: the Office International des Epizooties

PAMPs: pathogen-associated molecular patterns

PBMCs: peripheral blood mononuclear cells

PD_{50}: 50% protective dose

pDCs: plasmacytoid dendritic cells

poly I:C: polyinosinic: polycytidylic acid

PRRs: pattern-recognition receptors

PRRSV: Porcine reproductive and respiratory syndrome virus

ROS: reactive oxygen species

SI: Stimulation Index

ssRNA: single-stranded RNA

TAK1: Transforming growth factor beta-activated kinase 1

TLR9: toll-like receptor 9

TRAF6: TNF receptor associated factor 6

VLPs: virus-like particles

ABSTRACT:

CpG motif is described as a type of Immunostimulatory Sequence (ISS)，in which an unmethylated cytosine-phosphate-guanosine (CpG) motif is flanked by two 5′ purines and two 3′ pyrimidines. Since its discovery in 1995, several studies have shown that ODNs containing unmethylated CpG motifs (designated as CpG ODN) can activate host immune defense mechanisms, leading to both innate and acquired immune responses through toll-like receptor 9 (TLR9)–mediated recognition.

Synthetic CpG ODNs trigger an immunomodulatory cascade that involves professional antigen presenting cells, natural killer cells, B cells, and T cells, thereby underscoring their potential for use as immunoprotective agents as well as vaccine adjuvants. To date, the CpG ODNs used in most of the research were synthesized with a nuclease-resistant phosphorothioate backbone, while research on the adjuvanticity of plasmids containing multiple CpG motifs has rarely been reported.

In our study, a swine-specific CpG motif enriched plasmid (pUC18-CpG) was constructed. The immunostimulant property of pUC18-CpG was tested *in vitro* via lymphocyte transformation assay using swine peripheral blood mononuclear cells (PBMCs). Our results showed that the recombinant plasmid showed higher Stimulation Index (SI) compared to the positive control lipopolysaccharides (LPS).

Then pUC18-CpG as an adjuvant of FMD killed vaccine was tested for immunization and vaccination—challenge in a porcine model. Our results showed that co-administration of pUC18-CpG with the traditional FMD killed vaccine could significantly enhance the humoral immune response and generate higher levels of antibodies against FMDV

structural proteins. In addition, the PD_{50} value of the CpG adjuvanted vaccine was much higher than that of the traditional FMD killed vaccine.

The efficacy enhancement property of pUC18-CpG was further investigated on a PRRS MLV vaccine. After vaccination, PRRSV-specific antibodies, PRRSV-specific cytokines and clinical parameters were studied and compared between different vaccinated groups. During the following challenge study, co-administration of pUC18-CpG with the vaccine could confer higher protection rate. Our results have shown that co-administration of pUC18-CpG with the vaccine could elicit more potent adaptive immune response and provide better protection.

In summary, our research showed that pUC18-CpG could be a potent immunoadjuvant for the two swine vaccines and could greatly enhance the efficacy when administered in combination with either of them.

Resumen:

1. Introducción

La secuencia citosina-fosfato-guanosina (CpG) fue descrita como un tipo de inmunoestimulante de la respuesta inmune tanto humoral como celular. La secuencia CpG está flanqueada por dos purinas en 5’ y dos pirimidinas en el extremo 3’ (CpG—ODN). Desde su descubrimiento en 1995, varios estudios han demostrado que ODN, que contiene la secuencia no metilada de CpG (señalada como CpG ODN), puede activar mecanismos de defensa inmune, y estimular la respuesta inmune tanto innata como adquirida.

La secuencia CpG—ODN es reconocida por el receptor Toll–like 9 presente en ciertos tipos de células del sistema inmune de los mamíferos. CpG-ODN es capaz de estimular el sistema inmune, siendo específico para las distintas especies animales. Los CpG ODNs provocan una cascada de inmunomodulador que involucra a las células presentadoras de antígenos profesionales, las células NK, las células B y células T, subrayando así su potencial de uso como inmunoestimulante, así como adyuvantes de las vacunas.

La secuencia óptima para la estimulación de las células mononucleares de sangre periférica (PBMC) de cerdos fue descrita como GTCGTT . Hasta la fecha, la secuencia CpG-ODN con un fosforotionato resistente a la nucleasa ha sido empleada en la mayoría de los estudios previos. Sin embargo, pocos estudios han evaluado el funcionamiento, como adyuvante en cerdos, del plásmido que contiene la secuencia CpG.

La fiebre aftosa (FA) es una de las enfermedades de notificación obligatoria a la OIE, y de las más importantes que afectan a diferentes

especies ganaderas. La vacunación es considerada como una estrategia muy importante para el control y erradicación de la enfermedad en áreas endémicas, así como en casos de brotes en áreas normalmente libres de la enfermedad. Sin embargo, en algunos casos la vacunación no ha logrado evitar la difusión y transmisión de la enfermedad, en parte debido al fracaso a la hora de originar una respuesta inmune eficaz tras la vacuna empleada. Por lo tanto, el diseño de nuevas vacunas contra la fiebre aftosa debería incluir el uso de un adyuvante más eficaz.

Por otra parte, el síndrome reproductivo y respiratorio porcino (PRRS) se ha extendido en todo el mundo y sigue causando grandes pérdidas económicas en la industria porcina cada año. Las vacunas actuales contra la enfermedad están basadas en vacunas inactivadas y vacunas vivas atenuadas, realizadas con diferentes cepas de virus. Hasta la fecha, las vacunas vivas atenuadas han demostrado mejor eficacia de protección, especialmente contra la infección homóloga, pero su eficacia contra la infección heteróloga es variable. La utilización de adyuvantes inmunes más eficaces podría mejorar las vacunas vivas atenuadas.

2. Objetivos

Este trabajo tiene como principal objetivo la utilización de la secuencia CpG como inmunoestimulador en vacunas frente a la fiebre aftosa (FA) y el síndrome reproductivo y respiratorio porcino (PRRS), incluyendo:

(1) Construcción del plásmido que contiene la secuencia de CpG específica de cerdos y el estudio de sus efectos inmunoestimulantes in vitro;

(2) Evaluación del efecto inmune del plásmido que contiene el CpG, como adyuvante de una vacuna viva atenuada contra el síndrome

reproductivo y respiratorio porcino;

(3) Evaluación del efecto inmune del plásmido que contiene el CpG como adyuvante en una vacuna inactivada frente al virus de la fiebre aftosa.

3. Resultados

El plásmido que contiene las secuencias CpG específicas para cerdos (pUC18-CpG) se logró a través de la inserción en tándem de 5 copias de CpG ODN 2006 en los múltiples sitios de clonación del vector del plásmido pUC18. La propiedad inmunoestimulante de pUC18-CpG fue probada *in vitro* mediante el ensayo de la transformación de linfocitos, utilizando células mononucleares de sangre periférica de cerdos. Nuestros resultados mostraron que el plásmido recombinante mostró mayor índice de estimulación (SI) en comparación con el control de lipopolisacáridos (LPS).

En el estudio siguiente, el pUC18-CpG fue probado como coadyuvante en una vacuna inactivada frente al virus de la fiebre aftosa, en un modelo porcino con inmunización, vacunación y desafío. Nuestros resultados mostraron que la co-administración del pUC18-CpG con la vacuna inactivada de FA podría mejorar significativamente la respuesta inmunitaria humoral y generar mayores niveles de anticuerpos (más de 1,5 veces) contra las proteínas estructurales del VFA. Durante la prueba de potencia, se mostró que la co-administración de pUC18-CpG con la vacuna inactivada de FA mejoró la potencia de esta última (PD50 values: 12.51, 6.47).

Por otra parte, el pUC18-CpG también se probó en una vacuna viva atenuada contra el síndrome reproductivo y respiratorio porcino.

Después de la vacunación, diferentes parámetros fueron evaluados: anticuerpos, citoquinas específicas para PRRS, y parámetros clínicos, comparándo los grupos vacunados con los controles. La co-administración de pUC18-CpG con la vacuna PRRS ayudó a inducir una respuesta inmune humoral más elevada, y dio lugar a un tipo de respuesta inmune Th1. Durante el siguiente estudio de desafío, la co-administración del pUC18-CpG con la vacuna viva atenuada del PRRS redujo las síntomas clínicos e incrementó la tasa en protección (protección completa en el Grupo B con el pUC18-CpG).

4. Conclusión

Nuestra investigación ha demostrado que pUC18-CpG puede actuar como un potente inmunoadyuvante para las dos vacunas porcinas utilizadas en este estudio y mejorar la eficacia de ambas cuando se administra en combinación con ellas.

1. INTRODUCTION

1.1 CpG ODN: discovery, mechanisms and therapeutic applications

1.1.1 Innate immunity, PAMPs and PRRs

The immune system of vertebrates comprises two branches: the innate immunity and the adaptive immunity. The innate immune system is the first line in defending against the invasion of pathogenic microorganisms (Akira, Uematsu, and Takeuchi, 2006). The exposure of pathogens first triggers the innate immune response that is characterized by the production of type I interferons and other inflammatory cytokines (Medzhitov and Janeway, 1997). Subsequently, the adaptive immune response is triggered to eliminate pathogens in the late phase of infection as well as to generate immunological memory.

The innate immune system recognizes pathogen-associated molecular patterns (PAMPs) expressed by the infectious microorganisms (Janeway, 1989). PAMPs are conserved microbial structures such as LPS, flagellin or bacterial CpG DNA, etc. Recognition of PAMPs is mediated by the germline-encoded pattern-recognition receptors (PRRs) (Janeway and Medzhitov, 2002). So far, the PRRs are broadly categorized into 3 classes: secreted PRRs, transmembrane PRRs and cytosolic PRRs (Iwasaki and Medzhitov, 2010).

Toll-like receptors are members of the transmembrane PRRs family, and are among the most studied PRRs. Up to now, 12 members of the TLR family have been identified in mammals (Kumar, Kawai, and Akira, 2009b). Among them, TLR1, TLR2, TLR4, TLR5 and TLR6 are located on the cell surface, and detect PAMPs such as lipopolysaccharide (LPS) of Gram-negative bacteria (TLR4), lipoteichoic acids of Gram-positive bacteria (TLR1/TLR2), bacterial lipoproteins (TLR2/TLR6) and flagellin

(TLR5) (Hayashi et al., 2001; Ozinsky et al., 2000; Poltorak et al., 1998). TLR3, TLR7 and TLR9 are located on intracellular vesicles, and recognize double-stranded RNA (dsRNA) (TLR3), single-stranded RNA (ssRNA) (TLR7), and dsDNA (TLR9) (Alexopoulou et al., 2001; Diebold et al., 2004; Hemmi et al., 2000).

1.1.2 The discovery of CpG ODN

In the genomes of vertebrates, CpG dinucleotides exist at lower frequency, only about one-quarter of the prevalence exist in bacterial DNA, and are often highly methylated when appear (Bird et al., 1987; Yamamoto et al., 1992). During the early stage of antisense ODN research, unexpected stimulation of lymphocyte proliferation and immunoglobulin production were reported (Branda et al., 1993; McIntyre et al., 1993; Tanaka, Chu, and Paul, 1992). In 1995, Krieg and his colleagues reported that it was the CpG motif existed in the antisense ODNs that is responsible for the immune stimulation (Krieg et al., 1995).

A CpG motif is described as an ODN in which the CpG dinucleotides are flanked by two 5’ purines and two 3’ pyrimidines. The immunostimulatory properties of the CpG ODN are affected by the number of CpG motifs it contains, the space between the CpG motifs, additional flanking sequences and the ODN backbone (Hartmann and Krieg, 2000; Hartmann et al., 2000; Wloch et al., 1998). And the immune stimulatory effects of the CpG ODN could be further enhanced if the ODN contains a TpC dinucleotide on the 5’ end and is pyrimidine rich on the 3’ side (Hartmann et al., 2000).

