Comparison of IPMA and IFA Methods for Determination of Porcine Circovirus Type 2

Feng WEI[1], Yan WANG[1], Nana ZHAO[2], Yu GUAN[1], Weiqin MENG[1], Feng LI[1], Qingqing XU[1], Changjiang WANG[1]*

1. Shandong Binzhou Institute of Animal Husbandry and Veterinary Science, Binzhou 256600, China; 2. Binzhou Center for Disease Control and Prevention, Binzhou 256600, China

Abstract [**Objectives**] This study was conducted to find a convenient and reliable method for detecting the virus titer of porcine circovirus type 2 (PCV2). [**Methods**] The reaction conditions of the immunoperoxidase monolayer assay (IPMA) method for detecting the viral titer of PCV2 were optimized, and the results were compared with those obtained by indirect immunofluorescence assay (IFA). [**Results**] PCV2-infected cells exhibited brownish-red staining in either the nucleus or cytoplasm when detected by IPMA, and the detection results were largely consistent with IFA detection. [**Conclusions**] Both IPMA and IFA methods can be effectively used for determination of PCV2 viral titer, providing reliable support for assessing viral content during PCV2 vaccine development and validating virus inactivation efficacy.

Key words Porcine circovirus type 2; Viral titer; IPMA; IFA

DOI:10.19759/j.cnki.2164-4993.2025.02.008

Porcine circovirus (PCV) is one of the smallest viruses among animal viruses and belongs to circovirus of Circoviridae[1]. Based on genomic sequence analysis, PCV can be classified into four genotypes: PCV1, PCV2, PCV3, and PCV4[2]. Among these, PCV2 is the primary pathogen responsible for porcine circovirus disease[3]. PCV2 is pathogenic to pigs but does not cause cytopathic effect. After infecting host cells, the virus is mainly dispersed in the nucleus and cytoplasm[4].

PCV2 has a wide prevalence and high incidence rate, and pigs of all breeds are susceptible to it. Clinically, it is often found in co-infections with other pathogens such as porcine reproductive and respiratory syndrome virus, classical swine fever virus, pseudorabies virus, porcine epidemic diarrhea virus, congenital tremors, fetal myocarditis, and enteritis[5-9], leading to exacerbated clinical symptoms. Since its outbreak in Canadian pig herds in 1991, the disease has caused significant economic losses to the global pig farming enterprises.

Vaccination is currently the most effective means of preventing and controlling porcine circovirus disease. Effective immunization helps pigs resist the virus, reduces viremia, and shortens the time of carrying virus[10].

Before vaccine preparation, the viral titer must be determined, and the inactivation efficacy must be verified after virus inactivation. However, since PCV2 infection does not produce CPE in cells, this complicates both viral titer determination and inactivation verification. PK15 cells are the optimal cell line for culturing PCV2. Although PCV2 infection in PK15 cells does not cause CPE, it leads to the formation of numerous inclusion bodies in the cytoplasm[11]. Currently, the primary method for quantifying PCV2 viral content is indirect immunofluorescence assay (IFA). Previously, our laboratory mainly used IFA to measure PCV2 viral content. In this study, to explore one or more alternative detection methods, the immunoperoxidase monolayer assay (IPMA) and IFA for determination of PCV2 viral titer were compared, hoping to identify a more suitable detection method for PCV2 detection. This method can also be applied to vaccine antibody level detection and epidemiological investigations.

Received: January 2, 2025 Accepted: March 5, 2025

Supported by General Project of Shandong Provincial Natural Science Foundation (ZR2022MC173); Binzhou Young Science and Technology Rising Star Program (QMX2023004); Binzhou Comprehensive Experimental Station of Shandong Swine Industry Technology System Project (SDAIT-08-13).

Feng WEI (1979-), female, P. R. China, master, devoted to research about cell engineering and virology.

* Corresponding author.

