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Development of an Indirect-Elisa to Detect Antibodies against Porcine Reproductive and Respiratory Syndrome Virus Nucleocapsid Protein in Gansu China

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV), causes reproductive failure in sows and boars, and is an important pig pathogen that is responsible for respiratory symptoms in swine of all ages. PRRSV is divided into two genotypes, type1 (European) and type 2 (North American), on the basis of genetic and antigenic characters. Type 2 PRRSV, especially the highly-pathogenic (HP) virus, is widely circulating in China recent years and causes huge economic loss in swine industry. Therefore, development of the rapid and effective methods for epidemiological surveys are particularly important to control the plague. PRRSV nucleocapsid protein (N protein) is highly conserved among different strains, and it is widely used as a diagnostic antigen for the development of serologic diagnostic tools. In this study, we then developed an indirect enzyme linked immunosorbent assay (iELISA) using *E. coli* expressed recombinant N protein of type 2 PRRSV as the coated antigen to detect type 2 virus antibodies. N protein encoding gene was amplified from HP-PRRSV QH-08 strain, isolated from Qinghai, the neighbour province of Gansu, Northwest China. There were no any cross-reactions with porcine sera against type 1 PRRSV, CSF, PCV2, PPV, PRV, FMD serotype O, A and AsiaI. The specificity and sensitivity of the iELISA were evaluated with 53 negative, 60 infected and 314 vaccinated porcine sera, and further compared with two commercial ELISA kits. The results showed that the specificity of all three ELISAs was 100% and the sensitivity of our iELISA was 94.12%, which was a little lower than that of the commercial kits, 98.40% for LSI and 96.52% for IDEXX. The newly developed iELISA was applied to detect 979 serum samples from the non-industrialized pig farms located in different areas of Gansu province. There were no any infectious cases according to the result interpretation we set while the positive rate was generally low due to some specific reasons in the undeveloped regions. The

iELISA based on the fusion N protein antigen, encoded by ORF7 of type 2 HP-PRRSV QH-08 strain, is economical and easy to operate, and could be suitable and useful for the epidemiological studies of type 2 PRRSV in Northwest China, especially in Gansu and Qinghai provinces.

Abbreviations

PRRSV: Porcine Reproductive and Respiratory Syndrome Virus; IFA: Immunofluorescence Assay; IPMA: Immunoperoxidase Monolayer Assay; FBS: Fetal Bovine Serum; CAAS: Chinese Academy of Agricultural Sciences

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), a single-stranded, positive-sense RNA virus belonging to the family of the Arteriviridae, Nidovirales [1] is an important pig pathogen [2-4]. It can cause reproductive failure in sows and boars, and is responsible for respiratory failure in porcine of all ages [5-7]. PRRSV is divided into two genotypes, type1 or European and type 2 or North American, on the basis of their genetic and antigenic characters. Lelystad and VR-2332 are the classical prototype strain of type 1 and 2, respectively. Type 2 PRRSV are widely circulating in China recent years. A highly-pathogenic (HP) PRRSV of type 2, firstly emerged in Jiangxi province, China in May 2006, brought about serious economic losses and then rapidly spread nationwide. PRRSV genome is approximately 15Kb in size and encodes ten overlapping open reading frames (ORFs), designated as ORF1a, ORF1b, ORF2a, ORF2b, and ORF 3 through 7, including ORF5a. The viral structural proteins contain GP2a/2b, GP3, GP4, GP5a/b, membrane (M) and nucleocapsid (N). Among them, GP5, M and N, encoded by ORF5, ORF6 and ORF7, respectively, are the major immunogenic proteins of PRRSV [8].