Due to the divergence in the molecular structure of TLR9 in different species throughout evolution, the optimal CpG ODN sequence for

stimulating immune cells may differ among species (Rankin et al., 2001). For example, the TLR9 molecules of mice and human differ by 24% at the amino-acid level (Hemmi et al., 2000). Hence the optimal CpG ODN sequence for activating mouse immune cells is GACGTT, while the optimal sequence for activating human immune cells is GTCGTT (Krieg, Hartmann, and Yi, 2000; Rankin et al., 2001).

Cell populations that could express TLR9 may also differ among species. In mice, the immune cells of monocytes, macrophages and myeloid DCs could express TLR9 and respond to CpG stimulation, whereas in human, it is the B cells and pDCs that express TLR9 and mediate CpG activation (Hartmann and Krieg, 2000; Krug et al., 2001).

1.1.3 Mechanisms for immunostimulation by CpG ODN

It is reported that members of the phosphoinositide (PI) 3-kinase family play an important role in the uptake of CpG ODN by immune cells (Ishii et al., 2002). After internalization, CpG ODN is shuffled to and interacts with TLR9 that is present on endosomes. Interaction of TLR9 with CpG ODN triggers the swelling and acidification of the endosomes and the release of reactive oxygen species (ROS), which is very crucial to the following steps of the TLR9-mediated immunostimulation signaling pathway (Hacker et al., 1998; Yi et al., 1998).

The TLR9-mediated cellular activation involves a signaling cascade that is MyD88-dependent. In macrophages and conventional dendritic cells (cDCs), the signal transduction proceeds via the recruitment and activation of the IRAK family proteins, TRAF6, TAK1 and the IKK complex. The activation of the IKK complex activates the NF-κB subunits to initiate the transcription of inflammatory cytokine genes (Kawai and Akira, 2010;

Lin, Lo, and Wu, 2010; Tseng et al., 2010).

In pDCs stimulated with CpG ODN, the MyD88-mediated signaling pathway involves proteins such as IRAK4, TRAF6, TRAF3, IRAK1, and IKKa, which in turn phosphorylate IRF7 to initiate the transcription of type I interferons. In addition, proteins such as osteopontin, phosphoinositol 3 kinase (PI3K), mTOR (downstream of PI3K) and p70S6K also play critical roles in IRF7 activation (Kumar, Kawai, and Akira, 2009a; Takeuchi and Akira, 2010).

All the above-mentioned immunostimulatory effects are highly CpG specific, since ODN lacking the corresponding CpG motifs does not stimulate the secretion of proinflammatory cytokines and type I interferons (Gursel et al., 2002; Klinman et al., 1996).

1.1.4 Immunotherapeutic applications of CpG ODN

Immunoprotection and therapy. During evolution, the innate immune system of vertebrates could recognize and respond to the PAMPs of infectious pathogens, indicating that PAMP-mediated immune activation may be vital for host survival. In fact, the treatment of mice with a species-specific CpG ODN did provide immune protection against intracellular infectious challenges. CpG-treated mice could resist a wide range of infection by bacteria and viruses, and even by parasites (Ashkar et al., 2003; Klinman, Conover, and Coban, 1999; Ray and Krieg, 2003).

Post-exposure therapy with CpG ODN is generally less effective against acute viral infections compared to the effectiveness against chronic viral infections, suggesting its potential utility in the treatment of the latter. TLR9 activation leads to the inhibition of replication of hepatitis B virus *in vivo*, and improves the survival in a Friend leukaemia virus model when

given four days post-infection (Isogawa et al., 2005; Olbrich et al., 2002). Moreover, it has been reported that the administration of CpG ODNs could improve the protection of the immunosuppressed, the pregnant and the newborn against infection (Ito et al., 2004; Verthelyi et al., 2003).

***Vaccine adjuvants*.** CpG ODN stimulates the expression of inflammatory cytokines and type I interferons, and activates professional APCs, suggesting that CpG ODNs may be utilized as vaccine adjuvants. Up to 2011, over 600 preclinical studies have been carried out in examining the immunogenicity of CpG-adjuvanted vaccines (Bode et al., 2011). Co-administration of vaccines with CpG ODN could enhance antigen-specific humoral and cellular responses to the antigens, including subunit vaccines, killed or live attenuated vaccines, dendritic cell vaccines, etc.

In order to achieve the optimal antigen-specific immune response, it is necessary that the CpG ODN be maintained in close proximity to the antigen. Thus, conjugation of CpG ODN to the antigen by physical or chemical methods, or incorporating them in lipid emulsions or vesicles, could greatly enhance the specific IgG responses (Davis et al., 1998; Gursel et al., 2001). The mechanisms responsible for the strong adjuvant effects may include: 1) CpG-induced activation and maturation of APCs; 2) inhibition of B cell apoptosis; and 3) CpG-induced Th1-biased cytokine microenvironment. Meanwhile, conjugation of CpG ODN with the antigen can improve antigen uptake and thus reduce antigen dose (Tighe et al., 2000; Weeratna, Comanita, and Davis, 2003).

The ability of CpG ODN in boosting mucosal immunity has also been

evaluated. Multiple studies have proved that CpG ODN is an effective mucosal adjuvant for the respiratory tract, vaginal mucosal, oral, intrarectal and conjunctival vaccination, as well as transcutaneous immunization (Berry et al., 2004; McCluskie and Davis, 2000; Nesburn et al., 2005). CpG-adjuvanted vaccination through mucosal routes presented enhanced protection against infectious challenge.

In human clinical trials, CpG ODN has been studied as adjuvant for hepatitis B surface antigen, alone or in combination with alum. During a randomized double-blind controlled trial in HIV-infected populations, addition of CpG ODN to the Engerix-B vaccine significantly enhanced both the anti-HBs antibody titers and the antigen-specific T cell proliferative responses (Cooper et al., 2005; Cooper et al., 2004). Moreover, the ability of CpG ODN to induce a Th1-biased immune response and support $CD8^+$ T-cell responses suggests its potential use as adjuvant for allergy vaccines and cancer vaccines.

Treatment of cancer and allergic diseases. By activating the innate immune system and inducing IFN-gamma production, CpG ODN manifests antitumor activity in mouse models and human preclinical trials. In mice, CpG ODN-mediated immune activation could result in tumor eradication, but this therapy only applies to relatively small tumors. For the treatment of large tumors, CpG ODN has shown better results when administered in combinations with other types of anti-tumor therapies, like monoclonal anti-tumor antibodies, surgery, radiotherapy and chemotherapy (Krieg, 2004).

In human, CpG ODN has shown promising results in cancer immunotherapy. Several pharmaceutical companies, including Pfizer,

Idera and Dynavax, are developing CpG ODNs for the treatment of cancer. CpG ODN monotherapy has entered clinical trials for the treatment of non-Hodgkin lymphoma (NHL), renal cell carcinoma, melanoma, and non-small cell lung cancer. And several TLR9 agonists have been developed and have entered clinical trials to evaluate their safety and efficacy for the treatment of several hematopoietic and solid tumors (Murad and Clay, 2009).

Asthma is an allergic disease of the respiratory systems, which is resulted from over Th2-type immune response against the harmless antigens in the environment. The disease is mediated by the over-production of Th2-type cytokines like IL-4 and IL-5, and the increased differentiation of B cells into IgE secreting cells (Robinson et al., 1992). CpG ODN stimulates Th1-type immune response and elicits the production of IFN-γ and IL-12, which in turn suppress the Th2-type immunity and IL-4 production. Hence, by administration of CpG ODN, the allergic asthma may be reduced or eliminated. In fact, the anti-allergic properties of CpG ODN were reported when it was administered alone, mixed with allergen or physically linked to allergen (Horner et al., 2002).

1.2 Foot-and-mouth Disease

1.2.1 Etiology and current situation

Foot-and-mouth disease (FMD) is one of the notifiable diseases listed by the OIE. The causative agent of FMD is the foot-and-mouth disease virus (FMDV), a member of the Genus *Aphthovirus* in the Family *Picornaviridae*. There are seven serotypes of FMDV—A, O, C, SAT1, SAT2, SAT3 and Asia1, with each serotype containing several subtypes (Doel, 2003; Sumption, Rweyemamu, and Wint, 2008). During an outbreak, FMD causes high morbidity, with low to moderate mortality. Typical infection by FMDV is characterized by a vesicular condition of the feet, buccal mucosa and, in females, the mammary glands.

Different serotypes of FMDV are currently existed in different regions and countries, with the potential of rapid international spread (Zhang et al., 2011). Being one of the most contagious animal diseases, FMD affects millions of cloven-footed animals worldwide annually, causes severe economic losses and remains the main sanitary barrier in the international and national trade of animals and animal products. Therefore, it is of great importance to develop efficient strategies for the prevention, control and eradication of FMD.

1.2.2 Recent progress in FMD vaccine development

Vaccination has been proven to be a powerful strategy for control and eradication of animal infectious diseases. The first inactivated FMD vaccine was developed in the 1930's, through the inactivation of vesicular fluid obtained from deliberately infected cattle. For the present, FMD killed vaccine is produced by growing the live virulent strains of FMDV in BHK-21 cell lines under bio-secure conditions, inactivation by

chemicals such as binary ethyleneimine, and then formulated with oil-adjuvants (Doel, 2003; Lombard, Pastoret, and Moulin, 2007).

However, the manufacturing process of FMD killed vaccine may risk the release of the live virus to the environment because of incomplete inactivation, causing biosafety issues. Also, FMD killed vaccine exhibits other disadvantages such as the need of adequate cold chain of formulated vaccines , necessity of multiple vaccinations, short shelf life, etc., which calls for a better FMD vaccine design strategy.

During the past decades, several novel FMD vaccines have been developed in the lab and tested through animal experiments, and show very promising results. Among them there are:

Synthetic peptide vaccines are designed to include both T and B cell antigenic epitopes optimized for both immunogenicity and antigenicity. The synthesized peptide spans the variable G–H loop region, the carboxy-terminal part of VP1 and regions that represents FMDV T-cell epitopes (Wang et al., 2002). The efficacy of peptide vaccines is restricted by representing only a limited number of antigenic sites and/or T-cell epitopes of the virus. Rational designs of advanced structure of antigen peptide may provide more powerful FMD peptide vaccines.

Empty capsid vaccines are virus-like particles (VLPs) lacking the nucleic acid. The earliest VLPs were naturally produced in infected cells and were as immunogenic as virions (Rweyemamu, Terry, and Pay, 1979). Currently a number of expression systems have been utilized for the production of VLPs, including virus vectors, prokaryotic expression system and the baculovirus expression system. The delivery of the FMDV capsid sequence via a recombinant, replication-defective human adenovirus

type 5 (Ad5) provided early protection against challenge during a clinical study (Pacheco et al., 2005). And FMDV VLPs obtained through the silkworm-baculovirus expression system also showed good immunogenicity (Li et al., 2008).

Live attenuated vaccines are nowadays engineered with the infectious cDNA technologies, which allow the introduction of specific changes in the FMDV genome. A genetically engineered FMDV serotype A12 lacking a leader proteinase was attenuated and was not transmissible between cattle or swine, while induced protective immune response (Chinsangaram, Mason, and Grubman, 1998; Piccone et al., 1995). The discovery of more virulence determinants will contribute to the development of new types of attenuated vaccines, with the background of a better understanding of the virus-host interactions and mechanisms of virus pathogenesis.

DNA vaccines have several advantages over traditional vaccines, such as long-term protection, long shelf life and the ability to induce both humoral and cellular immune responses (Fowler and Barnett, 2012). Plasmids encoding antigenic epitopes of FMDV structural protein VP1 could elicit immune responses in mice and protected swine against viral infection (Wong et al., 2000). Moreover, several cytokines have proven to be efficient immunoadjuvants of DNA vaccines, including INF-gamma, IL-2, IL-6, etc.

