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ORIGINAL RESEARCH

Combined Antibody Tagged HRP Gold Nanoparticle Probe for Effective PCV2 Screening in Pig Farms

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Introduction: Porcine circovirus type 2 (PCV2) causes immune repression and intercurrent infections in pigs, resulting in a huge economic loss to the pig breeding industry. Additionally, the spread of PCV2 in pig farms can pollute the living environment of the residents in the farm's vicinity, which increases the rate of infections. Therefore, rapid and sensitive detection methods are needed for disease prevention and timely environmental cleaning.

Methods: This research describes a highly sensitive sandwich enzyme-linked immunosorbent assay (ELISA) that utilizes gold nanoparticles (AuNPs) in a functional, specific antibody labeled probe for the detection of PCV2. Due to their high specific surface area and histocompatibility, AuNPs were used as carriers of HRP labeled anti-PCV2 antibodies to amplify the detection signal.

Results: Compared to conventional sandwich ELISA procedures, this method resulted in higher sensitivity (51-fold) and a shorter assay time with a limit of detection of 195 $TCID_{50}$/mL. The cross-reactivity assay demonstrated that this assay was PCV2 specific.

Conclusion: The amplified Ab (HRP) labeled AuNPs probe provides a sensitive analytical approach for the determination of the traces of the PCV2 antigen in early diagnosis.

Keywords: PCV2, gold nanoparticles, ELISA, amplification, detection

Introduction

Porcine circovirus type 2 (PCV2), which belongs to the genus *Circovirus* in the family *Circoviridae*, is a major etiological agent of the post-weaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), and porcine respiratory disease complex (PRDC).[1,2] PCV2 infection is a significant immunosuppressive disease that greatly harms the pig breeding industry and hinders the industry's development in China and the whole world. Normally, PCV2 infection has no obvious seasonality and can occur throughout the year. While the PCV2 infection rate is high, it is mainly a recessive infection. Whether the pig is infected or not is not easy to detect; however, the virus can facilitate secondary invasions of other pathogens.[3] In recent years, the prevalence of PCV3, which demonstrates symptoms similar to that of PCV2, adds further difficulty to the early diagnosis of PCV2 infection.[4,5]

Furthermore, as one of the smallest animal viruses, PCV2 exhibits strong resistance to an external environment. Extensive research has shown that PCV2 can exist in pig farms for a long time, polluting the pig farm and the surrounding environment, tainting the surface and the groundwater, and endangering the water quality. In this regard, a high positive rate of PCV2 in the water and liquid samples of pig feces was observed in Brazil through a qPCR method.[6] Moreover, PCV2 was detected in bioaerosol and worker nasal wash samples in Malaysia.[7] The constant presence of PCV2 in the above environments facilitates the spread of infections among the pig population. Furthermore, Gilliland detected trace amounts of PCV1 and PCV2 in the DNA from human rotavirus vaccines, highlighting the issue of vaccine contamination.[8] Thus, it is crucial to develop simple, quick, and accurate methods for PCV2 detection in the environment at the early infection stage.

In the present study, a series of diagnostic procedures for PCV2-related disease have been performed. The most common methods for PCV2 pathogen diagnosis include virus isolation, indirect immunofluorescence assay (IFA), immunoperoxidase monolayer assay (IPMA), in situ hybridization (ISH), immunohistochemistry (IHC), polymerase

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chain reaction (PCR), and enzyme linked immunosorbent assay (ELISA).[9,10] IFA and IPMA are the classic methods for detecting antigens and antibodies due to their high sensitivity and good specificity; however, they are time-consuming and laborious, requiring high technology and equipment, and hence cannot be promoted and applied. The ELISA method has become the first choice for routine diagnostic reagents, and it is widely employed for antigen or antibody detection due to its simple operation, high sensitivity, ability to use many samples at a time, and easy standardization.[11] Presently, commercialized antibody detection kits are already in use. Yet, there is still a lack of research on the downsides of kits for antigen diagnosis, such as bad repeatability and false positives.[12] Therefore, a sensitive ELISA assay is useful for the detection of PCV2, particularly for the identification of quantitative analysis.

Gold nanoparticles (AuNPs) have attracted great interest in biomolecular and toxin detection due to their unique chemical and physical properties.[13–15] In particular, their large specific surface area and favorable biocompatibility can provide a suitable and promising platform for immobilizing biomacromolecules such as enzymes without affecting their own catalytic activity.[16] The use of AuNPs allows the attachment of multiple enzyme molecules, which can generate an amplified optical signal. Studies have shown that the AuNPs conjugated with several HRP labeled antibodies through their large specific surface area and then linked to more antigens produce an immunocomplex that realizes a signal amplification strategy for cancer biomarker detection. In our previous study, a dual-signal amplification strategy for PCV2 detection was developed using gold nanolayer modified microplates and tyramide. Additionally, several studies have employed AuNPs as antibody carriers to develop a highly sensitive, colorimetric enzyme immunoassay due to their high histocompatibility. Most of these methods focus on tumor markers or proteins instead of pathogens, such as viruses or bacteria.

