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Characterization and potential use of truncated PCV2 capsid protein and its polyclonal antibody for diagnosis of PCV2 infections

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Abstract: As porcine circovirus 2 (PCV2) ORF2 encodes the major structural protein (capsid) that is closely related to the pathogenesis, the capsid (Cap) protein could be used as a target antigen for serological analysis. The immunoactivities of the truncated capsid proteins containing immunogenic epitopes of PCV2 (Cap2s) or PCV1 (Cap1s) expressed in *Escherichia coli* were described, as well as the characteristic of their polyclonal antibodies in diagnosis of PCV2 infection. Western blot analysis revealed that both Cap2s and Cap1s gave strong signals on nitrocellulose membranes to their corresponding polyclonal antibody. Furthermore, either PCV2-positive sera from PMWS cases or PCV1-positive swine sera could only recognize Cap2s or Cap1s, respectively. There was also no cross-reactivity between the two polyclonal antibodies when reacted with natural Cap proteins of viral particles on cells by immunofluorescence assay (IFA). Thus, an ELISA was then developed using PCV2 Cap as coating antigen to evaluate the sero-prevalence of PCV2 infection in pigs. The PCV2-positive rate ranged from 48.28% to 100% among different herds (n=13) with an average of 80.69% (209/259). These results indicate that Cap2s was type-specific and could be used as a discriminative antigen for monitoring PCV2 antibody in serum. The polyclonal antibodies were also useful for differential identification of PCV1 and PCV2 infection by immunohistochemistry.

Keywords: Porcine circovirus; capsid protein; immunoactivity; immunofluorescence assay; sero-prevalence
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1 INTRODUCTION

Porcine circovirus (PCV), which belongs to the *Circoviridae*, is the smallest DNA virus with nonenveloped, single stranded circular genome[1]. Two types of PCVs, PCV1 and PCV2, have been characterised. PCV1 was identified as the persistent, non-cytopathic contamination of the porcine kidney cell line (PK-15)[2]. However, PCV2 is believed to be the causative agent of the infectious disease called postweaning multisystemic wasting syndrome (PMWS)[3, 4] which was first described in 1991 in Saskatchewan of western Canada [5] and have rapidly spread to all the major pig industry countries of the world[6-8]. Although the genomes of both PCV1 and PCV2 consist of 11 open reading frames (ORF) and the genomic organizations are similar, their nucleotide sequences only share less than 80% homology [9, 10]. Two major ORFs, ORF1 and ORF2, are oriented in opposite directions. With more than 85% identity of nucleotide sequence between two types of PCV, ORF1 is essential for viral DNA replication[11]. In contrast, the nucleotide sequence of ORF2 is highly variable (less than 60% homology) between PCV1 and PCV2, suggesting that the type-specific features of PCV might be determined by the respective ORF2 encoded proteins [12]. Since PCV2 is pathogenic in pigs with its Cap protein related to the pathogenesis, ORF2 and its encoded protein has been the focus of recent studies. The immuno-dominant epitopes of PCV2 cap protein are believed to be within amino acid residues from 47 to 84, 165 to 200 and the last 4 amino acids[13]. The Cap proteins of both PCV1 and PCV2 contain type specific epitopes[14]. PCV2 Cap protein is prone to variation within isolates from different geographic regions，especially in its epitopes[12, 15]. Thus, it is necessary to develop serologic assays based on isolates from local areas. This study was aimed to characterize the immunoactivity and the application in ELISA of truncated Cap proteins containing all the antigenic epitopes

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of PCV2 ORF2 isolated locally from Zhejiang province as well as the properties of its polyclonal antibody for diagnostic purposes by immunohistochemistry.

2 MATERIALS AND METHODS

2.1 Construction of the recombinant expres sion plasmids

DNA fragments containing all the putative antigenic epitopes of PCV2 cap protein were amplified and cloned into the expression vector pET-30a by the primer pairs in Table 1. PCV1 ORF2 was cloned into pET-32c. The recombinant plasmids named pET-Cap2s and pET32-Cap1s, respectively, were confirmed by digestion analysis and sequencing.