1.3 Porcine Reproductive and Respiratory Syndrome: current situation and challenges in vaccinology

1.3.1 Etiology and current situation

Porcine reproductive and respiratory syndrome (PRRS) is a swine disease that first emerged in the United States during the 1980s (Madsen et al., 1998). The causative agent of the disease is the PRRS virus (PRRSV), which is a small, enveloped virus belonging to the family *Arteriviridae*, genus *Arterivirus*. PRRSV is believed to replicate in macrophages, and adopt several mechanisms to evade the host's immune surveillance (Yoo et al., 2010).

PRRSV is an enveloped, single-stranded, positive sense RNA virus. The genome of PRRSV is about 15.4kb, which encodes for at least 10 open reading frames (ORFs) (Dokland, 2010). Based on nucleotide sequence analysis and comparison, PRRSV is categorized into type 1 and type 2 genotypes, representing the European and North American isolates, respectively. There is about 70% of homology at the nucleotide level between the two genotypes (Nelsen, Murtaugh, and Faaberg, 1999; Wootton, Yoo, and Rogan, 2000).

Due to the high error-rate inherent in PRRSV replication and recombination between different strains, there is an increasing diversity among strains of the two genotypes (Chang et al., 2002; van Vugt et al., 2001). Investigations have shown regional differences within each genotype. There have been reports on the high degree of polymorphism of the type 1 PRRSV in east Europe (Stadejek et al., 2006). Nowadays, type 2 PRRSV has been introduced into Europe and type 1 virus has appeared in North America, which may accelerate the evolution and

polymorphism of the virus.

Most isolates of PRRSV discovered in South America and Asia belong to the type 2 genotype, probably due to the movement of swine and/or semen. In China, a highly virulent strain of type 2 PRRSV appeared in 2006, characterized by a discontinuous 30 amino acids deletion in the NSP2 region (Tong et al., 2007).

1.3.2 Recent progress in PRRSV vaccine development

Inactivated PRRSV vaccines have been developed and usually administered in combination with immunoadjuvants, with the purpose of eliciting specific anti-PRRSV humoral responses. In fact, killed PRRSV vaccines are the only permitted vaccines in UK and some other countries，for use in sows and gilts (Kimman et al., 2009). However, Inactivated PRRSV vaccines have been proved to provide only partial protection upon challenge and therefore are considered to be ineffective or of limited efficacy at best, which signifies the importance of cell-mediated immunity in PRRSV prevention (Zuckermann et al., 2007).

Modified-live (MLV) PRRSV vaccines are derived by gradual attenuation of virulent field isolates by serial passage on culturing cells. During homologous virulent strain challenge, MLV vaccines have shown better efficacy than inactivated PRRSV vaccines in reducing disease occurrence and severity (Labarque et al., 2003). However, MLV vaccines are only partially effective in providing protection against infections by heterologous field strains of PRRSV (Okuda et al., 2008). Moreover, there have been reports over the safety issues of the MLV vaccines, concerning the reversion of virulence during its multiplication in pigs (Nielsen et al., 2001).

Other experimental PRRSV vaccines include DNA vaccines, vectored

vaccines, and DIVA (differentiating infected from vaccinated animals) vaccines (Barfoed et al., 2004; Cruz et al., 2010; de Lima et al., 2008). But so far there hasn't been a commercialized vaccine available from any of these candidates. Meanwhile, efforts have been made in utilization of immunoadjuvants for enhancing the efficacy of current PRRSV vaccines. These adjuvants include recombinant cytokines, synthesized reagents, and bacterial products. Among them, some interleukins (IL-2, IL-12 and Interferon-Alpha), poly I:C and CpG ODN show strong adjuvanticity in enhancing the CMI response to PRRSV vaccines, while the use of CpG ODN also enhance the PRRSV-specific antibody response after vaccination (Charerntantanakul, 2009).

2. PROBLEM STATEMENT

Foot-and-mouth disease (FMD) is a high-ranked disease in the Office International des Epizooties (OIE) list of notifiable diseases, and is of great socio-economic consequence due to its potential of rapid international spread. Vaccination has proven to be a powerful strategy for disease control and eradication in FMD-endemic areas and during emergency outbreaks in areas normally free from the disease. However, in some cases vaccination against the FMD has failed to prevent the establishment and transmission of the disease, partly due to the failure in eliciting a fast and effective immune response by the vaccine used, which calls for a better FMD vaccine design strategy.

Porcine reproductive and respiratory syndrome (PRRS) is a major swine disease that has spread worldwide and continues to cause great economic losses to the swine industry every year. Currently available vaccines against the disease mainly include killed virus (KV) vaccines and live attenuated vaccines. KV vaccines have been reported of limited efficacy, while live attenuated vaccines have shown better protection efficacy, especially against homologous infection. But the effectiveness of the live attenuated vaccines against heterologous infection is variable. Therefore, options as to the change of vaccination modes or the use of more powerful immunoadjuvants may help to enhance the efficacy of current PRRS live attenuated vaccines.

3. OBJECTIVES

Due to the ever-increasing knowledge in the field of innate immunity, the past decade has witnessed a great number of studies in the development of novel immunoadjuvants, which are targeted at the direct activation of the innate immune system. Among those, CpG ODNs as one of the pathogen-associated molecular patterns (PAMP), have shown great potential in inducing strong Th1—biased immune response. In order to better the efficacy of current swine vaccines and develop novel adjuvants that are applicable to farm animals, the research in this study includes:

1. The construction of plasmid containing multiple swine-specific CpG motifs and the study of its immunostimulatory effects *in vitro*;
2. The study of the adjuvanticity of the CpG-enriched plasmid on a traditional foot-and-mouth disease killed vaccine that is currently used in China;
3. The study of the adjuvanticity of the CpG-enriched plasmid on a porcine reproductive and respiratory syndrome live attenuated vaccine that is currently in use in China.

4. PUBLICATIONS

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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(Received 7 December 2011/Accepted 6 July 2012/Published online in J-STAGE 20 July 2012)

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.

doi: 10.1292/jvms.11-0543; *J. Vet. Med. Sci.* 74(12): 1647–1650, 2012

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.

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

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

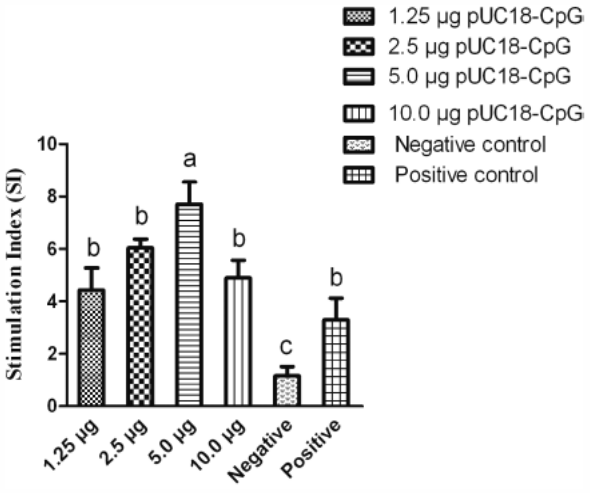

Fig. 1. Stimulation Index (SI) of different doses of pUC18-CpG. For each dose, lymphocyte transformation assay was performed in triplicates. SI was calculated per well and then expressed as mean ± SD (n=3). LPS served as a positive control, pUC18 as a negative control. Different letters mean statistical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

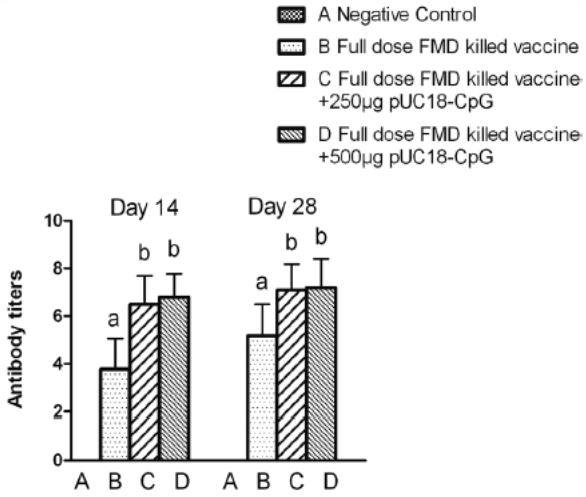

Fig. 2. The average valence of anti-FMDV antibody at different time intervals. Antibody titers were determined by calculating $\log_2$ of the highest serum dilution. For each group, antibody titers were expressed as mean ± SD (n=5). The difference between groups with different letters at each time period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

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Microbiology and Immunology

Microbiol Immunol 2012; **56**: 332–337
doi:10.1111/j.1348-0421.2012.00438.x

ORIGINAL ARTICLE

CpG-enriched plasmid enhances the efficacy of the traditional foot-and-mouth disease killed vaccine

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ABSTRACT

A CpG-enriched recombinant plasmid (pUC18-CpG) as an adjuvant of FMD killed vaccine was tested for immunization and vaccination challenge in a porcine model. Our preliminary results had indicated that the recombinant plasmid could enhance the humoral immune response triggered by the traditional oil-adjuvant vaccine after the initial inoculation. A subsequent vaccination-challenge test showed an increased PD_{50} value. Thus, coadministration of the recombinant plasmid with the oil-adjuvant vaccine helped illicit an immune response earlier than that elicited by giving the vaccine alone. Our results showed that pUC18-CpG can be a potent immunoadjuvant for the traditional FMD killed vaccine and can greatly enhance the traditional vaccine's efficacy when given in combination with it.

Key words CpG motif, efficacy, foot-and-mouth disease (FMD) killed vaccine, immunoadjuvant.

Foot-and-mouth disease, which is a highly contagious disease to cloven-footed animals, is a high-ranked disease in the Office International des Epizooties (OIE) list of notifiable diseases and is of great socioeconomic consequence because of its potential of rapid international spread (1,2). The causative agent of FMD is FMDV, a member of the genus *Aphthovirus* of the family *Picornaviridae* (3). There are seven serotypes of FMDV: A, O, C, SAT1, SAT2, SAT3, and Asia1, each containing several subtypes (4). Vaccination is a proven powerful strategy for disease control and eradication in FMD-endemic areas and in case of emergency outbreaks in areas normally free from the disease (5). However, vaccination has failed to prevent the establishment and transmission of the disease in some cases, partly because of the failure in eliciting an effective immune response by the vaccine used (6), which calls for a better FMD vaccine design strategy.

Since the discovery of ODN in 1995, several studies have shown that ODN containing unmethylated CpG motifs can activate host defense mechanisms, leading to innate and acquired immune responses through TLR9-mediated recognition (7,8). Synthetic ODN that contain immunostimulatory CpG motifs trigger an immunomodulatory cascade that involves professional antigen-presenting cells, natural killer cells, B cells, and T cells, thereby underscoring their potential for use as immunoprotective agents and vaccine adjuvants (9). Although, the CpG-ODN used in most of the research to date have been artificially synthesized with a nuclease-resistant phosphorothioate backbone (10,11), research on the adjuvanticity of plasmids containing multiple CpG motifs has been rarely reported.

Our previous study showed that plasmids containing CpG motifs can augment humoral immune responses in pigs inoculated with a killed-virus vaccine against

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Received 23 October 2011; revised 13 January 2012; accepted 5 February 2012.

Abbreviations: BEI, binary ethylenimine; DPI, day post infection; FMD, foot-and-mouth disease; FMDV, foot-and-mouth disease virus; LPB-ELISA, liquid phase block ELISA; LPS, lipopolysaccharide; MCS, multiple cloning sites; ODN, oligodeoxynucleotides; PAMP, pathogen-associated molecular patterns; PBMC, peripheral blood mononuclear cells; PD_{50}, 50% protective dose; TLR, Toll-like receptor.

porcine reproductive and respiratory syndrome (12). In the present study, a recombinant plasmid containing 20 copies of CpG ODN 2006 (pUC18-CpG) was evaluated for its ability to enhance the efficacy of the traditional FMD killed vaccine. Our results showed that coadministration of pUC18-CpG with the traditional vaccine can significantly enhance the humoral immune response and generate antibodies against FMDV structural proteins. In addition, the PD_{50} value of the adjuvant vaccine was much higher than that of the traditional FMD killed vaccine.