In this study, we proposed an enhanced sandwich ELISA based on highly sensitive AuNPs in a functionalized HRP labeled PCV2 antibody probe for the detection of PCV2 pathogens. AuNPs were used as carriers of the HRP-labeled PCV2 antibodies to amplify the detection signal. To the best of our knowledge, this study is the first to use a sandwich ELISA for the highly sensitive detection of PCV2 pathogens utilizing AuNPs in a functionalized HRP labeled PCV2 antibody probe.

Materials and Methods

Preparation and Characterization of AuNPs

Citric acid-capped AuNPs were synthesized by a previously reported approach with a few minor changes.[17] Briefly, a 4 mL 1% (w/v) $HAuCl_4$ and 98 mL ultrapure water solution were heated, and then 11.4 mL of 5% (w/v) sodium citrate was rapidly added into the boiling $HAuCl_4$ solution with vigorous stirring. The solution changed color from pale yellow to blue and then to wine. After it had cooled, the synthesized AuNPs were filtered through a 0.22-μm cellulose membrane and stored at 4°C. The wavelength of the AuNPs was scanned in visible light (400–800nm) with a UV-Vis spectrophotometer to obtain the AuNPs' visible light absorption spectrum. Afterward, 10 μL of the prepared AuNPNs solution was added to a copper mesh covered with a carbon film. After the AuNPs were dried, transmission electron microscopy (TEM, Hitachi HT-7700, Japan, 120kV) was used to characterize them and calculate their diameter.

Conjugated HRP and PCV2 Antibody (Anti-PCV2-HRP)

The HRP-labeled anti-PCV2 antibodies (anti-PCV2 replicase antibodies) were prepared using sodium metaperiodate ($NaIO_4$) by using a FastLink HRP Labeling Kit (KA1551, Abnova, Taipei, China) as per the manufacturer's instructions and the method described previously.[18]

Preparation of the Ab (HRP)-AuNPs Probe

The Ab (HRP)-AuNPs probe was prepared by following a published procedure with a minor modification.[19,20] Briefly, 10 μL of the freshly prepared Ab (HPR) solution at a concentration of 100 μg/mL was added to 200 μL of the AuNPs solution (containing 0.04% trisodium citrate, 0.26 mM K_2CO_3, and 0.02% sodiumazide, pH = 9.0) under agitation, followed by gentle mixing and incubation at room temperature for 1 hour. Then, 220 μL of 2% BSA solution was added while stirring for 30 minutes at room temperature for blocking. The mixture was then centrifuged at 15,000 rpm for 20

minutes at 4°C. The precipitate was washed with a BR buffer (containing 1% BSA and 0.05% Tween 20) and resuspended in 220 μL of 1% BSA. The obtained Ab (HRP)-AuNPs probe can be used directly or stored at 4°C for months.

Catalytic Activity of Ab (HRP)-AuNPs

To measure the catalytic activity of the probe, a 10 μL Ab (HPR) solution (100 μg/mL) was added to 220 μL of 2% BSA solution, and a 200 μL AuNPs solution was added to 220 μL of 2% BSA solution. Then, the two mixtures were centrifuged at 15,000 rpm for 20 min at 4°C. The precipitate was washed with a BR buffer (containing 1% BSA and 0.05% Tween 20) and resuspended in 220 μL of 1% BSA, respectively. Then, 100 μL of the freshly prepared Ab (HRP) solution, AuNPs solution, and Ab (HRP)-AuNPs probe were collected (2.3 section) into three micro-wells of 96-well plates and incubated at 37°C for 1 hour, followed by washing three times. Then, the TMB enzyme substrate (100 μL) was added to each well. After 15 minutes of incubation at room temperature, the enzymatic reaction was stopped with 50 μL of a stop solution (2 M H_2SO_4), and the reaction product was quantified at A450 (OD450) by using an automatic plate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The data were measured in triplicate, and the mean OD450 value and standard deviation for each sample were calculated.