2.2 Expression and purification of recombinant proteins

The *E. coli* BL21 strain containing plasmid pET-Cap2s or pET32-Cap1s was induced by isopropyl-thio-β-D-galactoside (IPTG) at a final concentration of 1 mM. After 3 hours, the bacteria were centrifuged, resuspended with lysis buffer (50 mM PBS, 8 M Urea, pH 8.0) and lysed by sonication on ice. The supernatants were then loaded into the columns containing Ni-NTA agarose (Invitrogen Co. Ltd., USA) for purification. Finally, the proteins of PCV2 ORF2 (Cap2s) and PCV1 ORF2 (Cap1s) were eluted (50mM PBS, 300mM imidazole) after washing (50mM PBS, 50mM imidazole) for 3 times.

Table 1 Primers used for amplification of PCV1 and PCV2 ORF2

	Primer pair	Sequences (5′→3′)	Restriction site	Product length/bp
PCV2	Ps5	TA*GAATTC*ATGACCCGCCTCTCCCGCACCTTC	*Eco*R I	480
	Ps6	AT*GCGGCCGC*TTAAGTGCCGAGGCCTACGTGG	*Not* I	
PCV1	Pj7	CA*GAATTC*GTATGAAGACGGGTATCTT	*Eco*R I	597
	Pj8	CG*CTCGAG*TTATTTATTTAGAGGGTC	*Xho* I	

2.3 Preparation of polyclonal antibodies

Protein concentration was determined by the Bradford assay with bovine serum album as standard. Two SPF rabbits were immunized subcutaneously with 3 mg Cap2s or Cap1s in Freund's complete adjuvant (Pierce, USA). Two weeks post-immunization (PI), the rabbits were boosted with 1.5 mg proteins in incomplete adjuvant. Two types of polyclonal antibodies were collected 6-weeks PI and titrated by ELISA using the method of checker board titration [16].

2.4 Analysis of immuno-activities of the recombinant proteins by western blot:

To analyze the immuno-reactivity to the polyclonal antibodies or swine sera, the proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Pall Co. Ltd., USA) in Semi-dry transfer cell (Bio-Rad) at 20V for 45 minutes. The membranes were then blocked with 5% (w/v) skim milk diluted in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-T) for 2 hours at 37℃. After washing, the membranes were probed respectively with two types of polyclonal antibodies (1:500 dilution) or swine sera (1: 100) for 1 hour, followed by incubation with horseradish peroxidase conjugated goat IgG (KPL Inc., USA) for 45 minutes. Finally, colors were developed with 4-chloro-1-naphthol.

2.5 Characterization of the polyclonal antibodies by immunofluorescence assay (IFA)

The reactivities of polyclonal antibodies to viral proteins and their specificities were identified by IFA according to the procedure described by Fenaux *et al* (2003)[17]. Briefly, cells were seeded on 6-well plates (Corning, U S A). PCV free-PK15 cell monolayer at about 80% confluence was infected with the PCV2 SH isolate. Three days later, the infected cells and PCV1-contaminated PK-15 cells were fixed with a pre-cooled solution containing 80% acetone at –20℃ for 15 minutes. After washing with PBS-T, the cells were incubated at 37℃ for 1 hour with 1:500-diluted polyclonal antibodies. Then, the plates were washed three times with PBS-T and incubated with the secondary fluorescein isothiocyanate-labeled goat anti-rabbit IgG (KPL Inc., USA) at 37℃ for 45 minutes. Finally, the plates were examined under a fluorescence microscope (Olympus IX71 inverted research microscope).

2.6 Optimization of an ELISA for monitor of PCV2 sero-prevalence in the swine herds

To evaluate the potential use of PCV2 Cap protein in serological survey of PCV2 infection, serum antibodies to PCV2 of 259 samples collected from 13 different

herds located in 4 regions of Zhejiang province were detected by a modified ELISA based on the Cap2s [16]. Briefly, 100 μl purified Cap2s diluted with 50 mM sodium carbonate buffer (pH9.6) to a final concentration of 50μg/ml was added into each well of 96-well microtiter plate (MaxiSorp, Nunc, Denmark) for incubation at 4℃ for 18 hours. Then the plates were blocked with 5%(w/v) skim milk diluted in PBS-T for 2 hours at 37℃. Serum samples diluted by 100-fold in PBS-T containing 0.5% (w/v) skim milk were added to corresponding wells and the plates were incubated for 1 h at 37℃. After washing for three times, plates containing 100μl per well of goat anti-pig IgG conjugated with horseradish peroxidase (KPL Inc., USA) at a dilution of 1:2000 were incubated for 45 min at 37℃. Thereafter, plates were washed and added with 100μl/well of the substrate solution (o-phenylenediamine dihydrochloride, Sigma). 30 min later, reaction was stopped by adding 50μl of 2M H_2SO_4. The OD_{492nm} values were measured using SpectraMax@ M2 microplate reader (Molecular Devices Corp. USA).

