CHARACTERIZATION OF KTY-06 FIELD PRRS STRAIN ISOLATED IN VIETNAM AND EVALUATION OF ANTIBODY PRODUCING OF THE INACTIVATED VIRUS IN PIG

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ABSTRACT

KTY-06 virus strain was isolated from 2-week-old piglet in a PRRS outbreak in Northern Vietnam in 2015 that had clinical signs, gross findings, and microsopic lesions specific with HP-PRRS. Results of a phylogenetic analysis of the open reading frame 5 region of the KTY-06 strain showed it to be a North American genotype and classified into sublineage 8.7, in which highly pathogenic Chinese PRRSV strains are also clustered. Passaging in Marc-145 cells, KTY-06 gradually induced 35% up to 100% CPE between 36 to 60 hours post inoculation. The titer of KTY-06 was 1.74 x 10^5 $TCID_{50}$/25µl. Of which, titer of the free viruses in the supernatant was higher than the titer of the cell associated virusese. Formalin-inactivated KTY-06 was able to stimulate pigs to produce specific antibodies against PRRSV. The immune response was detectable 14 day post immunization (dpi) (S/P ratio was 0.697 ± 0.271), peaked at 42 dpi (S/P ratio was 1.197 ± 0.256), and then declined at 49 dpi. The results suggest that KTY-06 was a candidate for strain selection for producing PRRS vaccine.

Keywords: Biology, antigenicity, PRRSV, KTY-06, Vietnam.

Đặc tính sinh học của chủng virus PRRS KTY-06 phân lập tại Việt Nam và đánh giá đáp ứng miễn dịch của lợn khi tiêm hỗn dịch kháng nguyên virus vô hoạt

TÓM TẮT

Chủng virus KTY-06 được phân lập từ lợn con (2 tuần tuổi) có triệu chứng lâm sàng, bệnh tích đại thể và vi thể đặc trưng của lợn mắc PRRS độc lực cao; lợn bệnh được thu thập trong một đợt dịch bùng phát PRRS tại miền Bắc Việt Nam trong năm 2015. Kết quả phân tích nguồn gốc phát sinh loài dựa trên trình tự gene ORF5 của chủng KTY-06 đã chỉ ra chủng virus này thuộc genotype Bắc Mỹ và sublineage 8.7; nằm cùng trong nhánh phát sinh với các chủng virus PRRS độc lực cao phân lập tại Trung Quốc. Khi nuôi cấy trên môi trường tế bào Marc-145, chủng virus KTY-06 gây bệnh tích tế bào từ 35% tới 100% trong 36 - 60 giờ sau khi gây nhiễm. Hiệu giá virus của chủng KTY-06 là 1,74 x 10^5 ($TCID_{50}$/25 µl). Trong đó, hiệu giá virus giải phóng tự do ngoài môi trường tế bào cao hơn so với hiệu giá virus liên kết trong tế bào. Hỗn dịch kháng nguyên virus KTY-06 sau khi vô hoạt bằng formalin được tiêm cho lợn thí nghiệm nhằm kiểm tra đáp ứng miễn dịch. Đáp ứng miễn dịch được phát hiện sau 14 ngày tiêm (với giá trị S/P là 0.697 ± 0.271), và đạt cực đại sau 42 ngày tiêm (với giá trị S/P là 1,197 ± 0,256), sao đó giảm dần sau 49 ngày tiêm. Kết quả nghiên cứu đã chỉ ra chủng virus KTY-06 có tiềm năng trong việc lựa chọn để sản xuất vắc xin phòng bệnh PRRS.

Từ khóa: Đặc tính sinh học, kháng nguyên, virus PRRS, KTY-06, Việt Nam.

1. INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) was first reported in 1987 in U.S. [4]. Since then, PRRS has been considered to be one of the most devastating swine diseases. For example, in the U.S., the economic losses due to PRRS are estimated to be 560 million USD per year [10]. The causative agent of PRRS (PRRSV) is a member of genus Arterivirus, family Arteriviridae, order Nidovirales. PRRSV induces reproductive failures such as late-term abortions, stillbirths, and weak newborn piglets, and is involved in the porcine respiratory disease complex [11]. A vaccine against PRRSV was first applied in Europe in 1993 and a year later in North America. There are two kinds of PRRS vaccine: inactivated and attenuated vaccines [14]. For in vitro studies, PRRSV is cultured in cell lines such as MA104, CL2621, and Marc-145 [1], [8]. In particullar, Marc-145 is able to support the growth of the virus and is commonly used for virus isolation [6], [9]. In Vietnam, from 2007 to present, PRRS has occured in almost all provinces and has caused a great deal of damage in the swine producing sector. From the outbreaks in 2012- 2013, samples were collected and used for PRRSV isolation. In this report, the biology and antigen producibility of a field strain of PRRSV (KTY-06) that was isolated from a PRRS outbreak in Northern Vietnam in 2015 were examined.

2. MATERIALS AND METHODS

2.1. Materials

A field isolate of PRRSV (KTY-06) and a vaccine strain of PRRSV (re-isolated from commercial Ingelvac PRRS vaccine), which served as control, were used. Strains were stored at -80^{0}C at the main laboratory of veterinary biotechnology, Vietnam National University of Agriculture.

2.2. Methods

2.2.1. Sequence and phylogenetic analysis of open reading frame 5

Total RNA was extracted using the QIAamp Viral RNA Minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Specific primer pairs [2] were used to amplify 720 bp amplicons encoding the complete open reading frame 5 (ORF5) region. PCR products were purified using a QIAquick Extraction Kit (Qiagen, Hilden, Germany) and directly sequenced by CEQ-8000 (USA). Nucleotide analysis of the virus was conducted by GENETYX verion 5.0. A phylogenetic tree was constructed with Mega 6.0 using the neighbor-joining method [12]. A boot-strap value of 1000 replicates was applied for robustness of phylogeny with 38 PRRSV references obtained from Genebank.

2.2.2. Virus propagation

Marc-145 cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS under the conditions of 37^{0}C and 5% CO_2 using T25 flasks with a volume of 25 cm^2.

Virus suspension was inoculated into the Marc-145 cells and inoculated for 30 min at 37^{0}C and 5% CO_2. Subsequently, 2 ml of DMEM supplemented with 10% Tryptose Phosphate Broth (TPB) was added. Cytopathic effect (CPE) of the inoculated cells was observed daily. The cells and supernatant were harvested when the CPE was approximately 80% - 90%.

2.2.3. Determining TCID50

25 µl of 10 times serially diluted viral suspension was inoculated into 3 wells of a 96 wells plate each with a monolayer of Marc-145 cells. After 1 hour of inoculation, DMEM supplemented with 10% TPB was added. CPE was daily observed and recorded. 50% Tissue Culture Infectious Dose ($TCID_{50}$) was determined by the method of Behrens - Karber [7].

2.2.4. Determining one-step growth curve of virus

KTY-06 was inoculated into Marc-145 at the multiplicity of infection (MOI) of 0.01. After 1 hour, the inoculums were discarded, and the cells were washed with 0.5 ml phosphate buffer saline (PBS). Infected cells were maintained in DMEM containing 10% TPB. At 6, 12, 24, 36, 48, 60 and 72 hours post inoculation (hpi), the supernantant and the cells associated for the fractions of virus were collected separately. The titer of virus in each fraction was then determined and ploted at collection time.

2.2.5. Virus inactivation

A formaldehyde solution (35%) was added to the viral suspension for a final concentration of 0.3%. The mixture was then incubated at 37^0C overnight as described in a previous study [3].