Materials and Methods

Experimental materials

Virus and cells The PCV2 strain and PK15 cells were preserved by the Key Laboratory of Binzhou Institute of Animal Husbandry and Veterinary Science, Shandong Province.

Major reagents and instruments PCV2 monoclonal antibody was provided by Shandong Landu Biotechnology Co., Ltd. Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG was purchased from Beijing Boyotod Technology Co., Ltd. Horseradish peroxidase (HRP)-labeled goat anti-mouse secondary antibody was purchased from Wuhan Boster Biological Technology Co., Ltd. AEC (3-amino-9-ethylcarbazole) was from Sigma, while other chemical reagents were domestic analytical pure, including AEC color developing solution (AEC 10 mg, dimethylformamide 1.25 ml, 0.05 M sodium acetate 25 ml, 30% H_2O_2 3.75 ml). The inverted fluorescence microscopes and CO_2 incubators (MCO-18AC) used in this study were purchased from Jinan Qingyuan Medical Equipment Co., Ltd. The binocular biological microscopes (XSB-102B) used were acquired from Weifang Longtai Medical Equipment Co., Ltd.

Methods

Subculture of PK15 cells The revived cells were placed in a 37 ℃ constant-temperature incubator for continued culture. Cell

growth was observed daily under a microscope. When the cells formed a dense monolayer after 48 h, the culture medium was discarded, and the cell layer was gently washed twice with PBS. An appropriate amount of 0.25% trypsin was added for digestion. Under microscopic observation, once the cells became slightly rounded and gaps appeared between them, the trypsin solution was removed. DMEM supplemented with 7% newborn calf serum was added, and the cells were gently pipetted to prepare a cell suspension. The cells were then subcultured at a 1∶3 ratio.

IPMA Method

Preparation of IPMA reaction plates A freshly prepared PK15 cell suspension was used to serially dilute the PCV2 virus solution at ratios of 10, 10^2, 10^3, 10^4, 10^5, and 10^6. The diluted virus was then inoculated into a 96-well cell culture plate, with eight wells per dilution and 0.1 ml per well. The plate was incubated at 37 ℃ with 5% CO_2 for 24 h. After discarding the growth medium, a maintenance medium was added, and the cells were cultured for an additional 48 h. Control wells containing uninfected PK15 cells were also prepared.

IPMA staining procedure The cell culture medium in the 96-well plate prepared in "Preparation of IPMA reaction plates" was discarded, and the cells were gently washed three times with PBS (pH 7.2 – 7.4). Each well was fixed with 50 μl of fixative for 30 min. After removing the fixative and allowing the plate to stand for 2 min, the cells were washed three times with PBS (pH 7.2 – 7.4). A blocking buffer (50 μl/well) was added, and the plate was incubated at room temperature for 1 h. The blocking buffer was discarded, and 50 μl of PCV2 monoclonal antibody (diluted at 1∶300 with blocking buffer) was added to each well, followed by incubation at room temperature for 1 h. The primary antibody was removed, and the cells were washed three times with PBS (pH 7.2 – 7.4). Horseradish peroxidase (HRP)-labeled goat anti-mouse secondary antibody (50 μl/well) was added, and the plate was incubated at room temperature for 1 h. The secondary antibody was discarded, and the cells were washed three times with PBS (pH 7.2 – 7.4). The AEC color developing solution (50 μl/well) was added and incubated at room temperature until color development (incubation at 37 ℃ could accelerate the reaction). The results were observed under an inverted microscope. Result interpretation: In case of positive reaction, cells exhibited brown-red staining in the cytoplasm and nucleus, while no staining was observed in control cells.

Selection of optimal fixative Three IPMA reaction plates were prepared as described above. Different fixatives, including 80% cold acetone (4 ℃, 30 min), cold methanol (4 ℃, 30 min), and cold methanol∶acetone (1∶1) (4 ℃, 30 min), were tested, respectively. After fixation, the plates were stained following the standard IPMA procedure. The optimal fixative was selected based on the results of staining.