Generally, virus isolation is considered to be the "gold standard" for diagnosing diseases [9]. Other assays, including western immunoblot analysis [10-12], the indirect immunofluorescence assay (IFA), the immunoperoxidase monolayer assay (IPMA), and the gel-based or real-time reverse transcription polymerase chain reaction (RT-PCR) assays are also applied for virus detection [13-16]. In addition, ELISA is more applicable for detection of a large amount of samples than the assays mentioned above due to its low costs, rapid and convenient operation, and high specificity and sensitivity. ELISA for detection of PRRSV antibodies could also give a clear diagnosis if the pigs have not been vaccinated. Serum antibodies against PRRSV N protein appear as early as 14 days post infection and thereafter last for approximately 6 months. Therefore, N protein is often chosen for the coated antigen of ELISA. Here, we developed PRRSV type 2 indirect ELISA (iELISA) based on the recombinant N protein expressed in *E. coli* system to evaluate the level of antibodies elicited by PRRSV vaccine immunization and diagnose the infected pigs without vaccination. The N protein encoding gene was amplified from type 2 PRRSV strain QH-08 isolated from Qinghai, the

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neighbour province of Gansu, Northwest China, when HP-PRRS outbroke in the local pig farm without the application of any PRRSV vaccines. By comparison with IDEXX and LIS ELISA kits, the newly developed iELISA showed satisfactory identity with the commercial kits. Furthermore, the established iELISA was applied to evaluate antibody levels of the pigs raised in the non-industrialized farms in some areas of Gansu province. PRRSV type 2 iELISA developed in this study will be useful for minitoring immune antibodies against type 2 virus and diagnosing the virus infection of the non-immune pigs in China, especially in northwest regions.

Material and Methods

Ethical statement

The experimental protocol was approved and performed in compliance with all relevant Chinese ethical guidelines and laws, and the animal care procedures complied with the regulations detailed under the Animal Welfare Act and in the Guide of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (CAAS).

Viruses and cells

The PRRSV QH-08 strain (complete genome, genebank accession number KU201579) was isolated from Qinghai province and identified as type 2 HP-PRRSV by OIE/China National Foot-and-Mouth Disease (FMD) Reference Laboratory, Lanzhou Veterinary Research Institute, China. The positive swine sera against classical swine fever virus (CSFV), porcine circovirus type 2 (PCV2), porcine parvovirus (PPV), porcine pseudorabies virus (PRV), foot-and-mouth disease virus (FMDV) serotype O/A/AsiaI were kept in the laboratory for the cross-reaction experiments. Type 1 PRRSV inactivated sera were gifted by PRRS OIE Reference Laboratory, National Veterinary Research Institute, Poland, and RT-Europe Center, Budapest, Hungary. The Marc-145 cell line, which is highly permissive for PRRSV infection, is a derivative of an African green monkey kidney cell line MA-104, and was maintained in Dulbecco's Modified Eagle Medium (DMEM,Gibco) supplemented with 10% FBS (Gibco) and penicillin/streptomycin sulfate. PRRSV was grown in Marc-145 cells as originally described [9].

Construction of pET/ORF7 recombinant plasmid

HP-PRRSV QH-08 strain, maintained in Marc-145 cells, was used to isolate viral RNA which further served as the template for the amplification of the N protein encoding gene or ORF7 by RT-PCR. The primers for ORF7 amplification (forward primer with EcoR I site 5′-GC<u>GAATTC</u>-GAGTGGTAAACCTTGTCAAA-3′ , and reverse primer with Xho I site 5′-GC<u>CTCGAG</u>TGCTGAGGGTGATGCTGTG-3′) were used for priming RT-PCR reaction, and primers contained EcoR I and Xho I restriction site as shown above by underline. RT-PCR was performed in a 25-μL reaction containing PrimeScript 1 step Enzyme Mix 1 μL (Takara Bio), 2×1 step buffer 12.5 μL, RNase Free ddH$_2$O 8.9 μL, 0.8 μL of each primer with the final concentration of 0.6 mM, and RNA 1 μL. The procedures were as follows: 50℃ for 30 min, 95℃ for 8 min, 35 cycles of 95℃ for 1 min, 57℃ for 50s and 72℃ for 1 min, extension at 72℃ for 10 min [9]. Briefly, the amplified N gene fragments were purified and cleaved with EcoR I and Xho I, and the digested fragments were then inserted into pET-30a (+) vector to obtain pET/ORF7 recombinant plasmid. The reconstructed plasmid was sequenced and analyzed by using DNASTAR software.