2.2.6. Experimental design of vaccination experiment

Six 2-month-old, healthy, PRRSV negative pigs were selected for the experiment. All animals were kept under biosafety level II (BSL) conditions, and were examined for abnormalities for 14 days. The pigs were divided into 2 groups of 3 heads. Group 1 served as the experimental (vaccinated) group and group 2 was the control (non-vaccinated) group. In group 1, inactivated KTY-06 with adjuvant was muscularly injected twice in one day and the injections were repeated 21 days after the first inoculation. In group 2, pigs were injected with DMEM as a control following the same schedule as group 1. After vaccination, body temperature and abnormalities of pigs in both groups were measured. Serum was collected at a fixed time for serum extraction to measure for specific antibodies.

2.2.7. Measuring specific antibodies against PRRSV

Specific antibodies against PRRSV in sera were measured using the by ELISA kit Cat. No. PRRS-AB (Median, Korea) following the instructions of the manufacturer.

3. RESULTS AND DISCUSSIONS

3.1. Origin of KTY-06 strain virus

KTY-06 virus strain was isolated from pig that displayed the clinical signs, gross findings, and microscopic lesions listed in Table 1. Results of bacteria and virus co-infectious test in swine were listed in Table 2 and 3.

The phylogenetic analysis results of the open reading frame 5 (ORF5) region of the KTY-06 strain show that the strain is a type II PRRSV and is classified into sublineage 8.7 (Figure 7). Representative highly pathogenic Chinese isolates from 2006 to 2011 also clustered within this sublineage. These findings are consistent with a previous classification in which PRRSV isolates from Vietnam and China collected in 2007 were clustered into a subgroup of type II PRRSVs, which are distantly related to a subclade of VR2332. With respect to vaccines expressing the GP5 protein, PRRSV strains circulating in Vietnam showed greater nucleotide identity to JXA1R than to virus strains used in other vaccines (Ingelvac PRRS MLV and Besta BSL-PS). This would suggest that the JAX1R vaccine is more appropriate for use against PRRSV strains circulating in Vietnam. Results of this phylogentic analysis of the ORF5 region of KTY-06 were the same as the results of a previous study [13]. Several new PRRSV strains that were isolated in 2011, 2012, 2013, and 2015 grouped in same sublineage as the KTY-06 strain. Taken together, our results show that the Vietnamese isolates in this study were of the North American genotype and clustered into the unique sublineage 8.7 alongside several highly pathogenic Chinese PRRSV strains.

3.2. Biological characterization of KTY-06

KTY-06 was evaluated for its capability to induce CPE, titer, and replication kinetics on Marc-145 cells.

3.2.1. Capability of inducing CPE

The capability to induce CPE of KTY-06 was characterized and compared with the vaccine virus. The results were given in Table 4.

Table 1. Information about the KTY-06 strain virus

Strain	Pig	Origin	Clinical signs	Gross findings	Microsopical lession
KTY-06	2-week-old piglet	Lung	High fever >40,5^{0}C; anorexia; more diarrheoa; blotchy reddening of the skin (Figure 1); dyspnea	Lung are mottled, tan and red, fail to collapse (Figure 2). Hemorrhagic points in renal cortex (Figure 3). Tough and scabrous spleen. Hemorrhage in some section of intestine. Hemorrhage in mesenteric lymph node. (Figure 4) Largerment in liver.	Congestion and haemorrhaging in lungs, lung lymph nodes, and tonsils. Inflammatory exudate in alveoli (Figure 5). Infiltration of inflammatory cells and proliferation of lymphoid capsule (Figure 6).

Table 2. Results of test bacteria co-infectious in swine

Bacteria	Escherichia coli	Salmonella spp	Pasteurella multocida	Actinobacillus pleuropneumoniae	Streptococcus suis
Result of test	+	+	+	-	-