Selection of optimal blocking buffer Two IPMA reaction plates were prepared as described, fixed with the optimal fixative, and blocked with following buffers: PBS containing 5% newborn calf serum, and PBS containing 10% calf serum, 0.05% thimerosal, 0.5% Tween, and 3% sucrose. A control without blocking buffer was also set. The optimal blocking buffer was selected based on the results of staining.

Determination of optimal primary antibody dilution Three IPMA reaction plates were prepared according to the aforementioned protocol. Following fixation with the optimal fixative and blocking with the optimal blocking buffer, the plates were incubated with primary antibody at serial dilutions of 1∶50, 1∶100, 1∶150, and 1∶200. The standard IPMA staining procedure was then performed. The optimal antibody dilution was determined based on comparative analysis of the staining results.

Determination of optimal secondary antibody dilution According to the manufacturer's instructions, the recommended dilution range for the secondary antibody was from 1∶500 to 1∶1 000. In this experiment, dilutions of 1∶500, 1∶800 and 1∶1 000 were tested, and the optimal dilution was selected based on the results of staining.

Comparative application of IPMA and IFA methods Ten 96-well plates were prepared following the method described in section "Preparation of IPMA reaction plates". Five batches of PCV2 samples (20230701, 20230708, 20230722, 20230802, and 20230820) were randomly selected and analyzed for viral content using both IPMA and IFA detection methods. The correlation between these two analytical approaches was systematically evaluated based on the comparative detection results.

Results and Analysis

Proliferation culture results of PCV2 in PK15 Cells

After 72 h of culturing PCV2 in PK15 cells, no cytopathic effect (CPE) was observed. However, compared with the cells of the blank control, the PCV2-infected cells exhibited slightly poorer cell conditions with reduced brightness. IPMA detection revealed the presence of reddish-brown granules in PCV2-infected PK15 cells, while no such granules were observed in the control cells (Fig. 1). Using the established IFA method for identification, bright green fluorescence was detected in PCV2-infected PK15 cells, whereas no fluorescence was observed in the control cells (Fig. 2).

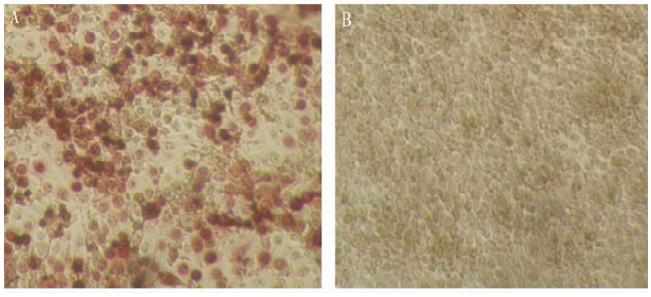
Selection of optimal fixative

The results of IPMA staining using different fixatives demonstrated that fixation with 80% cold acetone at 4 ℃ for 30 min yielded optimal results, showing the deepest staining intensity in positive cells.

Selection of optimal blocking buffer

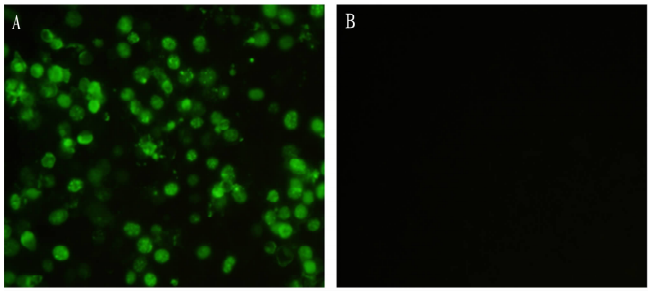
During the blocking step, two different blocking buffers were compared by 1 h of blocking treatment, while a control group without the addition of blocking buffer was also conducted. IPMA staining results demonstrated that the PBS containing 10% calf serum, 0.05% thimerosal, 0.5% Tween, and 3% sucrose provided optimal blocking performance, yielding intense staining of positive cells and a slightly higher measured titer for the same sample. In

contrast, the control group without the addition of blocking buffer showed faint positive-cell staining, bright background coloration, and lower viral titer.