Overexpression and purification of the fusion N protein

pET/ORF7 plasmid was transformed into competent *E. coli*

BL21 (DE3) strain and the recombinant *E. coli* was grown in LB medium containing 50 ug/mL kanamycin overnight at 37°C. Protein expression was induced by 1 mM isopropylthiogalactoside (IPTG) for 8hrs at 37°C. Cells were pelleted by centrifugation and resuspended to 1/25 of the initial culture volume in ice-cold phosphate-buffered saline (PBS, pH7.4). The fusion N protein containing 6xHis tag was further purified with a Ni-NTA purification kit (Merck). Finally, the purified N protein was analyzed by SDS-PAGE and western blot with porcine sera against type 2 PRRSV purchased from American VMRD company as the detection antibody and was then stored at −70°C for further use.

Development of the indirect ELISA (iELISA) for detection of type 2 PRRSV antibodies

The purified fusion N protein was coated onto 96-well microtiter plates (Nunc, Roskilde, Denmark) in 0.05M carbonate-bicarbonate buffer (pH 9.6) (Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C. The coated antigen was subsequently blocked with 3% (w/v) skim milk (Difco, Detroit, MI, USA) in PBST, containing 0.05% Tween 20, pH 7.4, for 2 hrs at 37°C after washing three times with PBST. The serum samples were firstly diluted in PBST and then added in duplicate (50 μL per well) and incubated for 30 mins at 37°C. Each plate contained the positive and negative control sera. Then, the plates were washed three times with PBST, and 50 μL of the anti-swine immunoglobulin HRP conjugate (diluted 1:20000 in dilution buffer, Sigma-Aldrich) was added into each well and incubated for 30 mins at 37°C. After washing, 50 μL of TMB (SurModics, Eden Prairie, MN, USA) was added per well. The optical density (OD) was measured at 450 nm after a 10∼12 min incubation at 37°C. The reactions were stopped by the addition of 1.5 M H$_2$SO$_4$ to the plate.

Evaluation of the sensitivity and specificity of the newly developed iELISA

To evaluate the sensitivity and specificity of the assay, the newly developed iELISA for detection of type 2 PRRSV antibodies was subject to the examination of 53 negative, 60 infected and 314 vaccinated serum samples. The iELISA was further compared with two commercial ELISA kits, IDEXX (USA, N protein-based iELISA) and LSI (France, M protein-based iELISA). In addition, all 113 serum samples (60 infected and 53 negative) were subjected to gel-based RT-PCR detection.

Detection of field sera collected from the non-industrialized pig farms in Gansu province, Northwest China, with the newly developed iELISA

979 field sera were collected from the non-industrialized pig farms located in different areas of Gansu province, Northwest China, to evaluate the antibodies against type 2 PRRSV by using the newly developed iELISA. Of these, 402 serum samples were from Center for Disease Control and Prevention (CDC) of Pingliang city. Other 256 sera were provided by CDC of Wuwei City, and 229 sera were from CDC of Gansu province. 92 sera collected from the region around Lanzhou, the capital city of Gansu province, were kept in our laboratory.

Results

Construction of pET/ORF7 recombinant plasmid and gene analysis

The N protein encoding gene or ORF7 of type 2 HP-PRRSV QH-

08 strain was obtained by RT-PCR. The amplicons were digested with EcoRI and XhoI, and then cloned into pET-30a (+) expression vector with 6xHis tag cleaved with the two same enzymes to obtain the recombinant plasmid pET/ORF7. After sequencing, analysis showed that N protein encoding gene shares about 99% homology with that of other type 2 HP-PRRSV deposited in Genebank. Type 2 HP-PRRSV QH-08 strain was isolated from a local non-industrialized pig farm, close to Xinin capital city of Qinghai province, Northwest China, in early 2008. PRRS widely and seriously outbroke out during that period in the farm where no any PRRSV vaccines were applied. Subsequent diagnosis showed that the pathogen was HP-PRRSV which firstly emerged in Southern China in 2006 summer and spread rapidly nationwide and led to serious economic losses to the victims, of which the majorities were the non-industrialized and lacked of biosecurity guarantee systems.