Note: +: Positive; -: Negative

Table 3. Results of virus co-infectious test in swine by PCR or RT-PCR

Virus	PCV2	CSFV	PEDV
Result of test	+	-	-

Note: +: Positive; -: Negative

Fig 1. Blotchy reddening of the skin of swine

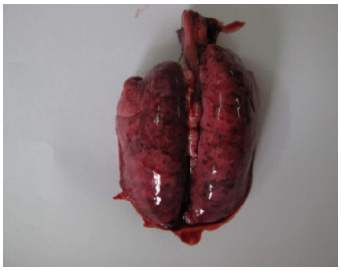

Fig 2. Lung are mottled, tan and red, fail to collapse

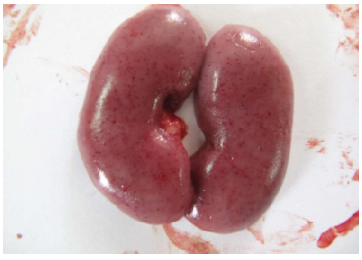

Fig 3. Hemorrhagic points in renal cortex

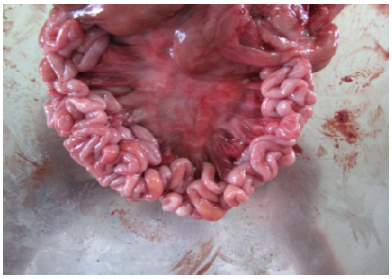

Fig 4. Hemorrhaging in mesenteric lymph node

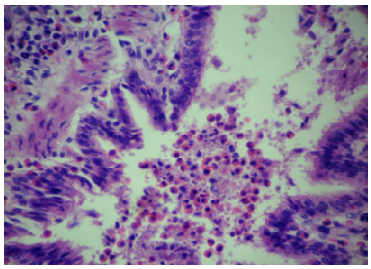

Fig 5. Inflammatory exudate in alveoli

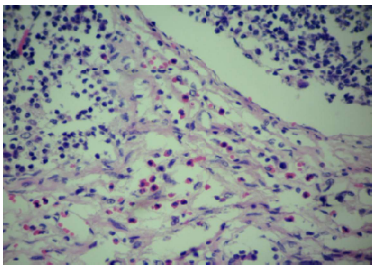

Fig 6. Infiltration of inflammatory cells and proliferation of lymphoid capsule

Fig 7. Phylogenetic tree of the KTY-06 strain virus and 38 reference strains obtained from Genebank

Note: The comparison was based on on the nucleotide sequence of PRRSV ORF5. The phylogenetic tree was generated by the neighbor-joining method using MEGA 6.0 with bootstrap values of 1000 replicates. Different lineages and sub-lineages are indicated. KTY-06 is marked with a green circle. The vaccine licensed for use in Vietnam is denoted with a red square.

Table 4. Capability to induce CPE of KTY-06 and vaccine strain

Virus	CPE (%) at each different time post inoculation					
	24 hpi	36 hpi	48 hpi	60 hpi	72 hpi	84 hpi
KTY-06	10	35	70	100	D	
Vaccine strain	10	30	50	100	D	

Notes: D: Complete detachment of the cell sheet. The number in each cell indicated percentage of CPE