A. PK15 cells infected by PCV2; B. PK15 cells.

Fig. 1 Detection of PCV2 by IPMA method



A. PK15 cells infected by PCV2; B. PK15 cells.

Fig. 2 Detection of PCV2 by IFA method

Determination of optimal monoclonal antibody dilution

The results of IPMA staining showed that the optimal dilution ratio of PCV2 monoclonal antibody was 1 ∶ 100.

Determination of optimal secondary antibody dilution

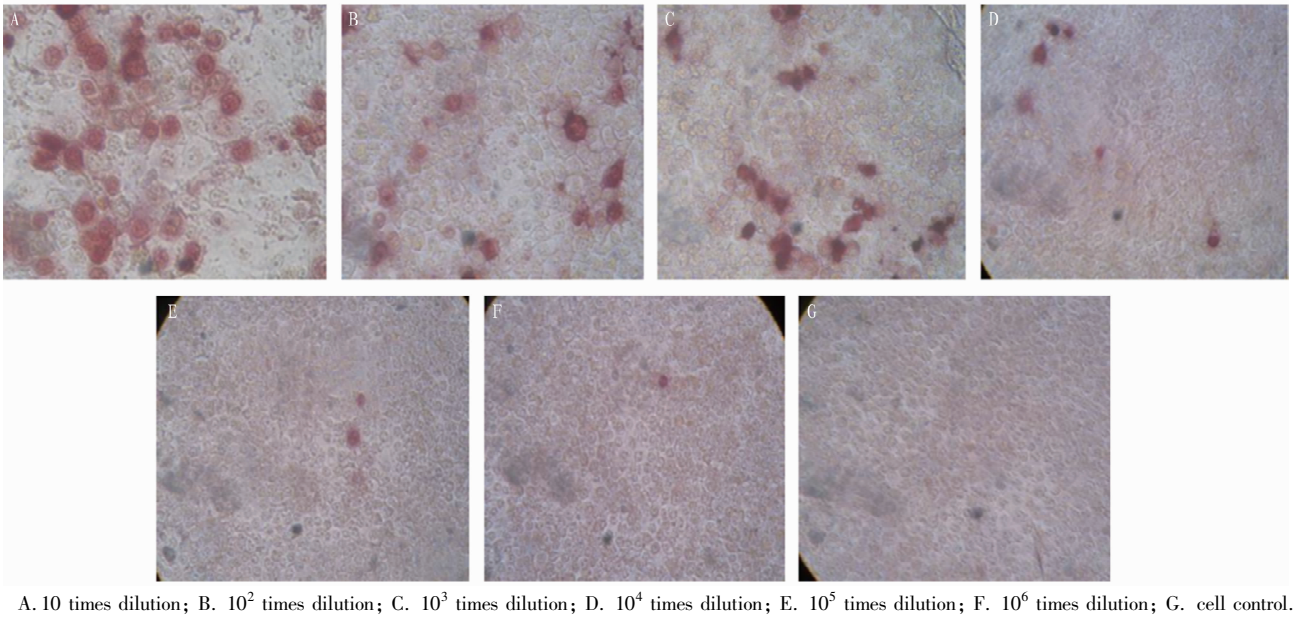
The results of IPMA staining showed that the optimal dilution ratio of HRP-labeled goat anti-mouse secondary antibody was 1 ∶ 1 000.

Comparative evaluation of IPMA and IFA methods

The virus contents of five samples were measured by both methods, and the results showed high consistency, as shown in Table 1. Visual staining results also demonstrated excellent correspondence (Fig. 3 – Fig. 4).

