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Establishment of Colloidal Gold Immunochromatographic Assay for RHDV Antibody

Lijun Zhou[1], Yilin Luo[1], Xiang Wang[1], Yong Song[2], Xulong Wu[1], Pengfei Zhang[1], Changyu Liao[1], Ruijiao Jiang[1], Xueping Yao[1], Yan Luo[1], Yin Wang[1], Yi Geng[1] and Zexiao Yang*

[1]Sichuan Agricultural University, Chengdu, China
[2] Tianjin Ringpu Biotechnology Co., Ltd, Tianjin, China
Corresponding author e-mail: yzxyang2003@126.com

Abstract. This study aims to establish a rapid detection method for antibody against rabbit hemorrhagic disease virus (RHDV) by using immunochromatography technology. The colloidal gold-labeled recombinant VP60 protein, anti-VP60 polyclonal antibody and goat anti-rabbit IgG were coated on quality control line (C line) and detection line (T line). The test strip preparation conditions were optimized by detecting positive serum and negative serum. The results showed that the T-line and C-line were all red when tested positive serums. But only the C-line was red on the negative strips, the T-line was not colored, the false positive rate and the false negative rate were all zero percent. The specificity, sensitivity and reproducibility tests results all indicated that the colloidal gold strip is a powerful testing tool for the epidemiological investigation and detection of the antibody level on viral hemorrhagic disease in rabbits.

1. Introduction

Rabbit hemorrhagic disease virus (RHDV) infection mainly causes an acute, severe, highly contagious and highly dead disease in rabbits. It may cause hemorrhage and congestion of multiple organs in rabbits. The virus was first reported in Jiangsu in 1984, and then spread to most parts of the country [1-3]. Under natural conditions, RHDV have a short incubation period, rapid spread and frequent epidemic, causing enormous economic losses to the rabbit industry [4]. RHDV is a single-stranded positive-strand RNA virus, and VP60 is the major structural protein of the virus, which relate to the antigenicity of RHDV. It can self-polymerize into virus-like particles in vitro, which is no different from natural RHDV virions in morphology and antigenicity [5]. The ELISA method using recombinant VP60 protein as coating antigen to detect RHDV antibody had high specificity and sensitivity and avoided the risk of direct viral dispersion [6]. Currently, the detection of RHDV antibodies mainly used hemagglutination inhibition test (HI) [7], indirect ELISA and competition ELISA [8]. But these methods were cumbersome, required professional equipment and technicians, and are not suitable for grassroots promotion. Therefore, it is particularly important to establish a method for rapid and accurate detection of RHDV antibodies. Colloidal gold test strips have been widely studied and applied in animal disease monitoring and food safety [9-12]. They are fast and very suitable for clinical applications. This study used purified VP60 protein as a gold-labeled antigen with good specificity and biosafety. On the NC membrane, the C-line and T-line were coated with anti-RHDV VP60 polyclonal antibody and goat anti-rabbit IgG, separately. The result can be judge based on the colors development of the T line and the C line. The test strip can be used for serum detection, and also can provides a rapid and convenient method for epidemiological investigation and

detection of viral hemorrhagic disease in rabbits.

2. Materials and Methods

2.1. Main Reagents and Materials
Recombinant VP60 protein was expressed by prokaryotic expression system, anti-RHDV VP60 recombinant protein polyclonal antibody, RHDV positive serum, RHDV negative serum, rabbit *Pasteurella multocida* serum, rabbit *B. bronchiseptica* serum, rabbit Clostridium *perfringens* type A bacterial serum was prepared by laboratory; IgG-free bovine serum albumin (BSA) and goat anti-rabbit IgG were purchased from Bioengineering (Shanghai) Co., Ltd.; PEG2000, K_2CO_3, chloroauric acid and trisodium citrate were purchased from Chengdu Haoboyou Technology Co., Ltd.; Commercial rabbit hemorrhagic tissue inactivated vaccine was purchased from Nanjing Tianbang Technology Co., Ltd; The nitrocellulose membrane, the sample pad, the gold standard pad, the absorbent paper, and the support plate were purchased from Beijing Jisen Biotechnology Co., Ltd.

2.2. Preparation of Colloidal Gold
Colloidal gold particles with a diameter of 20 nm was prepared by trisodium citrate method.

2.3. Preparation of Colloidal Gold Test Strips

2.3.1. Selection of optimum pH value for colloidal gold label VP60
1 mL colloidal gold solution and 0.2 mol/L K_2CO_3 solution were added to 9 tubes. The pH of the solution was adjusted to 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9 respectively. 40 µL 1mg/mL recombinant VP60 protein was added to each tube. After mixing for 30 min, 50 µL of 10% BSA was added and mixed at 4 °C for 1 h. The corresponding pH value of the red test tube is the optimum pH value of the colloidal gold labeled recombinant VP60 protein.