MATERIALS AND METHODS

Plasmid preparation and purification

The CpG-enriched pUC18 containing 20 copies of CpG ODN 2006 (sequence: 5′-TCGTCGTTTT GTCGTTTTGTCGTTTTGTCGTTTTGTCGTT-3′) was generated by tandem insertion of four copies of CpG ODN 2006 into the MCS of the pUC18 vector. pUC18-CpG was multiplied in *Escherichia coli*, which were then cultured by fermentation, and the recombinant plasmid was purified using the large-scale plasmid-purification method (13). After alkali lysis, the plasmid was selectively precipitated from the supernatant by using cetyltrimethylammonium bromide (CTAB). The plasmid was further purified using potassium acetate and Triton X-114 for removing the host protein and endotoxin, respectively. Limulus amebocyte lysate (LAL) tests were carried out to verify removal of endotoxin. Sequencing was carried out to confirm the CpG motif and its copy number. The purified plasmid was dissolved in endotoxin-free 10 mg/mL PBS and stored at −20°C until use.

MTT-lymphocyte transformation assay

Swine PBMC were separated and then stimulated by pUC18 vector alone or pUC18-CpG at different doses (2.5, 5.0 and 7.5 μg, respectively). Each dose was carried out in triplicate. LPS was added at 10 μg/mL and served as a positive control. Negative control cells were cultured without pUC18-CpG, pUC18 vector or LPS. The MTT assay was carried out at 72 hrs of cell culture, as described by Verma and his colleagues (14). Briefly, after 72 hrs culture, 20 μL MTT (5 mg/mL) was added per well. Cells were incubated for another 3 hrs. Colored crystals of formazan were dissolved with 100 μL SDS-isobutanol-HCl. Plates were kept on an orbital shaker for 5 min and optical density (OD) was read on a microplate reader (Bio-Rad, Philadelphia, PA, USA) at 570 nm. Stimulation index (SI) was calculated as described by Verma and his colleagues (14), in a revised manner as follows.

SI = (OD of test sample − OD of blank)/(OD of negative control − OD of blank)

Vaccine preparation

The FMDV serotype O (OS/99 strain) was propagated in baby hamster kidney cells (BHK-21). The culture medium was adjusted to a titer of 7.5 $TCID_{50}$ (50% tissue culture infective dose)/mL and inactivated with BEI at 37°C for 24 hrs. Different quantities of pUC18-CpG were added to the virus-inactivated medium, and vaccine samples were prepared by emulsification at an aqueous to oil phase (Montanide™ ISA 206 [ISA 206]) ratio of 46:54. Each dose (2 mL) of the prepared vaccine contained a total of 20 μg of 140S FMD virus. All the above procedures were carried out in the research laboratory of Xinjiang Tiankang Animal Science Bio-Technology Co., Ltd.

Animals and vaccination

Thirty-two 2-month-old Changbai piglets, all of which were seronegative for FMDV antibodies, were randomly divided into four groups with eight piglets per group. Group A was inoculated with 2 mL PBS and served as the negative control. Group B was inoculated with 2 mL laboratory-made FMD killed vaccine (serotype O, OS/99 strain) with ISA 206 as the adjuvant. Groups C and D were inoculated with an 2 mL inoculum containing the same dose of FMD killed vaccine and ISA 206 as used in group B, but with the addition of the pUC18-CpG plasmid by 250 μg/dose and 500 μg/dose, respectively. The inoculum in all the groups was injected i.m. in the musculi colli. A booster injection with the same dose was given 4 weeks later. All procedures involving animals conformed to the policies of the local animal care committee (20090803–2).

Sera collection and ELISA

Blood sample collection was carried out prior to the initial vaccination and then at 2-week intervals until 8 weeks after the booster injection. Serum was separated by centrifugation and stored at −20°C. The anti-O-type FMDV antibody LPB-ELISA kit (Lanzhou Veterinary Institute, Lanzhou, China) was used to measure the IgG antibody level of the anti-FMDV structural protein antibody.

Challenge study

The challenge study was carried out in a stepwise manner. After measuring antibody titer levels, pUC18-CpG containing the vaccine sample that induced the highest antibody level was chosen for the following potency test. Thirty 2-month-old piglets, having no FMDV antibodies were randomly divided into two groups with 15 piglets per

group. For carrying out the potency test, each group was subdivided into three groups (five piglets per subgroup). The first subgroup was vaccinated with a full dose (2 mL) of laboratory-made FMD killed vaccine, the second subgroup received 1/3 of the dose given to the first subgroup (0.67 mL), and the third group received 1/9 of the dose given to the first subgroup (0.22 mL). Two piglets having no FMDV antibodies were chosen as positive controls. The subgroups were separated from each other during the trial. Vaccination was carried out once during the potency test. On day 28 after vaccination, challenge was carried out by intradermal injection (injection volume, 0.2 mL) into the muscular part of the neck behind the ear with virulent FMDV strain OS/99 at 10,000 $TCID_{50}$.

PD_{50} calculation

The PD_{50} value of each vaccine sample was calculated on the basis of the number of animals protected from the challenge (no clinical signs of blisters in the mouth or on the feet for 10 days following challenge) in each subgroup. We used the Spearman–Kärber method for interpreting the PD_{50} estimates and concluded that the vaccine should contain at least three PD_{50} values per dose for commercial prophylactic use.

Statistical analysis

Data analysis was carried out by using the Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA), and the data were compared using Student's *t*-test. Antibody levels were presented as the geometric mean titer ± standard deviation (GMT ± SD). P value < 0.05 was considered statistically significant.

RESULTS

Lymphocyte transformation assay

Swine PBMC proliferation was measured by the MTT colorimetric assay method. SI values of the positive control (LPS) and each dose group of the recombinant or vector plasmid are shown in Figure 1. pUC18-CpG showed a strong stimulation effect at all the different doses, compared to the vector plasmid and LPS positive control. The SI value of pUC18-CpG at each dose was higher than that of the vector plasmid ($P < 0.05$ at each dose). The highest SI for pUC18-CpG was observed from the 5.0 μg dose group.

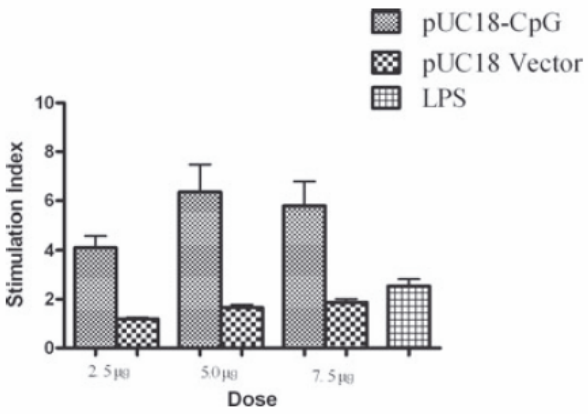

Fig. 1. Stimulation Index (SI) of different doses of pUC18-CpG and pUC18 plasmid vector. For each dose, lymphocyte transformation assay was carried out in triplicate. SI was calculated per well and then expressed as mean ± SD ($n = 3$). LPS served as a positive control.

pUC18-CpG enhanced the humoral immune response induced by the oil-adjuvant FMD-killed vaccine

The anti-FMDV structural protein antibody titers were retested under the same conditions used after the final blood sample collection. The average antibody titers of each group at different time points are described in Table 1 and Figure 2. During the entire experimental period, animals in the negative control group (Group A) remained seronegative for FMDV.

On day 14 after the initial vaccination, animals in group B showed lower average antibody titers than those in groups C and D, and only a few animals in group B showed 50% protection according to the criteria specified in the kit (antibody titers between 2 and 6.5). In contrast, the average antibody titers of the animals in groups C and D were remarkably higher than those of the animals in group B, and all the animals in groups C and D showed 50% protection. Differences between the average antibody levels of group B and those of groups C and D were statistically significant ($P < 0.05$ for both).

On day 28 after the initial vaccination, the average antibody level in group B had increased gradually but remained at a low level of below 3, whereas the antibody levels in groups C and D increased more rapidly to a value above 4.5. During this period, the differences between the average antibody levels of group B and those of groups C and D were statistically significant ($P < 0.05$ for both).

On day 14 after the booster injection, the average antibody titers in all the vaccinated groups had reached a peak level, and all the animals showed over 99% protection (average antibody titer levels above 6.5). The average antibody titer levels in each group then decreased gradually,

Table 1. Average valence of FMDV antibody

Group	Day post inoculation						
	Day 0	Day 14	Day 28	Day 42	Day 56	Day 70	Day 84
A	0	0	0	0	0	0	0
B	0	$2.32^{CD} \pm 0.16$	$2.79^{CD} \pm 0.08$	6.79 ± 0.11	6.59 ± 0.09	5.97 ± 0.16	5.62 ± 0.04
C	0	$3.44^{B} \pm 0.28$	$4.61^{B} \pm 0.25$	7.06 ± 0.18	6.87 ± 0.13	6.08 ± 0.16	5.80 ± 0.12
D	0	$3.89^{B} \pm 0.45$	$4.97^{B} \pm 0.19$	7.19 ± 0.21	6.93 ± 0.20	6.14 ± 0.14	5.86 ± 0.14

Antibody titers were determined by calculating $\log_2$ of the highest serum dilution.
Superscript capital letters indicate very significant differences between groups B and C and between groups B and D ($P < 0.01$).

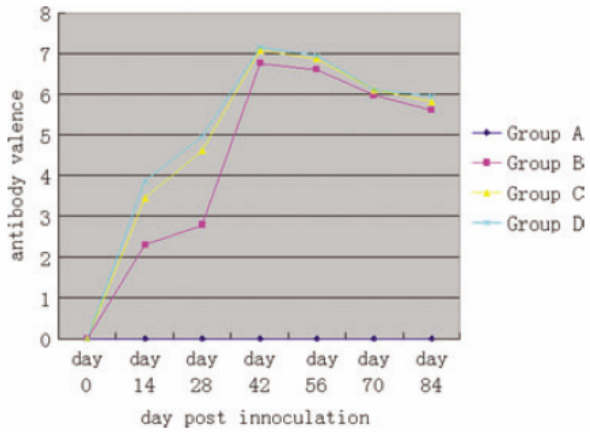

Fig. 2. Curve of recombinant Puc18-CpG plasmid to FMD killed vaccine corresponding to data from Table 1. Antibody titers were determined by calculating log2 of the highest serum dilution.

with the levels in groups C and D showing a higher value than that in group B for the remaining duration of the experiment. In this phase, no significant difference was noted between the average antibody titer levels in group B and those in groups C and D ($P > 0.05$ for both).

For the duration of the experiment, the average antibody titer levels in groups C and D showed no statistically significant difference ($P > 0.05$), although the level in the latter group was slightly higher.

Vaccine samples supplemented with pUC18-CpG showed better potency

After the comparing the antibody titer levels, the vaccine sample containing 250 μg pUC18-CpG was chosen for carrying out the potency test. Blood samples for serum separation were collected from all animals prior to carrying out the challenge. After the challenge, the animals were observed for clinical signs of FMD for up to 10 days. To avoid excessive challenge to the remaining animals, animals that developed clinical symptoms were removed immediately. Both control animals developed clinical signs of the disease in more than one foot. After completing the experiment, all the animals were killed and burnt to prevent spread of the virus into the environment.

The two piglets in the control group showed obvious manifestation of the symptoms of FMD infection on Day 4 and Day 5. In the oil-adjuvant vaccine group, one piglet from the 1/3 dose subgroup showed symptoms of FMD infection on Day 3, and the three piglets from the 1/9 dose subgroup were confirmed as infected on Days 3, 5, and 7, respectively. In the pUC18-CpG adjuvant vaccinated group, only one piglet from the 1/9 dose subgroup was confirmed as infected on Day 5.

PD_{50} was calculated on the basis of the number of animals protected within each subgroup by using the Spearman–Kärber method. Data obtained are shown in Table 2.