Expression and identification of the fusion N protein of type 2 PRRSV

The N protein encoding gene, i.e. ORF7, was expressed in *E. coli* BL21 (DE3) harbouring pET/ORF7 plasmid induced by IPTG. SDS-PAGE analysis revealed that the fusion N protein with approximate molecular weight 24 kilodalton (kDa) was mainly expressed in the form of inclusion bodies in *E. coli* BL21 (DE3). The fusion N protein could be purified by using Ni-NTA His Bind resin affinity chromatography due to the existence of 6xHis tag in pET-30a (+) expression vector (Figure 1). The reaction activity of the fusion N protein was detected by western blot with porcine serum against type 2 PRRSV produced by American VMRD company as the detection antibody. The result showed that the fusion N protein could very well react with the type 2 PRRSV sera and there was no or very weak reaction happened in mycoprotein of *E. coli* BL21 (DE3) habouring pET-30a (+) vector induced by IPTG served as the control (Figure 2).

Development of iELISA for detecting antibodies against type 2 PRRSV and evaluation of the specificity and sensitivity

Determination of the optimal concentration of the coated fusion N protein antigen and the dilution of the control sera was performed by checkerboard titration method. The purified fusion N protein can be coated at 0.2 μg/well and the optimal dilution was found to be 1:100 for the positive and negative serum. After iELISA for detecting type 2 PRRSV antibodies was developed based on the optimized conditions, it was used to react with porcine sera against type 1 PRRSV, CSFV, PCV2, PPV, PRV and FMDV serotype O/A/AsiaI. There were no cross reactions with those sera, indicating that the newly developed iELISA was specific for type 2 PRRSV antibodies. 427 serum samples consisting of 53 negative, 60 infected and 314 vaccinated were subjected to the detection by LSI and IDEXX ELISA kits, and our newly developed iELISA assay (Table 1). According to the comprehensive analysis, if the OD value is ≥ 0.4 at 450 nm, the serum sample can be regarded as positive; if OD <0.4, it shows a negative sample; if the OD value of the serum sample is >2.5, it means PRRSV infection happens. The results of Table 1 showed that the sensitivity of our iELISA was 94.12% and the specificity was 100%. The specificity was 100% both for LSI and IDEXX kits while the sensitivity was 98.40% and 96.52%, respectively. By comparison to the two commercial kits, there is still some room for our iELISA to improve in the aspect of sensitivity.

Detection of field sera collected from the non-industrialized pig farms in Gansu province, Northwest China, with the newly developed iELISA

The newly developed iELISA was applied to detect 979 porcine serum samples with unclear background collected from the non-industrialized pig farms located in different areas of Gansu province, neighbour of Qinghai, Northwest China. There were no any infectious cases according to the result interpretation we set because no serum sample's OD value was more than 2.5. The average positive
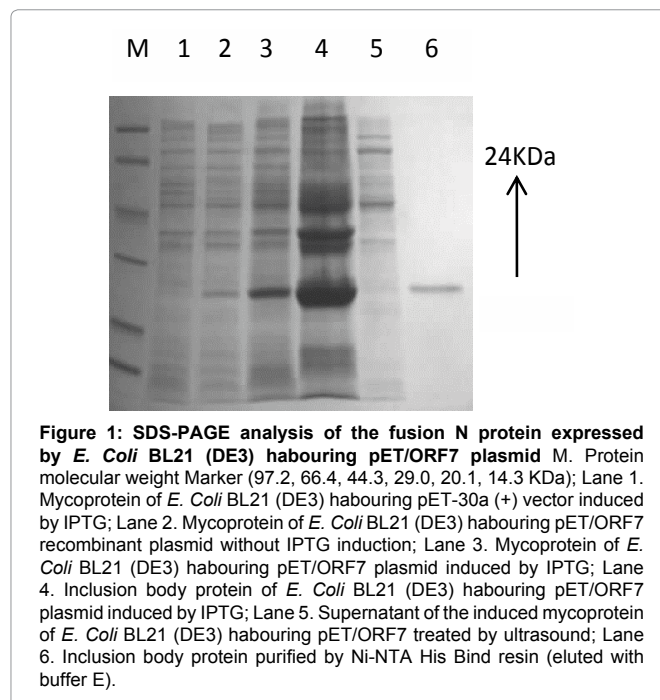