Procedure of the Amplified ELISA

In this method, 100 μL of the anti-PCV2 capsid antibodies (10 μg/mL) in a coating buffer was immobilized in each well and incubated at 4°C overnight. The solutions were discarded and washed three times to remove the unbound antibodies. Then, 100 μL of the blocking buffer (5% BSA) was injected into the wells and kept at 37°C for 2 hours, thereby blocking the nonspecific binding sites. The wells were washed again, and 100 μL of the PCV2 in the PBS was added to the wells. The plate was kept at 37°C for 1 hour and washed three times. Later, 100 μL of the Ab (HRP)-AuNPs probe was added to each well and incubated at 37°C for 1 hour, followed by three times washing. Finally, TMB enzyme substrate (100 μL) was added to each well. After 15 minutes of incubation at room temperature, the enzymatic reaction was stopped with 50 μL of a stop solution (2 M H_2SO_4), and the reaction product was quantified at A450 (OD450) by using an automatic plate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The data were measured in triplicate, and the mean OD450 value and standard deviation for each sample were calculated.

Results and Discussion

Basic Principles of AuNPs-Based Signal Amplification of ELISA for PCV2 Detection

The traditional ELISA immobilizes the PCV2 antibody on the surface of a solid phase carrier as a capture probe for PCV2, and the addition of the HRP-labeled PCV2 antibody to the antigen–antibody complex formed a specific sandwich immune structure. After the enzymatic substrate is added, a color reaction occurs, and quantitative detection of PCV2 antigen can be achieved by measuring the absorbance value of the product. Since protein molecules are easily adsorbed on the surface of gold particles without affecting the performance of the protein, we coated the HRP enzyme-labeled PCV2 antibodies on the surface of AuNPs, forming an Ab (HRP)–AuNPs complex by simple adsorption so that a single AuNP can support multiple enzyme labeled antibodies. We used the Ab (HRP)–AuNPs probe to replace the Ab (HRP) with a traditional ELISA method. As a single AuNP can efficiently couple multiple Ab (HRP) around its surface, the introduction of AuNPs revealed signal amplification of PCV2 antigen detection and improved the performance and sensitivity of the detection method.

Characterization of the Ab (HRP)–AuNPs Probe

To characterize the formation of the Ab (HRP)–AuNPs probe complex, we employed a variety of validation methods. The transmission electron microscopy displayed that the AuNPs had a uniform particle size about 16 nm in diameter and were slightly aggregated. After they were coated with Ab (HRP), the AuNPs demonstrated no change in particle size but exhibited better dispersion (Figure 1A), which suggests that protein adsorption is beneficial to the stability of AuNPs. Based on the results of dynamic light scattering (DLS) (Figure 1B), the hydrated particle size of the unmodified AuNPs