Table 1 Results of virus content detection in PCV2 samples by IPMA and IFA methods

Sample No.	Detection method	Virus content//LogTCID$_{50}$/ml
20180701	IPMA	7.250
	IFA	7.250
20180708	IPMA	7.375
	IFA	7.375
20180722	IPMA	7.000
	IFA	7.125
20180802	IPMA	6.875
	IFA	7.000
20180820	IPMA	7.125
	IFA	7.125



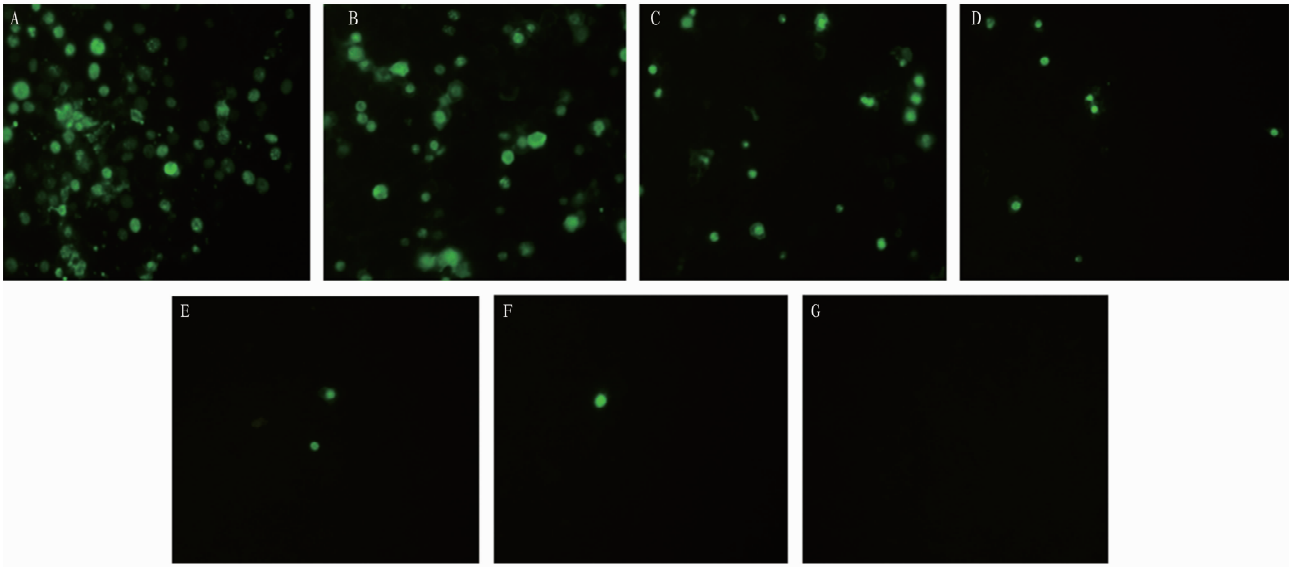
A. 10 times dilution; B. 10^2 times dilution; C. 10^3 times dilution; D. 10^4 times dilution; E. 10^5 times dilution; F. 10^6 times dilution; G. cell control.

Fig. 3 TCID$_{50}$ determination of PCV2 by IPMA method

Conclusions and Discussion

Porcine circovirus disease remains one of the most prevalent diseases threatening the swine industry, which has caused significant economic losses due to its widespread prevalence. In the prevention and control of this disease, vaccine preparation and quality testing are particularly critical, making the development of suitable and accurate detection methods for viral quantification and vaccine inactivation validation necessary. The indirect immunofluorescence assay (IFA), which combines antigen-antibody specificity with fluorescent antibody techniques, is widely used for PCV2 antigen and antibody detection due to its operational simplicity and high specificity. Cui *et al.* [12] established an IFA method for PCV2