2.3.2. Selection of the optimum protein amount for colloidal gold labeling
According to the pH value determined above, 1 mL colloidal gold of the optimum pH value was added to a 1.5 mL centrifuge tube. And the recombinant VP60 protein was sequentially added to the above gold gum solution to have a concentration of 5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL, 25 µg/mL, 30 µg/mL, 35 µg/mL, 40 µg/mL. Only 20 µL of the dilution was added to the control tube. After standing at 4 °C for 1 h. The lowest concentration of the protein, which the colloidal gold solution remains unchanged was used as the lowest stable protein content.

2.3.3. Recombinant VP60 Protein Gold Label
According to the above method, the colloidal gold with higher stability coefficient and optimum pH value was prepared. The colloidal gold solution and the recombinant protein were mixed gently. 50 µL 10% BSA and 50µL 20% PEG20000 were added to colloidal gold stable protective solution, standing at 4 °C for 1 h. The solution was centrifuged at 4 °C for 30 minutes at 10000r/min. The supernatant was absorbed carefully. The gold standard protein was precipitated and diluted with a stable solution.

2.4. Assembly of Test Strips
The sample pad, the gold standard pad, the nitrocellulose film, and the absorbent paper were sequentially adhered to the PVC substrate and cut into a 4 mm wide test strip by a slitter.

2.5. Test Strip Condition Optimization

2.5.1. Concentration selection of T line and C line
The goat anti-rabbit IgG was diluted to 1:2, 1:4, 1:6, 1:8, 1:10, separately and labeled the T line. The other conditions were unchanged. The positive rabbit anti-RHDV antibody was added to the PVC substrate. The rabbit anti-recombinant protein VP60 antibody was diluted to 1:2, 1:4, 1:6, 1:8, 1:10 and labeled the C line. Other conditions are unchanged. PBS solution was added to observe the test

results.

2.6. Sensitivity and Specificity of Colloidal Gold Test Strips

The gold standard test strips were prepared with optimized conditions. The sensitivity testing was performed by the positive anti-RHDV antibody, which was diluted with physiological saline by 1:2, 1:4, 1:8, 1:16, 1:32. Specific tests were performed using negative serum, Rabbit *Pasteurella multocida* serum, *B. bronchiseptica* serum, Rabbit *Clostridium perfringens* type A serum. The result was judged 10 minutes later.

2.7. Repeatability

10 samples were taken from each the 3 batches of test strips to detect positive and negative serum samples. And the test strip test results were observed.

2.8. Preliminary Application of Test Strips

5 serum samples and 100 serum samples of experimentally immunized RHDV inactivated vaccines were tested using gold standard test strips.

3. Results

3.1. Preparation of Colloidal Gold

The colloidal gold particles were prepared by the trisodium citrate method. The results showed that the colloidal gold solution was burgundy. The measurement by ultraviolet spectrophotometer showed that the prepared colloidal gold had a wavelength of 527 nm and the peak shape was narrow indicating that the colloidal gold particles were uniform and good dispersion.

3.2. Colloidal Gold Labeling Recombinant VP60 Protein Optimal pH Value and Optimal Protein Amount

The optimal pH value selection test of colloidal gold-labeled recombinant VP60 protein showed that the solution remained red when the pH value of it was 7.5. This pH value was the optimum pH value of labeled recombinant VP60 protein. The selection test results of the optimal protein amount of colloidal gold label showed that adding at least 20 μL of 1 mg/mL recombinant VP60 protein per ml of colloidal gold solution can keep the solution red. Increase 15% to 30% on this basis, 23~26 μL 1mg/mL recombinant VP60 protein was the optimal protein amount.

3.3. Test Strip Results

The gold standard recombinant VP60 protein was resuspended. The goat anti-rabbit IgG was diluted 4 times, and the rabbit anti-recombinant protein VP60 antibody was diluted 6 times. The prepared test strips were tested for positive and negative samples. The results are shown in Figure 1.
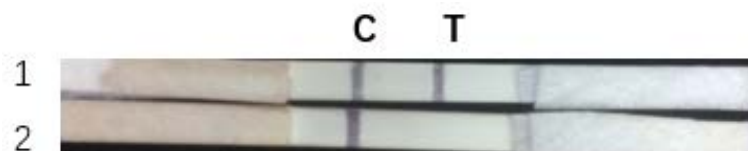

Figure 1. Test strip test negative and positive serum results
1: Positive serum. 2: Negative serum