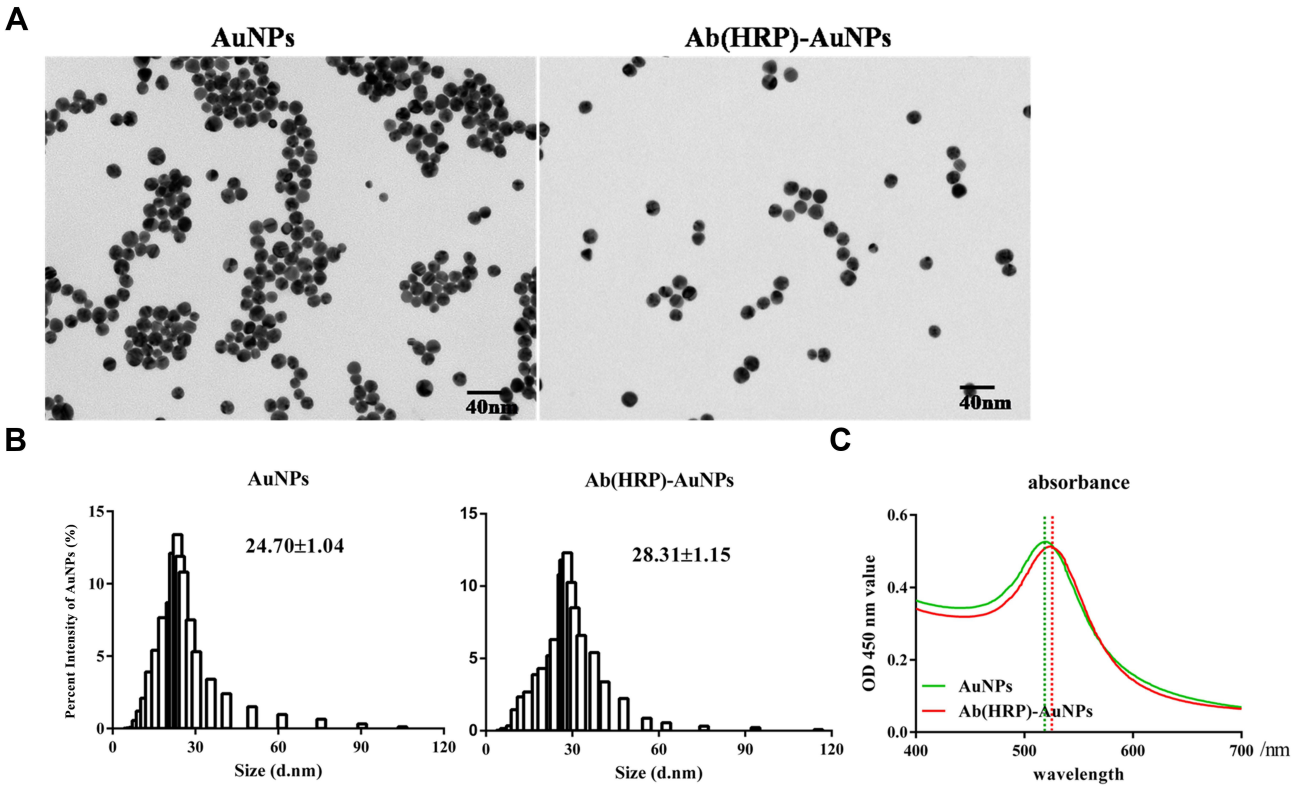

Figure 1 Characterization of AuNPs and Ab (HRP)-AuNPs complex. (**A**) Transmission electron microscopy (TEM) images of AuNPs and Ab (HRP)-AuNPs complex; (**B**) Dynamic light scattering (DLS) measurements of the average hydrodynamic diameters of AuNPs and Ab (HRP)-AuNPs complex; (**C**) UV-vis spectra of AuNPs and Ab (HRP)-AuNPs complex.

was 24.70 ± 1.04 nm. After they were coated with the protein, the hydrated particle size of the composite increased to 28.31 ±1.15 nm, further indicating that Ab (HRP) were bound to the surface of the AuNPs. The UV-vis absorption spectrum assay in Figure 1C demonstrates that the maximum absorption peak of the unmodified AuNPs is at 519 nm and that the probe complex, the AuNP coupled with HRP-labeled antibodies, exhibited the maximum absorption peak at 524 nm. Studies have reported that when AuNPs interact with the surrounding medium or when their particle size changes, it causes a redshift in the position of the absorption peak.[21] Therefore, it is determined that Ab (HRP) and AuNPs are mutually combined. Based on the experimental data of the above three methods, the Ab (HRP)–AuNPs probe complex was built successfully.

Characterization of the Catalytic Activity of Ab (HRP)–AuNPs Probe

To confirm the catalytic activity of the probe complex, the formation of blue products through the catalysis of TMB by AuNPs, Ab (HRP), and Ab (HRP)–AuNPs complex was investigated. As shown in Figure 2A, when the Ab (HRP)–AuNPs complex was added, the TMB substrate changed from colorless to blue and revealed an obvious absorption peak at 644 nm. When the acid was used to stop the reaction, the solution color changed from blue to yellow and revealed an absorption peak at 450 nm. This response followed the same trend as the HRP catalyzed reaction. At the same time, no color change was detected in individual AuNPs catalyzed reactions. These data suggest that the Ab (HRP)–AuNPs complex possesses the same catalytic activity as Ab (HRP) does.

Additionally, it has been reported that AuNPs coated with citric acid show peroxidase-like catalytic activity.[22] To investigate whether the system has a synergistic effect with HRP, the catalytic ability of AuNPs, Ab (HRP) and Ab (HRP)-AuNPs probes were compared at the same concentration. Figure 2B further confirms that the catalytic activity of AuNPs coated with citric acid was extremely low and when Ab (HRP) was adsorbed on the surface, the synergistic effect did not manifest, but the catalytic ability decreased slightly, which may be due to the loss during early centrifugation.