A panel of PCV2 negative sera (n=20) was also examined by ELISA to determine the cut-off OD_{490nm} value (mean ± 3SD) in the study. Serum samples with an optical density at 490nm greater than the cut-off value were considered as sera-positive for PCV2.

The immune sera of foot and mouth disease viruses (FMDV) type A, O, C and Asia I were bought from Lanzhou Veterinary Research Institute (Chinese Academy of Agriculture Sciences, Lanzhou 730046, China) with the value of 1:64. Sera of classical swine fever virus (CSFV) and porcine pseudorabies virus (PPV) (1:50) were screened by ELISA in our lab, as well as the PCV1 positive serum by IFA (1:50).

3 RESULTS

3.1 Construction of the recombinant expression plasmids pET-Cap2s and pET32-Cap1s

The recombinant expression plasmids containing fragment of PCV2 ORF2 epitopes or PCV1 ORF2 were confirmed by double digestion and sequencing. The nucleotide sequences of the inserted fragments were identical to that of PCV2 SH strain (Genbank accession number DQ195679) or PCV1 strain (Genbank accession number NC006266).

3.2 Expression and purification of the Cap2s and Cap1s

Expression of Cap2s and Cap1s in *E. coli* BL21 were induced by 1mM IPTG. The fusion proteins were successfully purified with NTA-agarose system (Fig. 1). The final concentrations of the eluted proteins were determined as 0.9 mg/ml and 1 mg/ml respectively by the Bradford method.

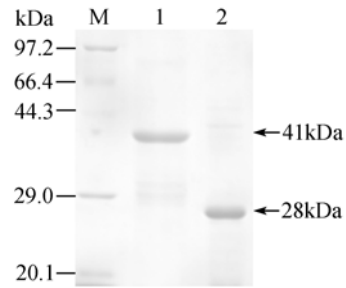

Fig. 1 Purification of the Cap1s (lane 1, 40 kDa) and Cap2s (lane 2, 28 kDa) by Ni-NTA agarose.

3.3 Characterization of the fusion proteins

The immunogenicity of Cap2s or Cap1s was examined by preparing polyclonal antibodies in rabbits. In the western blot, the Cap2s of 28-kDa and Cap1s of 41-kDa could react with its corresponding polyclonal antibody at 1: 500 dilution (Fig. 2, lane 2 and 3). But there were also cross-reactivities between the proteins and their polyclonal antibodies (Fig. 2, lane 1 and 4). However, PCV2 positive sera from diseased pigs only reacted specifically with Cap2s (Fig. 2, lane 5), while PCV1 positive sera could only recognize the band of Cap1s (Fig. 2, lane 7).

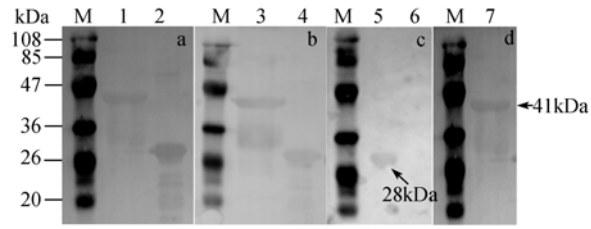

Fig. 2 Western blot analysis of immuno-reactivity of the purified proteins. Cap1s and Cap2s with molecular weight of 41kDa (lane 1 and 3) and 28kDa (lane 2 and 4) respectively that separated by SDS-PAGE and transferred to the nitrocellulose membranes could be recognized by both polyclonal antibodies. However, the Cap2s reacted specifically with PCV2-positive swine sera (lane 5), but could not recognize PCV1-positive seine sera (lane 6). In contrast, PCV1 positive sera could only recognize the band of Cap1s (lane 7). The pre-stained protein marker in lane M was introduced as standard.

3.4 Characterization of polyclonal antibodies by immunofluorescence assay (IFA)

In order to check the reactivity of polyclonal antibodies to the viral proteins and their specificity, PCV-free PK-15 cells infected with PCV2 SH isolate as well as PCV1 contaminated PK-15 cells were seeded and analyzed by IFA. In PCV2-infected PK-15 cells, positive signals were detected with anti-Cap2s polyclonal anti-

body (Fig. 3a), while no signal was found with antibody against Cap1s (Fig. 3b). On the contrary, the fluorescent signals were observed in PCV1-contaminated PK-15 cells incubated with anti-Cap1s polyclonal antibody (Fig. 3c), but there was no detectable signal when the cells were incubated with polyclonal antibody against Cap2s (Fig. 3d).