using PCV2-specific antibodies. Meanwhile, real-time quantitative PCR detects PCV2 genomic copy numbers at the nucleic acid level, while direct immunofluorescence qualitatively assesses Cap protein expression in infected cells, followed by viral titer calculation using the Reed-Muench method[13]. Lin *et al.* [14] developed an indirect ELISA method based on prokaryotically-expressed Cap protein for epidemiological detection of porcine circovirus disease. Xu *et al.* [15] established a sandwich ELISA method using eukaryotic-expressed Cap protein to detect PCV2 antibody. Walker *et al.* [16] introduced a competitive ELISA method for large-scale serological screening of PCV2 antibody. Tang *et al.* [17] employed immunohistochemistry to map PCV2 distribution in swine tissues, identifying positive signals in cardiac, hepatic, lymph node, and tonsil tissues. While numerous reports exist on PCV2 antigen/antibody detection, few describe IPMA for detection of PCV2 content. IPMA conjugates enzymes with antibodies (or antigens), and the labeled antibody (or antigen) molecules retain both immunological activity of binding to corresponding antibodies (or antigens) and enzymatic activity. The enzyme-labeled antibodies bind to antigens to form an enzyme-labeled antibody-antigen complex. The enzyme on the complex catalyzes substrate decomposition when encountering corresponding substrate, oxidizing hydrogen donors to produce colored substances[18]. The study by Gao *et al.* [19] demonstrated that the IPMA detection method of PCV2 exhibits excellent sensitivity and specificity, meeting the requirements for epidemiological investigations, clinical testing, and vaccine antibody level monitoring.



A. 10 times dilution; B. 10^2 times dilution; C. 10^3 times dilution; D. 10^4 times dilution; E. 10^5 times dilution; F. 10^6 times dilution; G. uninfected cells.

Fig. 4 $TCID_{50}$ determination of PCV2 by IFA method

The selection of fixative is crucial in immunocytochemical staining as fixation quality directly affects detection results. Effective fixation should preserve the natural morphology and activity of target antigens in cells to maintain the accessibility of labeled antibodies to antigens[12]. Appropriate fixation methods must be selected according to to-be-detected samples and selected cells. In this study, pre-chilled 80% acetone was adopted for fixation at 4 ℃ for 30 min, which achieved good results with transparent and clear cell morphology while avoiding corrosion to cell culture plates.

The operational procedures of both IPMA and IFA detection methods are essentially identical, with the only difference being their respective color developing systems. IPMA utilizes AEC for color development, which can be observed under an inverted microscope, where PCV2-infected positive cells appear reddish-brown while control cells remain unstained. In contrast, IFA requires fluorescence microscopy for result observation, where the excitation of green fluorescent protein carried by secondary antibodies emits green light for determining positivity or negativity. When observing fluorescence staining results under a fluorescence microscope, each observation should preferably not exceed 2 h, as prolonged continuous use may cause decreased brightness of the ultra-high pressure mercury lamp and weakened fluorescence intensity[20]. IFA staining results require observation in a darkroom, and prolonged viewing may cause eye fatigue, potentially leading inexperienced observers to misinterpret green impurities (non-specific staining) as positive signals, thus introducing errors. Therefore, the interpretation of IFA results demands experienced observers to minimize potential error.

The results of this study demonstrate that both IPMA and IFA methods can be effectively used for PCV2 titer determination. Compared with IFA, IPMA has lower experimental requirements, needing only a standard inverted microscope for observation with easily interpretable results, making it feasible for most laboratories while being more cost-effective. However, IPMA also has certain limitations, as inexperienced operators may occasionally misinterpret results. In conclusion, when using either method for detecting the content of PCV2 antigen, it is advisable to let experienced experimenters operate to minimize non-specific staining and ensure accurate results.