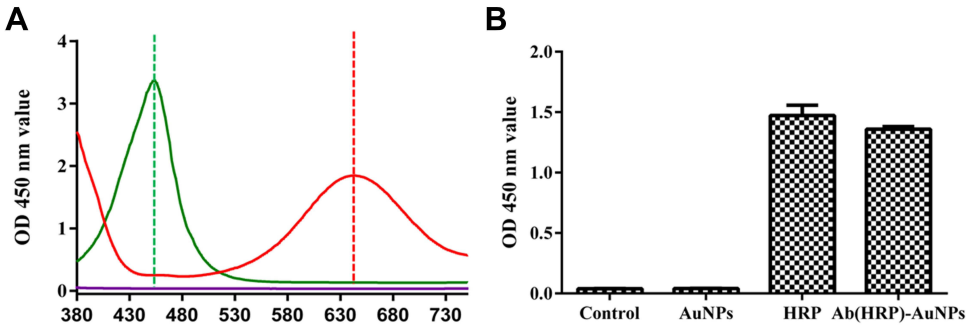

Figure 2 Confirmation of the catalytic function of Ab (HRP)-AuNPs complex. (**A**) The color reaction of TMB catalyzed by the Ab (HRP)-AuNPs complex in the presence of H_2O_2 (the purple line is the absorption spectrum of the reaction solution without the presence of Ab (HRP)-AuNPs complex; the red and green line are the absorption spectra of the reaction solution mixed with Ab (HRP)-AuNPs complex before and after addition of H_2SO_4 respectively). (**B**) Comparison of the catalytic activity of 0.95 nM pure AuNPs, 1.09 μg/mL pure HRP and Ab (HRP)-AuNPs complex (prepared from 0.95 nM AuNPs and 1.09 μg/mL HRP).

This decreased catalytic ability does not affect its signal amplification in immunoassays. The results showed that the AuNPs play the role of carriers in this system. The slight decrease in catalytic ability, caused by the coupling of multiple enzyme-labeled antibodies, can be ignored ($P > 0.05$).

Establishment and Evaluation of Enhanced Amplification Detection Method

To avoid non-specific adsorption of other proteins by AuNPs, BSA was used to block the remaining active sites of the Ab (HRP)–AuNPs complex in this experiment. By adjusting the amount of the BSA in the blocking buffer, it was found that the background signal decreased as the amount of BSA decreased until a plateau appeared (Figure 3A). Considering the detection signal, we chose 2% BSA as the optimal blocking concentration that demonstrated the highest signal-to-noise value.

Additionally, we also optimized the concentration of the Ab (HRP)–AuNPs complex in the immunoassay (Figure 3B). Without PCV2, the undiluted complex causes a higher background signal. With the increased dilution ratio of the probe complex, the PCV2 detection signal decreased, demonstrating its dependency on the dose. When the dilution reached 1000 times, the detection signal was very low. To balance sensitivity and specificity, the 100-fold dilution concentration was selected as the optimal concentration of the probe. At the above dilution, the OD450nm value was close to 1.0, and the P/N was up to the maximum.

Based on these data, 80 negative samples, including 40 PK-15 cell culture supernatant and 40 tap water samples collected in the laboratory, were statistically analyzed by this method. The average value was X = 0.165, the standard deviation was 0.022, and X + 3 SD = 0.231, and X + 2 SD = 0.209. Therefore, the sample OD450 < 0.200 was identified as negative in this method, and OD450 > 0.231 was considered a positive sample.

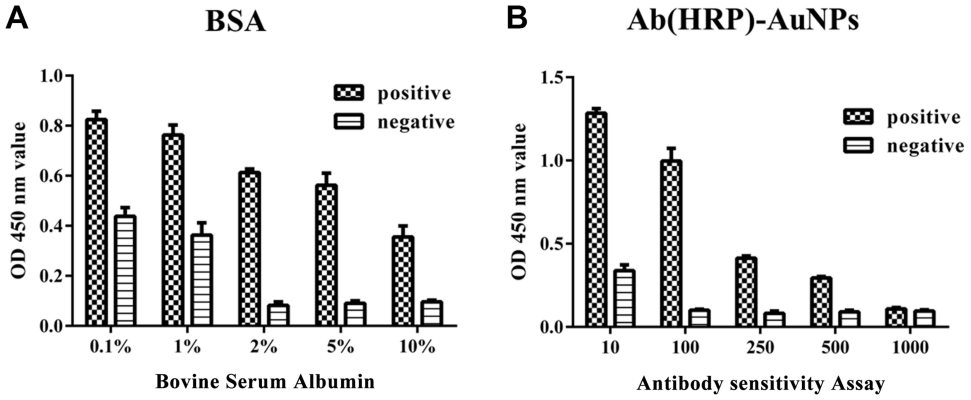

Figure 3 Optimization of the assay conditions. (**A**) Optimization of BSA concentration for blocking the nonspecific binding sites. (**B**) Optimization of the concentration of the Ab (HRP)-AuNPs complex to be used in the ELISA assay.

Evaluation of the AuNPs Amplified Detection Method

Based on the optimized procedures, the AuNPs amplified immunoassay with PCV2 detection was performed in terms of specificity, repeatability, linearity, and limit of detection (LOD) analysis. For the linearity, a series of concentration (from 25,000 $TCID_{50}$/mL, 12,500 $TCID_{50}$/mL, 6250 $TCID_{50}$/mL, 3125 $TCID_{50}$/mL, 1562.5 $TCID_{50}$/mL, 781.3 $TCID_{50}$/mL, 390.6 $TCID_{50}$/mL, 195.3 $TCID_{50}$/mL, 97.7 $TCID_{50}$/mL) of PCV2 standards were analyzed. The analyses demonstrated a good linear relationship between PCV2 and absorbance (Figure 4A) in the range of 25,000–195 $TCID_{50}$/mL. The regression equation of the line was Y = 0.729 ln(X)-3.872 with a correlation coefficient of 0.989, and the OD was 195 $TCID_{50}$/mL.