In this study, the vaccine sample containing pUC18-CpG showed a much higher PD_{50} value of 12.51, which was almost twofold that of the traditional oil-adjuvant vaccine (PD_{50} value, 6.47). Therefore, coadministration of pUC18-CpG with the traditional vaccine was shown to greatly enhance the potency of the latter, signifying the potential of pUC18-CpG as an effective adjuvant.

DISCUSSION

On infection with certain microorganisms, the host innate immune system detects PAMP to establish an early immune response (15). In addition, danger signals such as the CpG motif from bacterial or viral genomes can be recognized by TLR9 (16). Studies have shown that CpG motifs used for immunostimulation are highly conserved for different veterinary species (17), and the optimal nucleotide sequence for swine PBMC stimulation was found to be GTCGTT (18).

In the present study, we first used pUC18-CpG as an immune stimulator in a lymphocyte transformation test with swine PBMC. Our results showed that the stimulation index for pUC18-CpG at each selected dose was much higher ($P < 0.05$) than that of pUC18 vector alone, and

Table 2. PD_{50} value of each vaccine sample

Items/Animals	Dose	Total amount	Protected amount	Incidence	PD_{50} calculation	
Vaccine/ISA206	Subgroup 1	full	5	5	0	6.47
	Subgroup 2	1/3	5	4	1	
	Subgroup 3	1/9	5	2	3	
Vaccine/ISA206+CpG	Subgroup 4	full	5	5	0	12.51
	Subgroup 5	1/3	5	5	0	
	Subgroup 6	1/9	5	4	1	

Animals from the control group were vaccinated with the FMD-killed vaccine adjuvanted by ISA 206 alone, and animals from the Test group were vaccinated with the FMD killed vaccine adjuvanted by ISA 206 as well as pUC18-CpG plasmid. PD50 was calculated on the basis of the number of protected animals in each subgroup by using the Spearman–Kärber method.

that for the LPS positive control (Fig. 1), indicating that pUC18-CpG could be used as a potent immunoadjuvant to enhance the humoral immune response.

In the present study, we have shown (Fig. 2) that the pUC18-CpG plasmid containing swine-specific CpG motifs helped elicit an early immune response when coadministered with the traditional oil-adjuvant FMD killed vaccine. On day 14 after the initial vaccination, the average antibody titer in group D was 1.5-fold greater than that in group B. Although the induction of long-term enhancement of the antibody response is a key indicator of vaccine efficacy, unfortunately, this phenomenon was not observed in the present study. This may be due to the gradual degradation of the plasmids once the vaccination was carried out, hence limiting its adjuvant effect. Meanwhile, the early appearance of neutralizing antibodies is also important, especially when there is a need to carry out enforced immunization in a neighboring zone during an emergency outbreak of FMD in a disease-free area. (19).

In the current study, during the initial vaccination period, there were significant differences ($P < 0.05$) in the antibody levels between the group vaccinated with oil-adjuvant FMD-killed vaccine group (group B) and the group vaccinated with pUC18-CpG-adjuvant vaccine (groups C and D, immunized with FMD vaccine-oil adjuvant + pUC-18-CpG), with the antibody levels in the latter groups being much higher. During the experiment, the average antibody levels in groups C and D showed no statistically significant difference ($P > 0.05$), indicating that there is not a linear dose–effect relationship when the given plasmid dose is above 250 μg. Whether there is such a relationship when the given dose is below 250 μg remains to be further studied.

As killed vaccines mainly trigger humoral immune responses, the levels of elicited antibodies could be a useful indicator to evaluate the effectiveness of the vaccine used. After the booster vaccination, the average antibody titers in all vaccinated groups reached nearly the same high level and gradually declined to below 6 towards the end of the experiment. The adjuvant effect of pUC18-CpG during this period was not as obvious as that observed during the initial vaccination period, partly because of the immune regulatory effects of the host immune system (20).

To further validate the immunostimulatory effects of pUC18-CpG, a potency test was carried out to compare the PD_{50} values obtained with the traditional vaccine and those obtained with the pUC18-CpG adjuvant vaccine. According to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals issued by OIE, a commercial FMD vaccine for pigs should have a PD_{50} value of at least 3 per dose. In our study, the PD_{50} value of the pUC18-CpG adjuvant vaccine (12.51) was almost twice that of the traditional oil-adjuvant vaccine (6.47). The antibody titer values from the blood samples collected prior to performing the challenge also indicated a much higher level of anti-FMDV structural protein antibodies in the pUC18-CpG vaccine group (data not shown). From the above results and those given earlier, we conclude that pUC18-CpG is a potent immunoadjuvant for the traditional FMD-killed vaccine and that the efficacy of this vaccine can be greatly enhanced when it is given together with this adjuvant.

ACKNOWLEDGMENTS

We are highly grateful to the animal health worker of the company who carried out animal breeding and blood sample collection. This study was supported by Special Funds for Scientific Research on Public Causes (Research on Prevention and Control of Brucellosis and Bovine Tuberculosis, No. 200903027) and by Central Level Special Funds for Institute Fundamental Scientific Research on Public Causes (No. 2010js-1).

DISCLOSURE

The authors declare that no conflict of interest exists.

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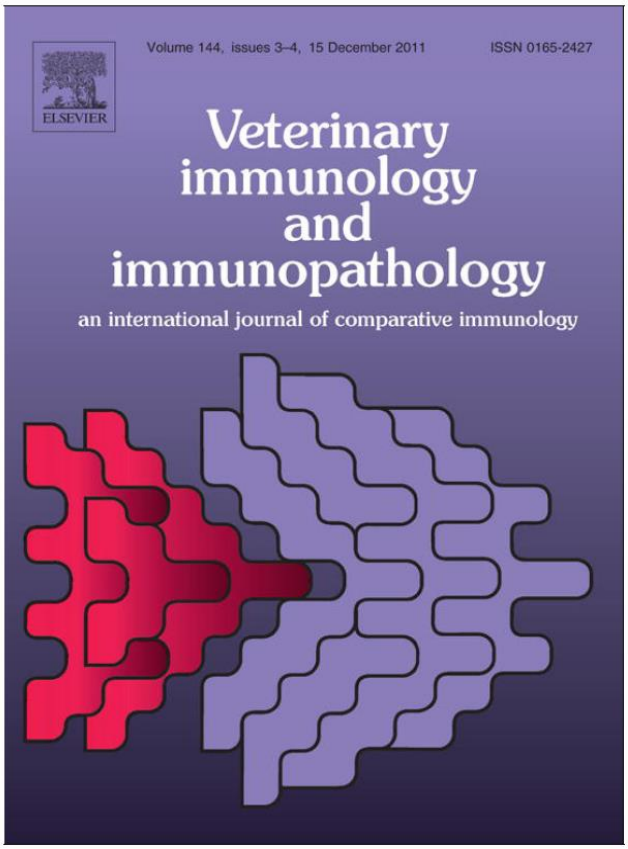

Veterinary Immunology and Immunopathology 144 (2011) 405–409

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Plasmid containing CpG motifs enhances the efficacy of porcine reproductive and respiratory syndrome live attenuated vaccine

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ARTICLE INFO

Article history:
Received 21 June 2011
Received in revised form 27 July 2011
Accepted 28 July 2011

Keywords:
Porcine reproductive and respiratory syndrome
CpG motif
Adjuvanticity
Immune response
Protection

ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) is now among the most important swine diseases that affect the Chinese swine industry. Both killed and live attenuated vaccines are currently used against the disease, but neither of them could provide full protection after vaccination. In the present study, the adjuvanticity of a plasmid containing CpG motifs (pUC18-CpG) was introduced to enhance the efficacy of a commercial PRRS live attenuated vaccine. After vaccination, PRRSV-specific antibodies, PRRSV-specific cytokines, and clinical parameters were studied and compared between different vaccinated groups. During a following challenge study, co-administration of pUC18-CpG with the vaccine could confer higher protection rate. Our results have shown that co-administration of pUC18-CpG with the vaccine could elicit more potent adaptive immune response and provide better protection.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a swine disease that first emerged in the United States during the 1980s and later in Europe in 1981 (Madsen et al., 1998; Mardassi et al., 1994). Nowadays it has spread worldwide and continues to cause great economic losses to the swine industry every year. The causative agent of the disease is the PRRS virus (PRRSV), which is a small, enveloped virus belonging to the family *Arteriviridae*, genus *Arterivirus* (Yang et al., 1998). PRRSV is believed to replicate in macrophages, and adopt several mechanisms to evade the host's immune surveillance (Yoo et al., 2010).

Currently available vaccines against the disease mainly include killed virus (KV) vaccines and live attenuated vaccines (Zuckermann et al., 2007). KV vaccines have been reported of limited efficacy, even against homologous challenge, possibly due to the antibody dependent enhancement (ADE) mechanism that facilitates the attachment and internalization of the virus into target cells (Cancel-Tirado et al., 2004). Up to now, live attenuated vaccines have shown better protection efficacy, especially against homologous infection, but their effectiveness against heterologous infection is variable (Kimman et al., 2009). Therefore, options as to the change of vaccination modes or the use of more powerful immunoadjuvants may help to enhance the efficacy of current live attenuated vaccine.

CpG motif was first described by Krieg AM in 1995 as an oligodeoxynucleotide (ODN) sequence in which an unmethylated CpG dinucleotide is flanked by two 5′ purines and two 3′ pyrimidines (Krieg et al., 1995).

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doi:10.1016/j.vetimm.2011.07.023

Being a ligand of the toll-like receptor 9(TLR9), CpG ODN can activate the host's innate immune system, especially antigen-presenting cells and B cells, signifying a potential use as vaccine adjuvant (Krieg, 2006). Synthetic CpG ODNs with a nuclease-resistant phosphorothioate backbone were found to be potent Th1 type immunoadjuvants. And our previous study has also shown that plasmids containing CpG motifs could function as immune potentiators to PRRS KV vaccines (Quan et al., 2010). In the present study, we further investigated the adjuvanticity of this plasmid to a PRRS live attenuated vaccine.

2. Materials and methods

2.1. Reagents

PRRS live attenuated vaccine (Freeze-dried) was purchased from Yangzhou VACBIO Bioengineering Co., Ltd. The vaccine was composed of an attenuated JXA1-R strain generated from serial passage of a high-pathogenic PRRS virus strain (JXA1). Each dose contains at least 10^5 $TCID_{50}$. PRRS Antibody Test Kit was purchased from the IDEXX Co., Ltd. Swine IFN-γ immunoassay kit was purchased from Invitrogen International Inc. Swine IL-6 immunoassay kit was from R&D International Inc. Heparin lithium tubes were purchased from HuNai Biotech Co., Ltd., China; 96-well cell culture plates were purchased from Corning Co., Ltd; Ficoll-Paque was from DingGuo Biotech, Beijing, China; The MiniBEST Viral RNA/DNA Extraction Kit and PrimeScript One Step RT-PCR Kit were purchased from TaKaRa, Dalian, China.

2.2. Plasmid preparation

The pUC18-CpG plasmid containing 20 copies of CpG Motifs (CpG Motif sequence: 5′-TCGTCGTTTTGTCGTTTTGTCGTTTTGTCGTTTTGTCGTT-3′) was generated through tandem insertion of the same motif into the multiple cloning sites (MCS) of pUC18 vector. pUC18-CpG was transformed into *Escherichia coli* (*E. coli*) and multiplied by fermentation. Then the recombinant plasmid was purified through a large scale plasmid purification method (Zhang et al., 2008). Briefly, after alkali lysis, the 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 the concentration of 10 mg/ml, and stored at −20 °C until use.

2.3. Animal selection and experiment design

Thirty 28-day-old Changbai piglets were selected from a pig farm, after being confirmed free of PRRSV infection by ELISA and of other swine diseases infection (classical swine fever, pseudorabies, porcine parvovirus disease, porcine circovirus disease) by specific PCR amplification. The piglets were randomly divided into five groups, 6 per group. Group A was immunized with a full dose PRRSV live attenuated vaccine; Group B was co-immunized with 500 μg pUC18-CpG and a full dose vaccine; Group C was co-immunized with 500 μg pUC18-CpG and 1/10 dose vaccine; Group D was co-immunized with 500 μg pUC18-CpG and 1/100 dose vaccine; Group E was immunized with PBS as a control group. All the vaccine and/or adjuvant were administered intramuscularly with a total volume of 2 ml. All procedures involving animals conformed to the policies of the local animal care committee.