(Continued on page 43)

[13] VENDAN RT, YU YJ, LEE SH, *et al.* Diversity of endophytic bacteria in ginseng and their potential for plant growth promotion[J]. Journal of Microbiology, 2010, 48(5): 559 – 565.
[14]TENG XM. Site selection, soil improvement, and pesticide residue degradation in farmland-cultivated ginseng[J]. Ginseng Research, 2005 (3): 32 – 33.
[15] BAO SD. Soil and agricultural chemistry analysis[M]. Beijing: China Agriculture Press, 1999.
[16] PAN JL, DAI WA, SHANG ZH, *et al.* Review of research progress on the influence and mechanism of field straw residue incorporation on soil organic matter and nitrogen availability[J]. Chinese Journal of Eco-Agriculture, 2013, 21(5): 526 – 535.
[17] WU AX, WANG X, LYU Y, *et al.* Progress of soil nutrients in cultivation of *Panax ginseng* in farmland[J]. Northern Horticulture, 2018 (22): 177 – 186.
[18] SONG CY, ZHANG XY, LIU XB, *et al.* Effect of soil organic matter on soil fertility and crop productivity[J]. Journal of Agricultural System Science and Comprehensive Research, 2008(3): 357 – 362.
[19] ZHAO SJ, YU DR, CAO XY, *et al.* Study on suitable soil conditions for ginseng cultivation[J]. Specialty Research, 1992(1): 16 – 19.
[20] JIN Y. Effect of different fertilization on the yield and quality of *Schisandra chinensis*[D]. Northeast Agricultural University, 2011.
[21] GONG JZ, ZHANG SJ, WANG YR, *et al.* Effects of nitrogen forms on the physiological characteristics of *Panax ginseng*[J]. Northern Horticulture, 2024(16): 96 – 101.
[22] ZHONGYL, FAN JB. Genotypic differences in grain Yield and physiological nitrogen use efficiency among riecultivars[J]. Pedos Phere, 2009, 19(6): 681 – 691.
[23] YANG JX, TIAN YX. Cultivation of medicinal plants[M]. Beijing: China Agriculture Press, 2004: 158 – 162.
[24] LYU L, ZHANG YY, SUN H, *et al.* Effects of phosphorus stress on nutrient elements and saponins in ginseng plants[J]. Journal of Jilin Agricultural University, 2023, 45(3): 307 – 315.
[25] MENG XY, LIU YY. Study on the effects of combined application of nitrogen, phosphorus, and potassium on ginseng quality[J]. Standardization Reports, 1996, 17(6): 36 – 37.
[26] ZHANG CG. Study on the effect of potassium fertilizer on ginseng growth [D]. Chinese Academy of Agricultural Sciences, 2018.
[27] ZHAO Y, WANG XQ, ZHENG YN, *et al.* Effect of inorganic fertilizers on yield properties of ginseng[J]. Journal of Jilin Agricultural University, 2001, 23(4): 56 – 59.
[28] XU YH, CAO ZQ, SONG XD. Discussion on scientific fertilization in ginseng production[J]. Ginseng Research, 2004(1): 22 – 25.

Editor: Yingzhi GUANG Proofreader: Xinxiu ZHU

(Continued from page 37)