To explore cross-reactivity with other porcine pathogens, the formation of the immuno-complex in the presence of PCV2, PCV1, PRRSV, PRV, CSFV, PEDV, PCV3, and PPV positive cell culture was detected. As seen in Figure 4B, no positive signal was detected except in the presence of PCV2, which suggests that the developed AuNPs amplified immunoassay is specifically applied for the detection of PCV2.

Additionally, Cap protein is widely used as the antigen protein for the commercial PCV2 detection kit. However, these kits can only detect Cap antibodies. Therefore, diagnosing PCV2 infections in vaccinated populations based on the existing diagnostic criteria becomes very difficult. With the worldwide use of whole-virus inactivated PCV2 vaccines and PCV2 Cap subunit vaccines, an ELISA against the proteins expressed during infections that are not present in vaccine compositions could be used to distinguish between infected and vaccinated animals.[5] Therefore, we employed Rep antibodies, which are non-structural proteins encoded by PCV2 ORF1 for virion capture to develop a sandwich ELISA.

The repeatability was evaluated by testing three positive samples at different times and under the same conditions, and the coefficient of variation of the value was calculated. The results in Table 1 show that the intra-assay coefficient of variation and the inter-assay coefficient of variation of three samples were between 4.65% and 6.84%, which suggested that the assay exhibited repeatability.

Application of the Detection in Clinical Samples

Four types of samples, including lymphatic tissues, groundwater, and treated and untreated water sources, from six suspected PCV2 infected pig farms were collected. Each type contained three samples, which totaled seventy-two samples. These samples were examined using an enhanced ELISA assay. At the same time, a PCR assay was employed to verify the enhanced ELISA data. We have compared the data from the two different detection methods in Table 2. The results of the application of the enhanced ELISA on these samples correlated well with the results of the PCR assay. The samples from the six pig farms that were PCR negative also tested negative for the enhanced ELISA. Similarly, in the infected pigs with the highest levels of PCV2 in the lymphatic tissues, the results of all of the tests were in agreement.

Under the above optimal experimental conditions, an enhanced enzyme immunoassay method based on AuNPs was established. A traditional ELISA can bind only one enzyme molecule to a virus antigen; however, when AuNPs are used

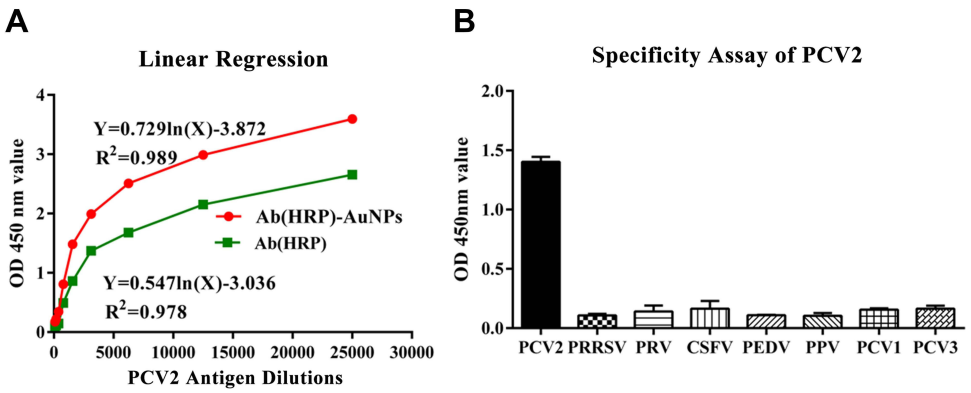

Figure 4 Linear analysis and specificity of Ab (HRP)-AuNPs probe based detection assay. (**A**) Linear analysis of the detection assay. (**B**) The specificity of the proposed enhanced immunoassay for PCV2.