2.4. Antibody titration and cytokine measurement

Blood samples were collected in evacuated test tubes with or without heparin lithium (150 USP units) by venipuncture of precaval vein following sterile procedures on days 7, 14, 21and 28 post-immunization, respectively.

For blood samples without anticoagulant treatment, serum was separated by centrifugation and stored at −20 °C until use. PRRSV-specific antibody level from each serum sample was titrated using IDEXX ELISA kit according to the manufacturer's instructions. PRRSV positive serum from the kit was used as positive control. PRRSV-specific antibody titers were reported as Sample/Positive (S/P) ratios, and the sample was considered positive if the S/P ratio was 0.4 or above.

For blood samples collected with anticoagulants, swine PBMCs were separated by density gradient centrifugation and used for cytokine analysis. Briefly, each sample was cultured in triplicates at a concentration of 1×10^6 cells/ml. After PRRSV antigen (10 μg/ml) was added as stimulus, cells were cultured at 37 °C for 72 h. After incubation, the supernatants from each well were harvested and stored at −70 °C until assay. Control stimuli were performed with RPMI 1640 medium alone or ConA at 5 μg/ml. The level of porcine IL-6 and IFN-γ in porcine PBMC culture supernatants was quantified by commercial swine IL-6 and IFN-γ immunoassay kits according to the manufacturer's instructions. Supplied standards were used to generate standard curves. The detection limits of the assay were 10 pg/ml for IL-6 and 2.0 pg/ml for IFN-γ. Geometric means from the replicates was calculated first, and then was used to generate geometric means among the animals within each group.

2.5. Challenge study

On day 28 post vaccination, animals in all groups were challenged with a virulent strain of PRRSV (JXA1 strain). Inoculation was performed by intramuscular administration of the virus in Eagle's Minimum Essential Medium within a total volume of 3 ml, containing $10^{4.05}$ TCID50. The mortality rate was observed each day until 21 days post challenge. Clinical signs were monitored based on the nervous and respiratory symptoms. Viraemia and virus excretion were observed and measured each week and autopsy symptoms were registered in the end of the experiment.

2.6. Statistical analysis

Data analysis was performed with Prism 5 software (GraphPad). Comparison was made by using the Student

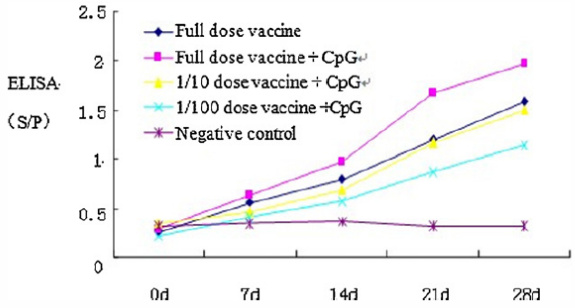

Fig. 1. PRRSV-specific antibody levels at different time intervals post vaccination. Antibody titers were reported as S/P. Sample was considered positive if the S/P ratio was 0.4 or above.

t-test. All values are presented as the mean ± SEM. A *p*-value < 0.05 was considered statistically significant.

3. Results

3.1. Comparison of humoral immune responses in different groups

PRRSV-specific antibody level was titrated by ELISA at one-week time intervals and described by S/P as shown in Fig. 1. During the whole process, animals in the negative control group (Group A) remained seronegative for PRRSV.

On day 7 post-immunization, PRRSV-specific antibodies were detectable in all vaccinated groups. The highest antibody level was observed in Group B (Full dose vaccine plus pUC18-CpG), which remained higher than antibody levels from the other groups during the following tests. On days 21 and 28, antibody level from Group B was significantly higher than that in the other vaccinated groups ($p < 0.05$). There were no significant differences between antibody titers in Group A (Full dose vaccine only) and Group C (1/10 dose vaccine plus pUC18-CpG). And the lowest level was observed in Group D (1/100 dose vaccine plus pUC18-CpG).

3.2. Quantification of PRRSV-specific cytokines from porcine PBMC supernatants

Samples from Group B showed the highest level of IL-6 on both day14 and day 28 post immunization ($p < 0.05$). Groups A and C showed no significant difference in IL-6 expression. Group D showed the lowest level of IL-6 in all vaccinated groups during the test (Fig. 2).

In terms of IFN-γ, the highest level was found on day 14 from Group B, which was significantly higher than that in the other groups at this time point ($p < 0.05$). On day 28, the IFN-γ levels in Group A and B dropped to a lower level, but IFN-γ levels from all vaccine plus CpG vaccinated groups were significantly higher than that from Group A ($p < 0.05$). On both occasions, cytokines levels from vaccinated groups were significantly higher than that from negative control groups ($p < 0.05$) (Fig. 3).

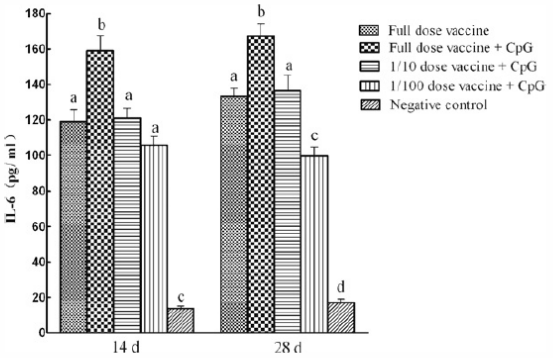

Fig. 2. Quantification ofIL-6 on days 14 and 28 post-inoculation in porcine PBMCs supernatants. Five groups of six 28-day-old piglets were immunized intramuscularly (i.m.). Data were expressed as mean ± SEM ($n = 6$). The difference between groups with different letters at each time period is significant ($p < 0.05$).

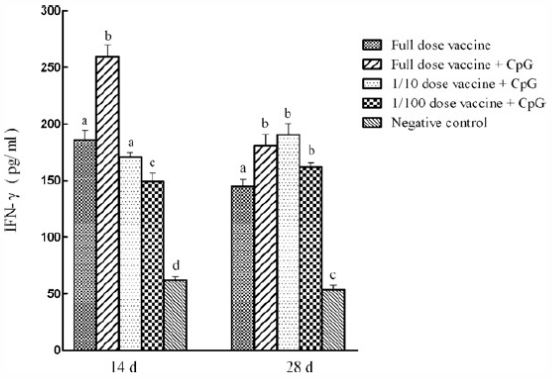

Fig. 3. Quantification of IFN-γ on days 14 and 28 post-inoculation in porcine PBMCs supernatants Five groups of six 28-day-old piglets were immunized intramuscularly (i.m.) Data were expressed as mean ± SEM ($n = 6$). The difference between groups with different letters at each time period is significant ($p < 0.05$).

3.3. Clinical observation, viraemia and virus excretion in vaccinated animals after challenge

After challenge, clinical signs were monitored, and the number of pigs showing nervous and respiratory symptoms was recorded. On day 21 post-challenge, the number of survived pigs in each group was counted (Table 1).

Virus excretion could be detected on days 7 and 14 in each group, while Group B showed the lowest percent-

Table 1
Clinical parameters post challenge.

Groups	Survivors on day 21 post-challenge	Number of pigs with clinical symptoms		
		DPC7	DPC14	DPC21
A	5/6	1/6	1/6	0/5
B	6/6	0/6	0/6	0/6
C	6/6	1/6	1/6	1/6
D	5/6	1/6	2/6	0/5
E	0/6	5/6	3/3	–

DPC: day post challenge.

Table 2
Viraemia and virus excretion in vaccinated animals after challenge.

Groups	Days post-challenge (DPC)							
	Virus excretion				Viraemia			
	0	7	14	21	0	7	14	21
A	0/6	4/6	2/6	0/5	0/6	4/6	3/6	0/5
B	0/6	3/6	1/6	0/6	0/6	3/6	1/6	0/6
C	0/6	6/6	3/6	1/6	0/6	6/6	4/6	1/6
D	0/6	6/6	3/6	2/5	0/6	6/6	4/6	2/5
E	0/6	5/5	3/3	–	0/6	5/5	3/3	–

age in detection rate (50% on day 7 and 16.7% on day 14, respectively). On Day 21, no virus excretion was detected in neither Group A nor B, but one animal died of PRRS on day 19 in group A (Table 2).

In the meantime, viraemia could be detected in all groups on days 7 and 14. Animals in Group B showed the lowest detection rate during the process (50% on day 7 and 16.7% on day 14, respectively), which is in accordance with the case of virus excretion. On day 21, animals in Group A and B showed no symptom of viraemia. All animal in the control group died of PRRS within the 21 days after challenge (Table 2).

3.4. Autopsy examination

At necropsy, animals in Group A showed obvious lesions in the lung and enlargement in the hilar lymph node, while there were no such symptoms observed in animals from Group B. However, pigs from both Groups C and D showed severe hemorrhagic kidneys, enlarged lymph nodes and spleen infarction. Moreover, two animals from Group D showed necrosis in the posterior segment.

4. Discussion

Ever since 2006, the emergence of highly pathologic porcine reproductive and respiratory syndrome (HP-PRRS) has cost great loss to the country's swine industry (Li et al., 2007). Although PRRSV killed vaccine can elicit rapid IgM and IgG production after immunization, neutralizing antibodies (NAs) are not detectable until 28 DPI (Diaz et al., 2005). Therefore, the protect efficacy of PRRSV killed vaccine is quite limited. The early emergence of non-neutralizing antibodies may facilitate the entrance of the viruses into targeted cell by means of ADE mechanism (Cancel-Tirado et al., 2004). In the present study, we chose a commercial PRRSV live attenuated vaccine, which showed better protection when co-administered with the plasmid containing multiple CpG motifs (pUC18-CpG).

The host innate immune system detects pathogen associated molecular patterns (PAMPs) to found an early immune response against the infection of microorganisms. As a danger signal, CpG motifs from the genome of bacteria and DNA virus could be recognized by TLR9. Multiple studies have shown that CpG motifs presented the characteristic of being species-specific (Rankin et al., 2001). The CpG motif for optimal stimulation of swine PBMCs was found to be GTCGTT (Linghua et al., 2006). During a lymphocyte transformation test, the Stimulation Index (SI) for the optimal dose of pUC18-CpG was 3 times higher than that for LPS (unpublished data), indicating that this plasmid could be used as a potent adjuvant for enhancing humoral immune response.

In this study, co-administration of pUC18-CpG with the commercial PRRS live attenuated vaccine helped to elicit a stronger humoral immune response. PRRSV-specific antibody titers in Group B remained higher than that from the other vaccinated groups during the test. Antibody titers from Groups A and C showed no significant difference during the process, while the immunizing dose in Group C was only 1/10 of that in Group A, indicating the antigen dose saving property of the adjuvant (Weeratna et al., 2003).

CpG ODN has been reported to trigger antigen presenting cells and B cell activation (Krieg et al., 1995), and induce a Th1 dominant cytokine profile (Corral and Petray, 2000). In the current study, cytokines as IL-6 and IFN-γ were assayed, respectively. Although IL-6 has been found to inhibit Th1 polarization (Diehl and Rincon, 2002), high levels of IL-6 found in Group B may help to overcome the suppression effect of regulatory T cells (Pasare and Medzhitov, 2003), allowing for a stronger humoral immune response, as indicated by the higher antibody titers from Group B. Higher IFN-γ levels in Group B on day 14 indicated that addition of pUC18-CpG to PRRSV live attenuated vaccine could enhance the immune response in a Th1 type biased response.