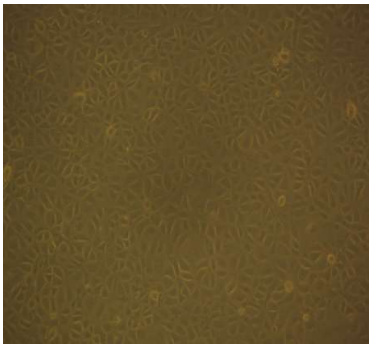

Figure 8. Mock Marc-145

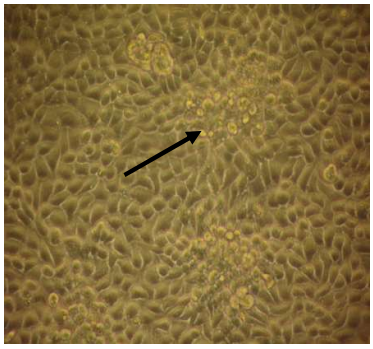

Figure 9. CPE at 24 hpi induced by KTY-06

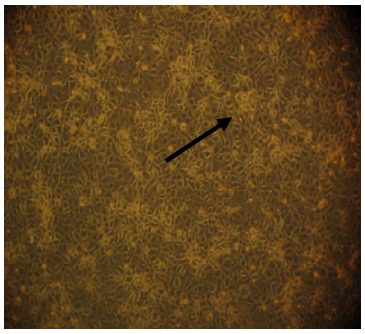

Figure 10. CPE at 48 hpi induced by KTY-06

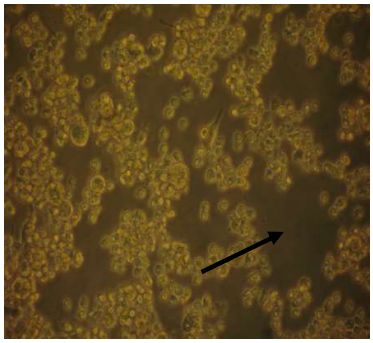

Figure 11. CPE at 72 hpi induced by KTY-06

Comparing the results shown in Table 4, it was observed that KTY-06 induced CPE is similar to the CPE of the vaccine strain. Infected cells were clumped and detached from the surface. CPE could be observed 24 hpi (Figure 9) with around 10% of the area affected. The percentage of cells showing CPE increased with time. Afer 48 hpi, 70% of the cells were affected (Figure 10), and the cell layer was affected 100% at 60 hpi (Figure 11). At 72 hpi, infected cells were detached completely. The specificity of CPE induced by KTY-06 was confirmed by immunohistochemistry staining (not shown). The viral titer was 1.74 x 10^5 $TCID_{50}$/25µl.

3.2.2. Replication kinetics of KTY-06

The one-step growth kinetics of the KTY-06 and vaccine viruses are shown in Figure 12. It was observed that the free viral fraction of KTY-06 and vaccine viruses had higher titers than the cell-associated virus. However, KTY-06 seemed to replicate faster than the vaccine virus. Of the KTY-06 strains, the titer of the

cell-associated virus continuously increased after 48 hpi to 72 hpi. Meanwhile, the titer of the cell-associated vaccine virus slightly increased after 24 hpi, but steadily increased at 60 hpi. Of the cell-associated fractions, KTY-06 had a maximum titer of 4.83 $TCID_{50}$/25 µl at 72 hpi while the vaccine virus had a maximum titer of 2.83 $TCID_{50}$/25 µl at 84 hpi. Of the free viral fractions, between 36 hpi to 60 hpi, the titer of KTY-06 tended to increase while the vaccine virus tended to slightly decrease. The above observations imply that the replication kinetics of KTY-06 are different from those of the vaccine virus.

3.3. Antigenic characterization of KTY-PRRSV06

ELISA analyses were done with the sera collected at different times post inoculation. The antibody responses are shown in Figure 13.

The ELISA results show that all the pigs of the mock inoculated group (control group, swine 1-3 in Figure 13) were negative (the S/P ratio < 0.4) at each sampling time (7 dpi to 49 dpi). In the inoculated pigs (experiment group, swine 4-6 in Figure 13), sero-conversion was observed between 7 dpi to 21 dpi. After boosting (21 dpi), the antibody titer was increased during the period