Table 1 Repeatability Test

Sample	A	B	C
Coefficient variation of inter-assay	4.65	5.13	4.89
Coefficient variation of intra-assay	6.35	6.84	5.71

Table 2 Detection of Virus in Clinical Samples

Positive/Negative / Sample	ELISA	PCR
Lymphatic tissues	17/1	17/1
Groundwater	4/14	4/14
Treated water	2/16	2/16
Untreated water	5/13	5/13

as carriers due to their high specific surface area, multiple enzyme molecules can be carried at the same time to enhance the immunoassay signal.[23] When the results of the traditional ELISA and the AuNPs enhanced immunoassay are compared, it can be seen that the signal improved significantly after the AuNPs were used as carriers of the enzyme-labeled antibodies.[24,25] As the virus concentration increased, the color of the substrate solution gradually darkened, and within this range, there was almost no obvious color change in the traditional ELISA. It should be noted that the traditional ELISA analysis generally takes 15–30 minutes with a limit of detection of $10^{4.}$ $TCID_{50}$/mL, while the AuNPs-enhanced immunoassay is performed with a limit of detection of 195 $TCID_{50}$/mL PCV2 within 15 minutes.[26–28] Therefore, in the early diagnosis of PCV2 infection, the high sensitivity and rapid color development of this method will have significant advantages.

Conclusion

We developed a novel, highly sensitive sandwich ELISA utilizing AuNPs in a functional specific antibody-labeled probe for the detection of PCV2. It was demonstrated that the AuNPs were used as carriers of HRP-labeled anti-PCV2 antibodies, which could enhance the performance of the immunoassay and achieve higher sensitivity and specificity, especially with a 195 $TCID_{50}$/mL detection limit of PCV2 antigen. Moreover, PCV2 antigens in environmental samples were also effectively detected using the enhanced immunoassay. In summary, the amplified Ab (HRP) labeled AuNPs probe-based immunoassay stands for high specificity, easy operation, and sensitive assessment for the rapid detection and reproducibility of monitoring the PCV2 in the environmental samples at an earlier stage.

Data Sharing Statement

The data sets and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflict of interest.