After challenge, animals in both Groups A and B showed mild clinical symptoms compared to that observed in Groups C, D and control group. Although the humeral immune response elicited in both Group A and Group C were comparable ($p < 0.05$), animals from Group C exhibited much severe clinical symptoms. This may due to the different cytokine profiles that each candidate stimulated in terms of type and quantity. Cytokines other than IL-6 and IFN-γ remain to be further studied, especially on the influence of cell mediated immune response.

Virus excretion could be detected on days 7 and 14 in all groups post challenge, while animals in Group B showed the lowest percentage in detection rate. On day 21, no virus excretion was detected in neither Group A nor B, while one animal from Group A died of PRRS on day 19 after challenge. Throughout the experiment, animals in Group B (full dose vaccine plus pUC18-CpG) showed the milder clinical symptoms and full protection against challenge. In the meantime, viraemia could be detected in all groups on days 7 and 14. Animals in Group B showed the lowest detection rate, which is in accordance with the case of virus excretion. On day 21, animals in Groups A and B showed no viraemia.

Taken together the results obtained above, a conclusion can be made that pUC18-CpG is a potent immunoadjuvant for PRRSV live attenuated vaccine and could greatly enhance its efficacy when administered together.

Acknowledgements

The work for animal breeding and blood sample collection by the animal health worker in the veterinary college of Yangzhou University is highly appreciated. The study was supported by grants from Key Projects in the National

Science & Technology Pillar Program during the Eleventh Five-year Plan Period of China (No. 2006BAD31B03) and also by Special Funds for Scientific Research on Public Causes (Research on prevention and control of Brucellosis and Bovine Tuberculosis, No. 200903027).

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5. DISSCUSSION

Adjuvants are administered with antigens to enhance the correspondent immune response stimulated by the latter. Since recombinant antigen proteins or synthetic peptides provide more biosafety than the crude inactivated micro-organism, while being less immunogenic, the specific and powerful adjuvants could play an important role in improving the efficacy of modern vaccines as the antigens become more purified (Aucouturier, Dupuis, and Ganne, 2001).

Currently approved adjuvants include Aluminum Salts, Freund's Adjuvant as well as certain oil and water emulsions (Reed et al., 2009). Oil-based adjuvants are nowadays in wide use for veterinary vaccines. However, they may cause local and general reactions, including fever, granuloma or abscesses, etc. Despite the impressive success of their adjuvanticity in generating immunity against viral and bacterial infections, there remains a need for the research and development of novel adjuvants that could enhance protective antibody responses, and those induce strong Th1 immune response.

Adjuvants are believed to take effects through a variety of mechanisms. Most of the traditional adjuvants serve as carriers or matrix for antigens (Petrovsky and Aguilar, 2004), while novel adjuvants are targeted at the direct activation of the innate immune system. During the past decade, many pattern recognition receptors (PRRs) and their corresponding signaling pathways in the innate immune system have been highlighted and the innate immune responses have proven to be capable of strongly influence the adaptive immune response (Schijns and Lavelle, 2011).

Most members of the PRR families are potential targets for adjuvants derived from the PAMP molecules. Among them, 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 (Klinman, 2004). Indeed, Incorporation of a higher number of CpG motifs into an FMD DNA vaccine candidate has proven to induce much stronger immune responses. During another investigation, treatment of the tight-skin mouse model of scleroderma with CpG-enriched plasmid has ameliorated the scleroderma-like syndrome in tight-skin mice (Shen et al., 2005; Zhang et al., 2005). In the current research, a swine-specific CpG motif enriched plasmid has been constructed and its immunostimulatory properties have been studied.

(1) The test of the immune stimulatory effect of pUC18-CpG *in vitro*—swine PBMCs lymphocyte transformation assay

Unmethylated CpG motifs are more prevalent in the bacterial genome than in vertebrate genomic DNAs. Oligodeoxynucleotides (ODN) containing CpG motifs could activate host immune defense mechanisms, leading to innate immune responses including direct B cell activation and type I interferons expression (Krieg, 2006). During the early empirical structure–activity relationship studies, species-specific differences in the optimal CpG motif have been discovered, which for mice is GACGTT but GTCGTT for humans (Hartmann and Krieg, 2000; Krieg et al., 1995).

Since the demand for safer and more powerful adjuvants in human and veterinary vaccinations is ever-increasing, as well as the application of animal models to human disease, it is worthwhile that CpG ODN be further investigated in veterinary species. During another study, optimal CpG motifs for veterinary and laboratory animals were screened via lymphocyte proliferation assay. And it has been shown that CpG motifs used for optimal immunostimulation are highly conserved among different veterinary species (Rankin et al., 2001).

It has been reported that peripheral blood mononuclear cells (PBMCs) from pigs respond to ODNs containing specific CpG motifs by proliferating and secreting IL-6, IL-12 and TNF-α, and the optimal nucleotide sequence for swine PBMCs stimulation was found to be GTCGTT (Kamstrup, Verthelyi, and Klinman, 2001; Linghua et al., 2006). Hence，it would be of great practical importance to determine whether plasmid containing multiple CpG motifs is immunostimulatory in husbandry animals. With large scale fermentation and purification

process of the recombinant plasmid at a relatively lower cost (Sun et al., 1994), CpG enriched plasmid may be of great value both as vaccine adjuvants (increasing both humoral as well as cell-mediated immune response elicited by protein, peptide-based antigens and DNA) and as immunoprotective agents.

In the present study, we used a CpG motif enriched plasmid (pUC18-CpG) as an immune stimulator for swine PBMCs lymphocyte transformation assay. Our results showed that the Stimulation Index (SI) for the optimal dose of pUC18-CpG was more than 2.5 times higher than that of LPS, indicating that pUC18-CpG could be used as a potent immunopotentiator for enhancing humoral immune response.

(2) The test of the immune stimulatory effect of pUC18-CpG *in vivo*—enhancement on the efficacy of the traditional foot-and-mouth disease killed vaccine

Foot-and-mouth disease is one of the notifiable diseases by the OIE and is of great socio-economic consequence because of its potential of rapid international spread. FMDV infection could cause high morbidity, with low to moderate mortality in most cases. Vaccination has proven to be a powerful strategy for disease control and eradication in FMD-endemic areas and in case of emergency outbreaks in areas normally free from the disease (Hutber et al., 2011).

The currently in use FMD killed vaccine is manufactured by growing the live virulent strains of FMDV in BHK-21 cell lines under bio-secure conditions, inactivation by chemicals, and then formulated with oil-adjuvants (Doel, 2003). The synthetic peptide vaccine is also commercially available in China for the prevention and control of the disease. Other vaccine candidates under research include the live attenuated vaccines, empty capsid vaccines and live vector vaccines, etc. (Rodriguez and Grubman, 2009).

Although the FMD killed vaccine has a number of positive characteristics, including the induction of immune protection upon challenge and thus the prevention and control of the disease, there are some major shortcomings of this vaccine and its production. The manufacturing process of FMD killed vaccine may risk the release of the live virus to the environment because of incomplete inactivation, causing biosafety issues. Also, FMD killed vaccine exhibits other disadvantages such as the need of adequate cold chain of formulated vaccines, necessity of

multiple vaccinations, short shelf life, etc. Moreover, the use of oil-based adjuvants may cause local and general reactions.

On the other hand, synthetic peptide vaccines are designed to include both T and B cell antigenic epitopes optimized for both immunogenicity and antigenicity. But the efficacy of these vaccines is restricted by representing only a limited number of antigenic sites and/or T-cell epitopes of the virus. On these regards, a better FMD vaccine design strategy may provide more powerful FMD vaccines.

Indeed, efforts as to the development of more efficacious adjuvants have been made during several previous studies. It has been reported that FMDV replication could be inhibited by type I interferons (Chinsangaram, Koster, and Grubman, 2001). Thus an Ad5 vector containing the porcine IFN-α gene expressed high levels of the biologically active interferon when infected the cells. And the pigs inoculated with a single dose of the recombinant vectored-vaccine were completely protected when challenged with FMDV (Chinsangaram et al., 2003).

Poly(I:C) is a synthetic double-stranded polyribonucleotide, which could induce the expression of type I interferons. During another research, co-administration of a multi-epitope protein with poly(I:C) can greatly increase the neutralizing antibody response against FMDV in mice. And the poly(I:C) adjuvanted multi-epitope protein vaccine candidate completely protected pigs against virulent foot-and-mouth disease virus challenge (Cao et al., 2012; Cao et al., 2013).

CpG ODNs are also strong inducers of type I IFNs. In a previous study, treatment of mice with CpG ODN alone significantly reduced viremia, the severity of disease and the death rate during the challenge with

foot-and-mouth disease virus (FMDV) (Kamstrup, Frimann, and Barfoed, 2006).

In our *in vivo* adjuvanticity test, pUC18-CpG was employed as vaccine adjuvant and co-administered with a commercial FMD killed vaccine. On day 14 after the initial vaccination, the average antibody titer in group D was 1.5 times higher than that in group B, implying that co-administration of pUC18-CpG may help bring forward the appearance of anti-structural protein antibodies. The early appearance of neutralizing antibodies is of great significance, especially when there is a need to perform enforced immunization in the neighboring zone during an outbreak of the disease (Patil et al., 2002). On day 28, the antibody titers of Groups C and D remained higher than that in Group B ($p<0.05$).

To reduce the cost of CpG ODN production, which is normally via chemosynthesis, the CpG motifs were inserted into a plasmid vector, as described in the first paper. The adjuvant effect of the recombinant plasmid was then compared with that of the synthesized CpG ODN, which contains a phosphorothioate backbone. Both forms of CpG ODN were found to enhance the humoral immune response when co-administered with the traditional FMD killed vaccine in a previous animal experiment，generating higher antibody levels than that of the vaccine alone.

During the initial vaccination period in the current study, there were significant differences ($P < 0.05$) in the antibody levels between the group vaccinated with the traditional oil-adjuvant FMD killed vaccine (group B) and the groups vaccinated with pUC18-CpG-adjuvant vaccine (groups C and D), with the antibody levels in the latter groups being

much higher. Since killed vaccines mainly trigger humoral immune responses, the levels of antibodies could be a useful indicator to evaluate the effectiveness of the vaccine used.

After the booster vaccination, the average antibody titers in all vaccinated groups reached nearly the same high level and gradually declined to below 6 at the end of the experiment. The adjuvant effect of pUC18-CpG during this period was not as obvious as that observed during the initial vaccination period, partly because of the immune regulatory effects of the host immune system(Lim et al., 2005).

According to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals issued by OIE, a commercial FMD vaccine for pigs should have at least 3 PD_{50} per dose for routine prophylactic use. To further validate the immunostimulatory effects of pUC18-CpG, a potency test was carried out to compare the PD_{50} values obtained with the traditional vaccine and that obtained with the pUC18-CpG adjuvanted vaccine. In our study, the PD_{50} value of the pUC18-CpG adjuvanted vaccine (12.51) was almost twice that of the traditional oil-adjuvant vaccine (6.47). Thus it is feasible to use the pUC18-CpG as a potent adjuvant for the traditional FMD killed vaccine, in order to achieve better immune protection effects.

(3) The test of the immune stimulatory effect of pUC18-CpG *in vivo*—enhancement on the efficacy of the porcine reproductive and respiratory syndrome live attenuated vaccine

Porcine reproductive and respiratory syndrome (PRRS) is a swine disease that first emerged in the United States during the 1980s and later in Europe in 1981 (Madsen et al., 1998). Nowadays it has spread worldwide and continues to cause great economic losses to the swine industry every year. Currently available vaccines against the disease mainly include killed virus (KV) vaccines and live attenuated vaccines (Zuckermann et al., 2007).

KV vaccines have been reported of limited efficacy, even against homologous challenge. Live attenuated vaccines have shown better protection efficacy, especially against homologous infection, but their effectiveness against heterologous infection is variable (Kimman et al., 2009). Other experimental PRRSV vaccines include DNA vaccines, vectored vaccines, and DIVA (differentiating infected from vaccinated animals) vaccines. But so far there hasn't been a commercialized vaccine available from any of these candidates.