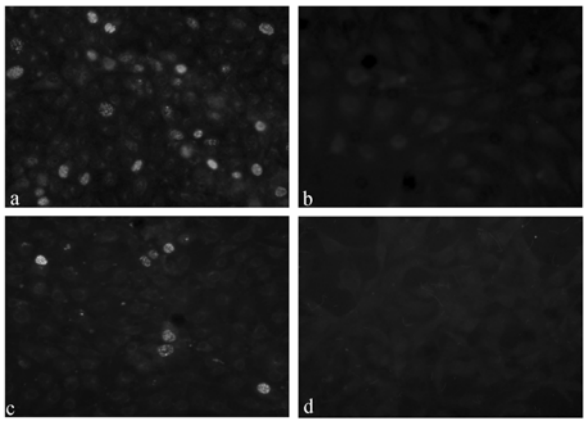

Fig. 3 Characterization of the polyclonal antibodies on PCV-free PK-15 cells infected with PCV2 SH isolate (a, b) or PCV1 contaminated PK-15 cells (c, d) by immunofluerescence assay (IFA). Signals were detected in PCV2 infected PCV-free PK-15 cells incubated with antibody against Cap2s (a), but it was negative when incubated with antibody against Cap1s (b). Contrarily, there were positive signals in the PCV1 contaminated PK-15 cells incubated with antibody against Cap1s (c), but no visible signals while incubated with antibody against Cap2s (d).

3.5 Application of the Cap2s as coating protein for investigation of PCV2 infection in fields by ELISA

The optimal dilutions of purified Cap2s (50μg/ml) and serum samples (1:100) were determined in preliminary assays by checker board titration. Moreover, the specificity and reproducibility of the ELISA were also evaluated. As a result, the positive sera of PCV1, CSFV and PPV and immune sera of FMDV were detected as negative in ELISA. Furthermore, the standard deviations (SD) of OD_{492nm} value of positive controls or negative controls in three reduplicative assays were between 0.0395 and 0.0571.

PCV2 antibodies of serum samples (n=259) of pigs from 13 herds were detected by ELISA to evaluate the potential application of the assay in fields. According to the average OD_{492nm} value of twenty PCV2 negative sera of 0.2122, the cut-off value for PCV2 sera-positiveness in this study was 0.3022 (mean ± 3SD). As shown in Table 1, the average sera-positive rate was 80.69% (209/259) and the prevalence of PCV2 infection was varied within different herds from 48.28% to 100%.

Table 2 Sero-prevalence of PCV2 antibody in pigs from different herds evaluated by ELISA with Cap2s as coating protein.

Herds	No. positive / No. samples	Rositive rate/%
002	27/32	84.38
003	28/31	90.32
006	30/30	100
011	4/7	57.14
016	10/10	100
030	9/10	90.00
101	14/29	48.28
103	10/10	100
104	10/11	90.90
105	18/23	78.26
106	22/26	84.62
301	18/30	60.00
306	9/10	90.00
Total	209/259	80.69

4 DISCUSSION

PMWS has rapidly spread to all major pig producing countries of the world [7, 8] and had an increasing impact on the swine industry. PCV2 is believed to be the major aetiological agent of PMWS [7, 18]. The availability of type-specific serological assays is therefore significantly important in investigating the prevalence of PCV2 infection. In this study, the truncated capsid protein of PCV2 (Cap2s) and PCV1 (Cap1s) were expressed and purified. The polyclonal antibodies were then prepared in SPF rabbits. Both of the Cap proteins could be recognized by their relative polyclonal antibodies. There was cross-reactivity between the two antibodies when reacted with the expressed proteins in western blot, which might be due to the antibodies to the His tag in recombinant proteins or some other proteins of *E. coli*. However, either the PCV2-positive sera from diseased pigs or PCV1-positive swine sera identified by IFA only reacted specifically with Cap2s or Cap1s, respectively. Moreover, cross-reactivity was not found on infected cells by IFA, since each polyclonal antibody reacted specifically with its corresponding viral Cap protein. Therefore, the truncated PCV2 Cap containing all the epitopes was actually type-specific in reaction with swine positive sera and was valuable for further serological diagnosis of PCV2 infection.