References

[1] MANKERTZ A, CALISKAN R, HATTERMANN K, *et al.* Molecular biology of porcine circovirus: Analyses of gene expression and viral replication[J]. Vet Microbiol., 2004, 98(2): 81 – 88.
[2] SUN W, DU Q, HAN Z, *et al.* Detection and genetic characterization of porcine circovirus 4 (PCV4) in Guangxi, China[J]. Gene, 2021, 773: 145384.
[3] SEGALES J. Porcine circovirus type 2 (PCV2) infections: Clinical sings, pathology and laboratory diagnosis[J]. Virus Res., 2012, 167 (1 – 2): 10 – 19.
[4] LIU CM, ZHANG CF, WEI YW, *et al.* Cultivation and identification of porcine circovirus type 2 (PCV2) cell culture-adapted strain[J]. Chinese Journal of Preventive Veterinary Medicine, 2006, 28(3): 248 – 251. (in Chinese).
[5] YI J, LIU C. Molecular characterization of porcine circovirus 2 isolated from diseased pigs coinfected with porcine reproductive and respiratory syndrome virus[J]. Virol J., 2010, 7: 286.
[6] PEREZ LJ, DIAZ DE ARCE H, PERCEDO MI, *et al.* First report of porcine circovirus type 2 infections in Cuba[J]. Res Vet Sci, 2010, 88 (3): 528 – 530.
[7] TANG Q, GE L, TAN S, *et al.* Epidemiological survey of four reproductive disorder associated viruses of sows in Human Province during 2019 – 2021[J]. Vet Sci., 2022, 9(8): 425.
[8] GUO Z, RUAN H, QIAO S, *et al.* Co-infection status of porcine circoviruses (PCV2 and PCV3) and porcine epidemic diarrrhea virus (PEDV) in pigs with watery diarrhea in Henan province, central China [J]. Microb Pathog., 2020, 142: 104047.
[9] ALLAN GM, MCNEILLY F, ELLIS J, *et al.* PMWS: Experimental model and co-infections[J]. Vet Microbiol, 2004, 98(2): 165 – 168.
[10] HE ZQ, XU C, WANG AF, *et al.* Quantitative method of porcine circovirus II vaccine antigen and its application[J]. China Animal Industry, 2024, 4: 43 – 44. (in Chinese).
[11] SANCHEZ RE JR, MEERTS P, NAUWYNCK HJ, *et al.* Characteristics of porcine circovirus-2 replication in lymphoid organs of pigs inoculated in late gestation or postnatally and possible relation to clinical and pathological outcome of infection[J]. J Vet Diagn Invest., 2004, 16 (3): 175 – 185.
[12] CUI SJ, JIN YP, LI X, *et al.* The development of an indirect immunofluorescent technique of porcine circovirus[J]. Chinese Journal of Preventive Veterinary Medicine, 2007, 29(1): 63 – 66. (in Chinese).
[13] YANG X. Study on the culture characteristics of PCV2 in vitro and the effect of HMGCR on the replication of OCV2[D]. Jilin: Jilin University, 2015. (in Chinese).
[14] LIN YX, SUN YW, LIU ZM, *et al.* Development of an indirect ELISA diagnostic method with the main antigentic region of PCV2 capsid protein expressed in *E. coli*[J]. Chinese Journal of Preventive Veterinary Medicine, 2007(3): 217 – 221. (in Chinese).
[15] XU ZJ, WEI RR, LI YA, *et al.* Development of a double antibody sandwich ELISA for quantitative detection of porcine circovirus type 2 Cap protein[J]. Animal Husbandry and Veterinary Medicine, 2018, 50 (10): 73 – 77. (in Chinese).
[16] WALKER IW, KONOBY CA, JEWHURST VA, *et al.* Development and application of a competitive enzyme-linked immunosorbent assay for the detection of serum antibodies to porcine circovirus type 2[J]. J Vet Diagn Invest., 2000, 12(5): 400 – 405.
[17] TANG N, LYU YL, GUO X, *et al.* Detection of distribution of porcine circovirus II in experimentally infected piglets with immunohistochemical method[J]. Acta Veterinaria et Zootechnica Sinica, 2008(3): 372 – 375. (in Chinese).
[18] WEI XM, REN RP. Handbook of laboratory testing of animal diseases [M]. Beijing: China Agriculture Press, 2006. (in Chinese).
[19] GAO J, HU JH, SUN FP, *et al.* Establishment and application of PCR method for detection of porcine circovirus II[J]. Acta Agriculturae Shanghai, 2007, 23(4): 102 – 104. (in Chinese).
[20] GUO QH, LIU XL. Establishment of immunoperoxidase monolayer cell assay for detection of porcine circovirus type 2[J]. Heilongjiang Animal Science and Veterinary Medicine, 2012, 6: 122 – 123. (in Chinese).

Editor: Yingzhi GUANG Proofreader: Xinxiu ZHU