3.4. Sensitivity Test of Colloidal Gold Test Strips

The results of the strip sensitivity test showed that the test strip can directly detect the positive sample. When the dilution was 1:32, the test line was visible, indicating that the test strip is sensitive. The results are shown in Figure 2.
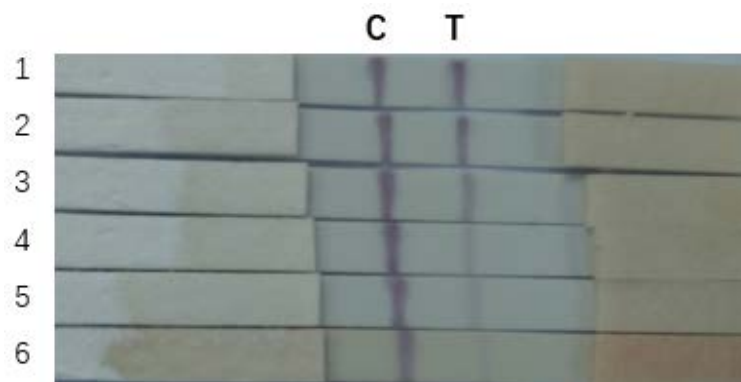
Figure 2. Sensitivity test result
1-6: Positive serum dilutions are 1:2, 1:4, 1:8, 1:16, 1:32, 1:64

3.5. Specificity Test of Colloidal Gold Test Strips

The results of the strip specific test showed that the positive serum and RHDV negative serum of *Pasteurella multocida*, B. *bronchiseptica*, rabbit *Clostridium perfringens* type A were negative, and the results of RHDV positive serum test were Positive, indicating that the test strip has good specificity. The results are shown in Figure 3.
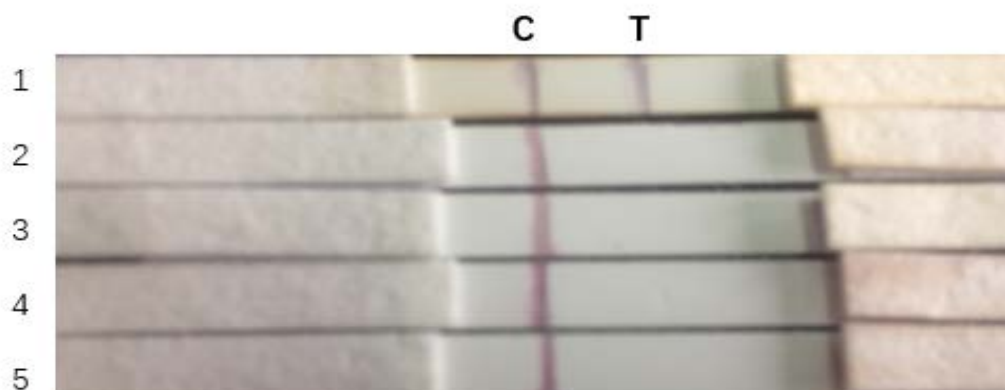

Figure 3. Specificity test result
1-5: RHDV-positive serum, RHDV-negative serum, rabbit *Pasteurella multocida* serum, *B. bronchiseptica* serum, rabbit Clostridium *perfringens* type A serum

3.6. Repeatability Test

3 batch test strips tested positive and negative results were consistent, indicating that the test strips have good repeatability and no error.

3.7. Initial Application of Test Strips

The clinical test of immunocolloidal gold test strips was carried out by using 5 samples of RHDV antibodies, 5 samples of RHDV-negative sera, and 100 clinical samples, and using a rabbit prion IgG antibody ELISA kit parallel test. The results showed that the sera of the 5 test immunized RHDV inactivated vaccines were all positive, 5 negative sera were negative, 100 clinical samples were positive, and the coincidence rate with the commercial kit was 100%.

4. Conclusion

Rabbit viral hemorrhagic infection has a short incubation period, short onset of acute disease, high infection rate, high mortality rate, rapid spread, and more explosive epidemics, causing serious economic losses and threats to the rabbit industry [4]. Currently, vaccination is mainly used to control

and prevent the disease. The detection methods mainly include hemagglutination inhibition test and ELISA [5,8], but these methods are time consuming and cumbersome. Therefore, it is particularly important to detect rabbit viral hemorrhagic antibodies quickly and accurately. Immunochromatography is an immunoassay that combines the advantages of chromatography and immunotechnology. It is fast and accurate, and is suitable for the operation requirements of grassroots veterinarians and farm technicians.

In this test, the goat anti-rabbit IgG was diluted 4 times, and the rabbit anti-recombinant protein VP60 antibody was diluted 6 times. Colloidal gold-labeled recombinant protein was gold standard pad, rabbit anti-recombinant protein polyclonal antibody was labeled on C-line, goat anti-rabbit IgG labeled on T-line. The test results were accurate. The immunocolloidal gold test strips for RHDV antibody detection was initial established. 5 serum samples of experimental immunization, 5 serum samples of non-immune negative antibody and 104 serum samples of clinical immunization were tested. The results showed that the colloidal gold test strip has good sensitivity and specificity, and its sensitivity can reach 1:16. Specific detection showed red bands on T-line in rabbit anti-RHDV serum. There were no color bands on the T line in serum of *Clostridium perfringens* type A and rabbits such as *Pasteurella bacillus*. All 5 positive samples were positive and 5 non-immunized sera were negative. 104 clinical samples were tested by designed RHDV antibody test strips. The results showed that 100 were positive and 4 were negative. This study provided a useful immunoassay strip for RHDV antibody level monitoring.

5. Acknowledgements

6. References

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