PMWS is most commonly diagnosed on the basis of characteristic histopathological lesions and clinical symptoms as well as the presence of PCV2 in the lesions [19]. Methods most commonly used for PCV2 detection include indirect immunofluorescence assays (IFA) for

PCV2-infected cells and PCR amplification [20]. PCR can provide an alternative confirmatory tool when combined with characteristic histopathological changes in tissues, but in situ hybridization and immunohistochemistry (IHC) should be considered as the better techniques to diagnose clinical PMWS cases [8]. Thus, type-specific antibody to PCV2 viral protein is on urgent to be developed for further diagnosis or study of PMWS. In this study, the characteristic of polyclonal antibody to PCV2 Cap was evaluated. It was confirmed as type-specific by IFA since only PCV2 viral protein was clearly detected in PCV2-infected cells but no signal was observed in PCV1-contaminated PK-15 cells, indicating that the polyclonal antibody against PCV2 capsid protein was useful for future diagnosis with the method of IHC as it cost much less than use of monoclonal antibody.

PCV2 ORF2 has been identified as a major viral structural protein that can form viral capsid-like particles in insect cells infected with ORF2-expressing recombinant baculovirus [21]. Because of the close relevance to pathogenicity and encoding the major immunogenic protein of the virus and the principal bearer of type-specific epitopes [21, 22], the characterization of PCV2 ORF2 has become more important and the Cap protein can be used as diagnostic antigen for serologic detection of PCV2 infection [23]. de Boisseson *et al* (2004) [24] have reported that the variation between the complete PCV2 sequences from different geographic areas was mainly due to the variability within ORF2, especially within its epitopes. Thus, it might be better to develop assays based on the local isolates. In this study, the ORF2-encoded PCV2 Cap protein containing all epitopes was used to develop an ELISA to monitor PCV2 infection in fields. The ELISA was found to be specific to PCV2 antibody and reproducible. Overall, 259 serum samples from 13 different herds were tested and 209 samples were found as positive，with a high positive rate of 80.69%, indicating that PCV2 infection was prevalent in Zhejiang province and it might be as the causative agent in developing PMWS. The results were consistent with findings of PCR detection of PCV2 in tissues (data not shown). Even higher PCV2 sero-prevalence was reported in numbers of serological surveys in other counties [25, 26, 27]. Though, no relationship between regions and sero-prevalence could be found, there were significant differences of prevalence of PCV2 infection within herds (varied from 48.28% to 100%), which could be due to the different health status, immunization strategies or even management. Conclusively, the ELISA based on the protein was specific and had a potential use in monitoring of PCV2 sero-prevalence in fields, which would be helpful to the early warning and control of PMWS.

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猪圆环病毒 2 型(PCV2)核衣壳蛋白免疫原性的鉴定和应用及其多抗在 PCV2 感染诊断中的应用

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摘要：猪圆环病毒 2 型 ORF2 编码与病毒毒力相关的结构蛋白——核衣壳蛋白(Cap)，该蛋白可以用于 PCV2 感染的血清学调查，但不同区域的 PCV2 分离株的 ORF2 特别是其抗原表位序列存在一定的突变。本研究将 PCV2 浙江分离株 ORF2 的主要抗原表位以及 PCV1 ORF2 进行了原核表达，将分别纯化的融合蛋白 Cap2s 和 Cap1s 免疫 SPF 兔后制备多抗，并进一步分析了纯化蛋白的免疫原性和多抗的特性。Western blot 结果表明无论 Cap2s 和 Cap1s 均能与两个多抗发生交叉反应，而 PCV2 或 PCV1 阳性猪血清只能分别特异性地识别 Cap2s 和 Cap1s。IFA 结果则证明两个多抗对于天然 Cap 蛋白无交叉反应性。利用 Cap2s 作为包被抗原对 13 个猪场的 259 份血清样品的 PCV2 抗体进行 ELISA 检测，平均阳性率为 80.69%(209/259)，而各猪场的阳性率差异较大(48.28%~ 100%)。以上结果表明 Cap2s 可作为一个型特异性抗原用于浙江省本地猪场猪群血清中 PCV2 抗体的监控，而其多抗也可用于免疫组化对 PCV2 感染进行有效诊断。

关键词：猪圆环病毒；核衣壳蛋白；免疫原性；间接免疫荧光；血清阳性率

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