References

1. Rose N, Opriessnig T, Grasland B, Jestin A. Epidemiology and transmission of porcine circovirus type 2 (PCV2). *Virus Res*. 2012;164(1–2):78–89. doi:10.1016/j.virusres.2011.12.002
2. Lopez-Lorenzo G, Diaz-Cao JM, Prieto A, et al. Environmental distribution of porcine circovirus type 2 (PCV2) in swine herds with natural infection. *Sci Rep*. 2019;9(1):14816. doi:10.1038/s41598-019-51473-6
3. Meng XJ. Porcine circovirus type 2 (PCV2): pathogenesis and interaction with the immune system. *Annu Rev Anim Biosci*. 2013;1:43–64. doi:10.1146/annurev-animal-031412-103720
4. Vargas-Bermudez DS, Vargas-Pinto MA, Mogollon JD, Jaime J. Field infection of a gilt and its litter demonstrates vertical transmission and effect on reproductive failure caused by porcine circovirus type 3 (PCV3). *BMC Vet Res*. 2021;17(1):150. doi:10.1186/s12917-021-02862-5
5. Wang Y, Noll L, Lu N, et al. Genetic diversity and prevalence of porcine circovirus type 3 (PCV3) and type 2 (PCV2) in the Midwest of the USA during 2016–2018. *Transbound Emerg Dis*. 2020;67(3):1284–1294. doi:10.1111/tbed.13467
6. Garcia LA, Viancelli A, Rigotto C, et al. Surveillance of human and swine adenovirus, human norovirus and swine circovirus in water samples in Santa Catarina, Brazil. *J Water Health*. 2012;10(3):445–452. doi:10.2166/wh.2012.190
7. Borkenhagen LK, Mallinson KA, Tsao RW, et al. Surveillance for respiratory and diarrheal pathogens at the human-pig interface in Sarawak, Malaysia. *PLoS One*. 2018;13(7):e0201295. doi:10.1371/journal.pone.0201295
8. Gilliland SM, Forrest L, Carre H, et al. Investigation of porcine circovirus contamination in human vaccines. *Biologicals*. 2012;40(4):270–277. doi:10.1016/j.biologicals.2012.02.002
9. Huang L, Wei Y, Xia D, et al. A broad spectrum monoclonal antibody against porcine circovirus type 2 for antigen and antibody detection. *Appl Microbiol Biotechnol*. 2019;103(8):3453–3464. doi:10.1007/s00253-019-09715-0
10. Segales J. Porcine circovirus type 2 (PCV2) infections: clinical signs, pathology and laboratory diagnosis. *Virus Res*. 2012;164(1–2):10–19. doi:10.1016/j.virusres.2011.10.007
11. Yin S-H, Yang S-L, Tian H, et al. An ELISA based on a truncated soluble ORF2 protein for the detection of PCV2 antibodies in domestic pigs. *Virol Sin*. 2010;25(3):191–198. doi:10.1007/s12250-010-3085-5
12. Pileri E, Cortey M, Rodríguez F, Sibila M, Fraile L, Segalés J. Comparison of the immunoperoxidase monolayer assay and three commercial ELISAs for detection of antibodies against porcine circovirus type 2. *Vet J*. 2014;201(3):429–432. doi:10.1016/j.tvjl.2014.05.025
13. Ambrosi A, Airo F, Merkoci A. Enhanced gold nanoparticle based ELISA for a breast cancer biomarker. *Anal Chem*. 2010;82(3):1151–1156. doi:10.1021/ac902492c
14. Chu X, Fu X, Chen K, Shen GL, Yu RQ. An electrochemical stripping metalloimmunoassay based on silver-enhanced gold nanoparticle label. *Biosens Bioelectron*. 2005;20(9):1805–1812. doi:10.1016/j.bios.2004.07.012
15. Wang WG, Li JM, Dong CZ, et al. Ultrasensitive ELISA for the detection of hCG based on assembled gold nanoparticles induced by functional polyamidoamine dendrimers. *Anal Chim Acta*. 2018;1042:116–124. doi:10.1016/j.aca.2018.08.038
16. Liu Y, Zhang L, Wei W, et al. Colorimetric detection of influenza A virus using antibody-functionalized gold nanoparticles. *Analyst*. 2015;140 (12):3989–3995. doi:10.1039/C5AN00407A
17. Freeman RG, Grabar KC, Allison KJ, et al. Self-assembled metal colloid monolayers: an approach to SERS substrates. *Science*. 1995;267 (5204):1629–1632. doi:10.1126/science.267.5204.1629
18. Camargo IF, Gaspar AM, Yoshida CF. Comparative ELISA reagents for detection of hepatitis B surface antigen (HBsAg). *Mem Inst Oswaldo Cruz*. 1987;82(2):181–187. doi:10.1590/S0074-02761987000200005
19. He Y, Zhang S, Zhang X, et al. Ultrasensitive nucleic acid biosensor based on enzyme-gold nanoparticle dual label and lateral flow strip biosensor. *Biosens Bioelectron*. 2011;26(5):2018–2024. doi:10.1016/j.bios.2010.08.079
20. Ruiz G, Tripathi K, Okyem S, Driskell JD. pH impacts the orientation of antibody adsorbed onto gold nanoparticles. *Bioconjugate Chem*. 2019;30 (4):1182–1191. doi:10.1021/acs.bioconjchem.9b00123
21. Jain PK, Lee KS, El-Sayed IH, El-Sayed MA. Calculated absorption and scattering properties of gold nanoparticles of different size, shape, and composition: applications in biological imaging and biomedicine. *J Phys Chem B*. 2006;110(14):7238–7248. doi:10.1021/jp057170o
22. Long YJ, Li YF, Liu Y, Zheng JJ, Tang J, Huang CZ. Visual observation of the mercury-stimulated peroxidase mimetic activity of gold nanoparticles. *Chem Comm*. 2011;47(43):11939. doi:10.1039/c1cc14294a
23. Wu L, Yin W, Tang K, et al. Highly sensitive enzyme-free immunosorbent assay for porcine circovirus type 2 antibody using Au-Pt/SiO 2 nanocomposites as labels. *Biosens Bioelectron*. 2016;82:177–184. doi:10.1016/j.bios.2016.04.001
24. Ge M, Li RC, Qu T, Gong W, Yu XL, Tu C. Construction of an HRP-streptavidin bound antigen and its application in an ELISA for porcine circovirus 2 antibodies. *AMB Express*. 2017;7(1):177. doi:10.1186/s13568-017-0473-3

25. Han S, Xiao Y, Zheng D, et al. Establishment and application of a competitive enzyme-linked immunosorbent assay differentiating PCV2 antibodies from mixture of PCV1/PCV2 antibodies in pig sera. *BMC Vet Res*. 2016;12(1):175. doi:10.1186/s12917-016-0802-9
26. McNeilly F, McNair I, Mackie DP, et al. Production, characterisation and applications of monoclonal antibodies to porcine circovirus 2. *Arch Virol*. 2001;146(5):909–922. doi:10.1007/s007050170124
27. McNeilly F, McNair I, O'Connor M, et al. Evaluation of a porcine circovirus type 2-specific antigen-capture enzyme-linked immunosorbent assay for the diagnosis of postweaning multisystemic wasting syndrome in pigs: comparison with virus isolation, immunohistochemistry, and the polymerase chain reaction. *J Vet Diagn Investig*. 2002;14(2):106–112. doi:10.1177/104063870201400203
28. Yang L, Zhang H, Chen J, Qiao X, Hou J, Zheng QS. Establishment of a sandwich ELISA for quantitative determination on porcine circovirus type 2b. *Chin J Prev Vet Med*. 2017;39(12):987–992.