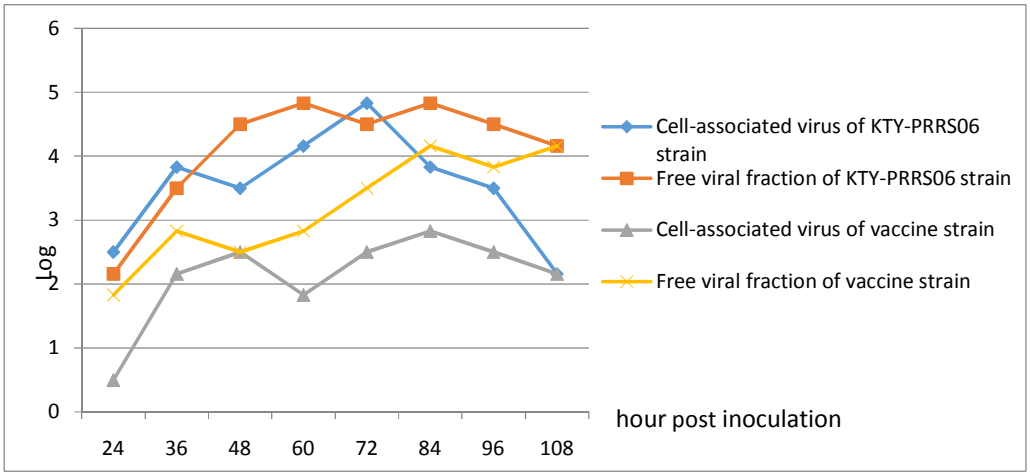

Figure 12. Replication kinetics of the KTY-06 and vaccine virus

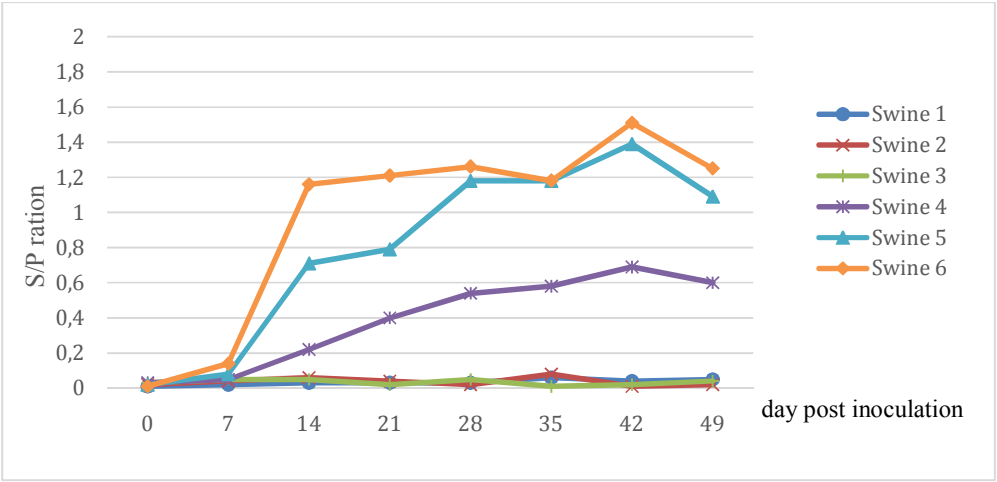

Figure 13. Kinetics of the antibody responses of inoculated and non-inoculated pigs

of 21-42 dpi, and then slightly decreased from 42-49 dpi. The average antibody titer (S/P ratio) of the experiment group at 14 dpi was 0.697 ± 0.271), it peaked at 42 dpi (1.197 ± 0.256), and was maintained above the cutoff value (S/P average at 49 dpi was 0.980 ± 0.196). The high levels of the antibody titer after the second inoculation, so called boosting, were caused by immunological memory. The kinetics of the immune responses of the inoculated pigs by inactivated KTY-06 was in line with a previous study [5].

4. CONCLUSIONS

KTY-06 virus strain was isolated from 2-week-old piglet that had clinical signs, gross findings, and microscopic lesions specific with HP-PRRS. Results of the phylogenetic analysis of the ORF5 region of the KTY-06 strain support grouping the strain with North American genotypes and its classification in sublineage 8.7, in which highly pathogenic Chinese PRRSV strains are also clustered. This virus strain was also evaluated for biological and antigenic characterizations. Pigs immunized with inactivated KTY-06 did not show any abnormalities, thus providing support that the pilot vaccine is safe. The specific immune responses against PRRSV were detected 14 dpi with the average S/P ratio of 0.697 ± 0.271, peaked at 42 dpi with the average S/P ratio were 1.197 ± 0.256, and then slightly declined at 49 dpi.

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