Figure 1: SDS-PAGE analysis of the fusion N protein expressed by *E. Coli* BL21 (DE3) habouring pET/ORF7 plasmid M. Protein molecular weight Marker (97.2, 66.4, 44.3, 29.0, 20.1, 14.3 KDa); Lane 1. Mycoprotein of *E. Coli* BL21 (DE3) habouring pET-30a (+) vector induced by IPTG; Lane 2. Mycoprotein of *E. Coli* BL21 (DE3) habouring pET/ORF7 recombinant plasmid without IPTG induction; Lane 3. Mycoprotein of *E. Coli* BL21 (DE3) habouring pET/ORF7 plasmid induced by IPTG; Lane 4. Inclusion body protein of *E. Coli* BL21 (DE3) habouring pET/ORF7 plasmid induced by IPTG; Lane 5. Supernatant of the induced mycoprotein of *E. Coli* BL21 (DE3) habouring pET/ORF7 treated by ultrasound; Lane 6. Inclusion body protein purified by Ni-NTA His Bind resin (eluted with buffer E).
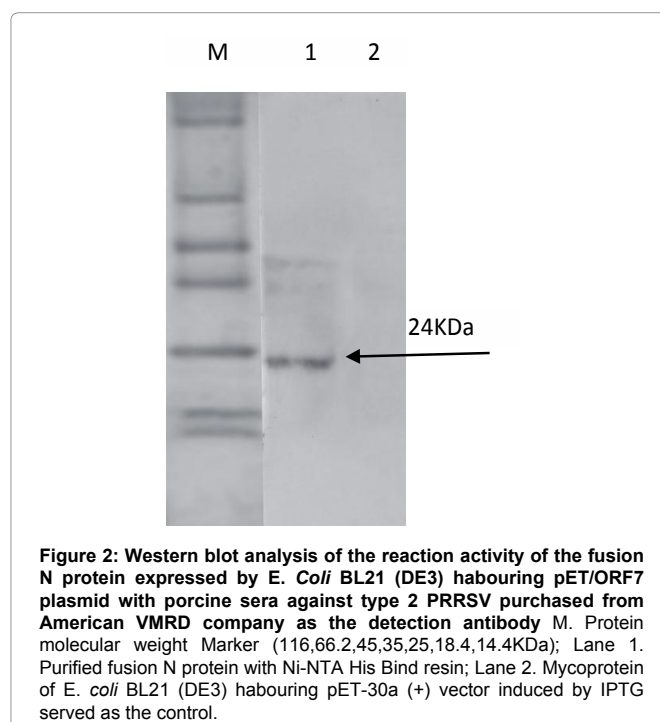

Figure 2: Western blot analysis of the reaction activity of the fusion N protein expressed by E. *Coli* BL21 (DE3) habouring pET/ORF7 plasmid with porcine sera against type 2 PRRSV purchased from American VMRD company as the detection antibody M. Protein molecular weight Marker (116,66.2,45,35,25,18.4,14.4KDa); Lane 1. Purified fusion N protein with Ni-NTA His Bind resin; Lane 2. Mycoprotein of E. *coli* BL21 (DE3) habouring pET-30a (+) vector induced by IPTG served as the control.

Table 1: Evaluation and comparison of the clinical specificity and sensitivity of the iELISA

Sample	Number	Results of RT-PCR	Detection results of PRRSV antibody		
			iELISA	LSI ELISA	IDEXX ELISA
Negative sera	53	53 (-)	53 (-)	53 (-)	53 (-)
Infected sera	60	60 (+)	60 (+)	60 (+)	60 (+)
Vaccinated sera	314		292 (+)	308 (+)	301 (+)
clinical specificity			100% (53/53)	100% (53/53)	100% (53/53)
clinical sensitivity			94.12% (60+292)/ (60+314)	98.40% (60+308)/ (60+314)	96.52% (60+301)/(60+314)

Table 2: The iELISA detection of 979 sera from the non-industrialized pig farms in Gansu, China.