Ever since 2006, the emergence of a highly pathologic porcine reproductive and respiratory syndrome (HP-PRRS) has cost great loss to China's swine industry (Li et al., 2007). Although PRRSV killed virus (KV) vaccine can elicit rapid IgM and IgG production after immunization, neutralizing antibodies (NAs) are not detectable until 28 DPI (Diaz et al., 2005). Therefore, the protection efficacy of PRRSV killed vaccine is quite limited. Moreover, the early emergence of non-neutralizing antibodies may facilitate the entrance of the viruses into targeted cells by means of

the ADE mechanism (Cancel-Tirado, Evans, and Yoon, 2004).

Efforts have been made in utilization of immunoadjuvants for enhancing the efficacy of current PRRSV vaccines. These adjuvants include recombinant cytokines, synthesized reagents, and bacterial products. Among them, some interleukins (IL-2, IL-12 and Interferon-Alpha), poly I:C and CpG ODN show great adjuvanticity in enhancing CMI response to PRRSV vaccines, while the use of CpG ODN also enhances the PRRSV-specific antibody response after vaccination(Charerntantanakul, 2009).

In previous studies, CpG ODN has been employed to enhance both Th1 and Th2 responses to the PRRSV KV vaccine, and was found to significantly increase both cell-mediated and humoral responses against PRRSV in pigs, as well as the protective efficacy in challenge models (Linghua, Xingshan, and Fengzhen, 2007; Linghua et al., 2006). During another research, plasmid containing swine-specific CpG motifs could also augment the immune responses of pigs immunized with the PRRSV KV vaccine (Quan et al., 2010).

In the present study, a commercial PRRSV live attenuated vaccine was chosen, and conferred better protection when co-administered with the plasmid pUC18-CpG. During the animal experiments, co-administration of pUC18-CpG with the commercial PRRSV live attenuated vaccine helped to elicit a stronger humoral immune response. PRRSV-specific antibody titers in Group B remained higher than that from the other vaccinated groups during the test. Antibody titers from Groups A and C showed no significant difference during the process, while the immunization dose in Group C was only 1⁄10 of that in Group A,

indicating the antigen dose saving property of the novel adjuvant (Weeratna, Comanita, and Davis, 2003).

CpG ODN has been reported to trigger direct activation of antigen presenting cells and B cells, and induce a Th1 dominant cytokine profile (Corral and Petray, 2000; Krieg et al., 1995). In the current study, cytokines as IL-6 and IFN-γ were assayed, respectively. Although IL-6 has been reported to inhibit the Th1 polarization (Diehl and Rincon, 2002), high levels of IL-6 found in Group B may help to overcome the suppression effect of regulatory T cells (Pasare and Medzhitov, 2003), allowing for a stronger humoral immune response, as indicated by the higher antibody titers observed in Group B. Higher IFN-γ levels in Group B on day 14 indicated that addition of pUC18-CpG to PRRSV live attenuated vaccine could enhance the immune response in a Th1-biased way.

After challenge, animals in both Groups A and B showed mild clinical symptoms compared to that observed in Groups C, D and the control group. Although the humoral immune response elicited in both Group A and Group C were comparable ($p<0.05$), animals from Group C exhibited much severe clinical symptoms. This may due to the different cytokine profiles that each vaccine stimulated in terms of type and quantity. Cytokines other than IL-6 and IFN-γ remain to be further studied, especially about their influences on the cell−mediated immune response.

Virus excretion could be detected on days 7 and 14 in all groups post challenge, while animals in Group B showed the lowest percentage in the detection rate. On day 21, no virus excretion was detected in either Group A or B, while one animal from Group A died of PRRS on day 19

after challenge. Throughout the experiment, animals in Group B (full dose vaccine plus pUC18-CpG) showed the mildest clinical symptoms and full protection against challenge. In the meantime, viraemia could be detected in all groups on days 7 and 14. Animals in Group B showed the lowest detection rate, which is in accordance with the case of virus excretion. On day 21, animals in Group A and B showed no viraemia. Based on the above results, it is possible that the pUC18-CpG could be used as a potent adjuvant for the PRRSV live attenuated vaccine.

6. CONCLUSIONS (CONCLUSIONES)

FIRST

pUC18-CpG containing 20 copies of CpG ODN 2006 was achieved through tandem insertion of 5-copy CpG ODN 2006 into the multiple cloning sites of the pUC18 plasmid vector.

PRIMERA

El pUC18-CpG que contiene 20 copias de CpG ODN 2006 se logró a través de la inserción en tándem de 5-copia CpG ODN 2006 en los múltiples sitios de clonación del vector-plásmido pUC18.

SECOND

pUC18-CpG stimulated swine PBMCs proliferation was measured by the MTT colorimetric assay at different doses. Compared to the LPS positive control, pUC18-CpG showed higher SI, implying the potential use as a potent vaccine adjuvant.

SEGUNDA

Estudios de transformación de linfocitos se realizaron para valorar el efecto inmune del plásmido a diferentes dosis, utilizando el método del MTT. El pUC18-CpG demostró mayor índice de estimulación que el control positivo de LPS en todas las dosis estudiadas, lo que implica su potencial uso como un adyuvante potente.

THIRD

When co-administered with a traditional oil-adjuvant FMD killed vaccine FMDV OS/99，pUC18-CpG helped to elicit an early immune response, and enhance the anti-FMDV structural protein antibody titers by more than 1.5 times higher during the first vaccination.

TERCERA

Cuando se co-administra una vacuna inactivada de FA (OS/99) con el pUC18-CpG，se genera una respuesta inmune más temprana, mejorando los títulos de los anticuerpos, frente a la proteína estructural del VFA, más de 1,5 veces que el control durante la primera vacunación.

FOURTH

During the potency test, co-administration of pUC18-CpG with the FMD killed vaccine FMDV OS/99 (Serotype O, registered by the XinjiangTiankang Animal Science Bio-Technology Co., Ltd.) was shown to greatly enhance the potency of the mentioned vaccine (PD50 values: 12.51, 6.47).

CUARTA

Durante la prueba de potencia, se mostró que la co-administración del pUC18-CpG con la vacuna inactivada del VFA (OS/99) mejoraba la potencia de esta última (PD50: 12.51, 6.47).

FIFTH

Co-administration of pUC18-CpG with a commercial PRRSV live attenuated vaccine JXA1-R helped to elicit a stronger humoral immune response. On day 21 post-vaccination, the PRRSV-specific antibody titers in the pUC18-CpG adjuvanted full-dose vaccine group was about 1.4 times higher in comparison with the control group.

QUINTA

La co-administración del pUC18-CpG con una vacuna viva atenuada contra el síndrome reproductivo y respiratorio porcino (vacuna JXA1-R) ayudó a inducir una respuesta inmune humoral mayor que con solo la

vacuna. En el día 21 después de la vacunación, los títulos de anticuerpos específicos frente al virus de PRRS, en el grupo de animales vacunados con la dosis completa y con el adyuvante pUC18-CpG, fue alrededor de 1.4 veces mayor que la del grupo control.

SIXTH

Co-administration of pUC18-CpG with the commercial PRRS live attenuated vaccine JXA1-R resulted in a Th1−type biased immune response. 14 days after the vaccination, the IFN-gamma levels in the pUC18-CpG adjuvanted full-dose vaccine group was almost 1.4 times higher in comparison with the control group.

SEXTA

La co-administración de pUC18-CpG con la vacuna viva atenuada del PRRS (JXA1-R) dio lugar a un tipo de respuesta inmune Th1. 14 días después de la vacunación, los niveles de IFN-gamma en el grupo vacunado con la dosis completa y el adyuvante pUC18-CpG fue 1,4 veces mayor que la del grupo control.

SEVENTH

Co-administration of pUC18-CpG with the commercial PRRS live attenuated vaccine could ameliorate the clinical symptoms and confer higher protection rate during challenge by the virulent PRRS virus JXA1.

SÉPTIMA

La co-administración de pUC18-CpG con la vacuna viva atenuada del PRRS podría conferir síntomas clínicos menos severos y una mayor tasa de protección durante el desafío por el virus PRRS virulento (JXA1).

EIGHTH

The pUC18-CpG is proven to be a potent immunoadjuvant for the traditional FMD killed vaccine and the commercial PRRS live attenuated vaccine used in this study.

OCTAVA

El pUC18-CpG ha demostrado ser un potente inmunoadyuvante para la vacuna inactivada del virus de la fiebre aftosa y la vacuna viva atenuada de PRRS utilizadas en este estudio.

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APPENDICES：

RESUME

PERSONAL INFORMATION

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EDUCATION

Universidad Complutense de Madrid	preventive veterinary medicine	2008-- present
Chinese Academy of Agricultural Sciences	preventive veterinary medicine	2004-- 2007
Jilin University	bioengineering	2000--2004

PUBLICATIONS

1. Construction of Swine-Specific CpG Motif Plasmid and the Study of Its Immunostimulatory Effects both In Vitro and In Vivo. **Guo X**, Jia H, Yuan W, Zhang Q, Hou S, Sun Y, Zhu G, Zhu H, Sánchez-Vizcaíno JM. J Vet Med Sci. 2012 Jul 20. [Epub ahead of print];
2. CpG-enriched plasmid enhances the efficacy of the traditional foot-and-mouth disease killed vaccine. **Guo X**, Jia H, Zhang Q, Yuan W, Zhu G, Xin T, Zhu H, Sánchez-Vizcaíno JM. Microbiol Immunol. 2012 Feb 15. doi: 10.1111/j.1348-0421.2012.00438.x. [Epub ahead of print] (corresponding author) ;
3. Plasmid containing CpG motifs enhances the efficacy of porcine reproductive and respiratory syndrome live attenuated vaccine. **Guo X**, Zhang Q, Hou S, Zhai G, Zhu H, Sánchez-Vizcaíno JM. Vet Immunol Immunopathol. 2011 Dec 15;144(3-4):405-9. Epub 2011 Aug 4. (corresponding author);

4. Expression and identification of African swine fever virus protein p72 expressed in insect cells. HOU Shaohua，BAI Lihua，**GUO Xiaoyu**，JIA Hong，JIANG Yitong，ZHU Hongfei. Chinese Journal of Preventive Veterinary Medicine, Vol. 34，No.1 Jan. 2012;

5. Development of a Novel RT-LAMP Assay for Porcine Reproductive and Respiratory Syndrome Virus. XIN Ting, HOU Shaohua, JIA Hong, **GUO Xiaoyu**, DING Jiabo, LI Yanpeng, DING Min, ZHU Hongfei. Scientia Agricultura Sinica, 2010,43(1):185-191;

6. Selection of Liquid Media for High-density Fermentation of Recombinant E. coli for Production of Plasmids. ZHANG Quan, **GUO Xiaoyu**, YUAN Weifeng, SUN Huaichang, ZHU Hongfei. China Biotechnology，2007，27（1）：102-105

7. Amplification of high-density fermentation of recombinant E. Coli for production of recombinant plasmid DNA. ZHANG Quan, YU Kexiang, **GUO Xiaoyu**, ZHU Hongfei, SUN Huaichang. Journal of Yangzhou University (Agricultural and life science edition)，2007，28（1）：17-20;

Research Contents

- Screening of CpG motifs with the highest stimulation index through lymphocyte transformation assay；
- Construction of recombinant plasmids containing different copies of the CpG motifs selected；
- Test of the stimulation effects of the constructed plasmids；
- Test of the stimulation effects of the constructed plasmids through animal experiments；
- Test of the biosafety of the constructed plasmids，including the

biodistribution, persistence and horizontal transmission of the recombinant plasmids in animals；

Other research directions including：

- Optimizing the process for large-scale fermentation of plasmid DNA by E.coli；
- Biosafety assessment, including intermedial, environment-release and production experiments；
- Enhancement of the stimulation effect of Foot-and-Mouth disease vaccine by CpG ODN；
- Construction and expression of subunit vaccine for acephalocystis granulosus and its purification；
- Research on molecular immunoadjuvant C3d；
- Identification of African Swine Fever virus by Realtime PCR or ELISA.