Sources of sera	Number of samples	Distribution of OD_{450nm} value of positive sera		Positive rate (%)
		≥0.4	between 1.0- 2.5	
Pinliang	402	86	5	22.64 (91/402)
Lanzhou	92	36	25	66.30 (61/92)
Wuwei	256	81	13	36.72 (94/256)
CDC of Gansu province	229	88	10	42.79 (98/229)
total	979	291	53	35.14 (344/979)

rate was 35.14% (344/979). Among the positive sera, OD values of the majorities were between 0.4 and 1.0. The positive percentage was 22.64% (91/402), 66.30% (61/92) and 36.72% (94/256) in Pingliang, Lanzhou (capital city of Gansu province) and Wuwei region, respectively. Of 229 serum samples from CDC of Gansu province, the positive rate was 42.79% (98/229).

Discussion

In this study, we developed an iELISA using the recombinant N protein expressed in *E. coli* as the coated antigen to detect antibodies against type 2 PRRSV. N protein encoding gene, ORF7, was amplified by RT-PCR with RNA template extracted from HP-PRRSV QH-08 strain isolated during PRRS outbreak in a local non-industrialized pig farm before vaccine application located in Qinghai Province, the neighbour of Gansu, Northwest China. Therefore, iELISA based on the N protein encoded by QH-08 strain's gene could be suitable for the detection of antibodies against type 2 PRRSV in Northwest China.

The newly developed iELISA showed good analytical specificity since there were no cross-reactions with other porcine sera against the reproductive failure related viruses, such as type 1 PRRSV, CSF, PCV2, PPV and PRV. Furthermore, there were no any reactions with porcine sera of FMDV type O/A/AsiaI which have or had (AsiaI) circulated in China. Clinical specificity of iELISA can reach 100% although its sensitivity was 94.12%, which was a little lower than that of LSI and IDEXX iELISA kits. Maybe the sensitivity can be further enhanced via improving the purification of the fusion N protein antigen and further optimizing the reaction conditions (Table 2).

The newly established iELISA was applied to detect porcine serum samples collected from the non-industrialized pig farms located in different areas of Gansu province, Northwest China. There were no type 2 PRRSV infection cases because no OD values of 979 sera were more than 2.5 at 450 nm absorbance. However, the average positive rate of the serum antibodies was 35.14% (344/979). Although there was no clear background of those sera, it was supposed that the following factors accounted for such a low percentage: (1)There existed a low percentage of the regional vaccination coverage although

HP-PRRS is listed as one of the compulsory vaccination programs by the government. Backyard and small-sized (dozens only) pig farms are still popular in China, especially in Gansu province, located in the undeveloped Northwest China. Vaccination is hard to fully cover those farms. (2)Vaccination efficacy was relatively low because the vaccines were not kept under appropriate temperature during transportation and storage process. Lack of timely boost was maybe another reason. Therefore, the vaccines characterized by thermal type and long-term duration of immunity are more suitable for the non-industrialized pig farms. (3) In addition, PRRSV undergoes rapid mutations which can cause changes of antigenicity [19-23]. It was reported that the antigenic variation is mainly responsible for the inability of the current vaccines to control PRRS very well. Vice verse, vaccines can speed up the rapid mutation due to the selective pressure to some extent [17-18]. Now in China, the majority of PRRSV vaccines are the live attenuated vaccines which have the risk of reversion to virulence. Therefore, controversy and divergence exist about whether it is necessary to continue to widely apply PRRSV vaccine in China.

Our iELISA for detecting type 2 PRRSV antibody developed based on the *E. coli* expressed fusion antigen does not require cell cultures to produce virus to work as the coated antigen. Therefore, it is an economic method and the test can be performed in the laboratory without biosafety facilities. The primary results show that the newly developed iELISA in this study will be suitable and useful for screening porcine sera antibodies against type 2 PRRSV in China, especially in Northwest regions although more samples are further needed for the accurate and full evaluation of the method.

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