Research Note: A novel peptide-based ELISA for efficient detection of antibody against chicken infectious anemia virus

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ABSTRACT Chicken infectious anemia virus (**CIAV**) is the pathogen of chicken infectious anemia. Currently, due to the lack of effective diagnostics technology and prevention approach, CIAV has spread globally and caused huge economic losses to poultry industry. In this study, a novel peptide-based ELISA (**pELISA**) for efficient detection of antibody against CIAV was developed. The peptide (25CRLRRRYKFRHRRRQRYRRRAF45) used in pELISA was highly conserved in VP1 protein of different CIAV isolates. The specificity and reproducibility showed that the pELISA only reacted with sera against CIAV, not with sera against other pathogens tested, and the CV of the intra-/inter-assay of the pELISA was 6.8 to 9.22%. Moreover, the comparison assay using 56 clinical samples showed that the positive rate of the pELISA and the commercial ELISA kit (IDEXX) was 85.7 and 80.4%, respectively. The pELISA generated here provides a rapid and efficient serological detection method for diagnosis of CIAV infection and evaluation of the efficacy of CIAV vaccination.

Key words: chicken infectious anaemia virus, peptide, indirect ELISA, CIAV antibodies

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INTRODUCTION

Chicken infectious anemia (**CIA**) is an important poultry disease caused by chicken infectious anemia virus (**CIAV**) (Eltahir et al., 2011). CIAV is a kind of DNA virus carrying a single stranded and circular genome with the size of about 2.3kb, which belongs to genus *Gyrovirus*, the family *Anelloviridae* (Rosario et al., 2017). CIAV transmits vertically and horizontally, and causes aplastic anemia and systemic lymphoid tissue atrophy in the infected chickens. Therefore, CIAV infection significantly affects the regulation of the entire immune system, which results in immunosuppression and secondary infection (Adair, 2000). The genome of CIAV encodes major 3 viral proteins VP1, VP2, and VP3 (Noteborn and Koch, 1995). VP1 is the capsid protein with dominant B cell epitopes. As nonstructural proteins, VP2 and VP3 play critical roles in viral assembly and the pathogenesis. Since its first report in 1979, CIAV has spread globally, and caused big economic losses to the poultry industry (Adair, 2000). Recently, the molecular epidemiology data reveal that CIAV is endemic in chicken farms, particularly in indigenous chicken flocks in China, and significantly restricts the sustainable development of domestic poultry industry. Therefore, the efficient detection approaches for CIAV is critical for better controlling the diseases caused by CIAV. Although several methods such as PCR, neutralization test and indirect immunofluorescence assay (**IFA**) and ELISA have been developed (Todd et al., 1990b; Wanganurakkul et al., 2020), these methods have individual advantages and disadvantages. Among these approaches, the ELISA is mostly applied in detection of the antibody against CIAV (Todd et al., 1990b). However, the antigen source of these ELISA assay was either from the CIAV in cell culture, or from the purified recombinant protein of VP1 (von Bulow, 1988; Todd et al., 1990a, 1990b, 1999; Lee et al., 2011;

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Wanganurakkul et al., 2020). Therefore, the preparation of the antigen for the current ELISA method used for CIAV serological assay is time consuming and costly. To develop an efficient ELISA with a reliable source of antigen, in this study, a synthesized peptide derived from VP1 protein was used as the coating antigen to generate a peptide-based ELISA for detection of antibody against CIAV.

MATERIALS AND METHODS

Viruses and Serum Samples

CIAV Haian strain (**CIAV-HA**) was isolated and stored in our laboratory. The positive sera against CIAV, avian reticuloendotheliosis virus (**REV**), avian infectious laryngotracheitis virus (**ILTV**), avian infectious bronchitis virus (**IBV**), Newcastle disease virus (**NDV**), avian influenza virus H5/H9 subtypes (**AIV-H5/H9**), and avian leukemia virus A/J/K subgroups (**ALV-A/J/K**), respectively, were preserved in our laboratory. The sera of the specific-pathogen-free (**SPF**) chickens were obtained from Harbin Guosheng Biological Co. LTD (Harbin, China). Hundred clinical sera samples were from a chicken flock vaccinated with CIAV. Rabbit sera and sheep sera were purchased from Lanzhou Minhai Biological Engineering Co. LTD (Lanzhou, China). The commercial CIAV Ab ELISA test kit was purchased from IDEXX.

Preparation of Positive Sera Against CIAV

Eleven 3-day-old SPF chicks were inoculated with 500 μL CIAV-HA strain ($10^{5.6}$ $TCID_{50}$) and then immunized every 10 d. The blood samples of the immunized chickens were collected at d 21, 35, 49, 63, 77, 91, and 105 post the first inoculation, and tested for the antibody against CIAV.

Synthesis and Selection of Peptide

The VP1 protein of the classical CIAV strain CUX-1 (GenBank: M55918; Nation: Germany) was analyzed for potential B cell epitopes by using the PROTEAN software in DNAStar. Three peptides with high antigenicity and hydrophilicity were selected and then synthesized either coupled with KLH or without KLH (Synpeptide Co. LTD, Shanghai, China). The sequences of the 3 peptides were listed in Table 1. The reaction profile of the 3 peptides was detected by indirect ELISA.

Table 1. The sequences of the 3 peptides derived from VP1 protein.

Peptides	Sequences	Positions
Peptide 1	CRLRRRYKFRHRRRQRYRRRAF	25−45 aa
Peptide 2	CTRRDDVKYSSDHQNRW	235−250 aa
Peptide 3	CPPGQRSVSRRSFNHHKAR	326−343 aa

Development and Optimization of Peptide-Based ELISA)

The optimal peptide was selected as the coating antigen. The parameters of the peptide-based ELISA (**pELISA**) were optimized as the following. Working dilutions of synthetic peptides (0.0625 μg/mL, 0.125 μg/mL, 0.25 μg/mL, 0.5 μg/mL, 1 μg/mL, 2 μg/mL, 4 μg/mL, 8 μg/mL) were prepared using 0.1 M carbonate/bicarbonate buffer (pH 9.6). The working dilutions were added to an ELISA plate (100 μL/well) and coated at 4°C for 12 h. After incubation with the coating antigen, the plates were washed 2 times with PBST and then incubated with different blocking buffer (300 μL/well) at 37°C for 30 min, 1 h, 2 h, respectively. The blocking buffers consisted of PBST containing 5% rabbit serum, 8% rabbit serum, 1% BSA, 2% BSA, 5% sheep serum, 8% sheep serum, 1% trehalose, and 5% skim milk, respectively. After 3 washes, serum samples were diluted to 1:100, 1:200, 1:300, 1:400, 1:500, 1:600, 1:700, and 1:800 in PBST (100 μL/well) with 5% rabbit serum, and then incubated at 37°C for 30, 45, 60, and 90 min, respectively. Following incubation, the plates were washed 5 times, and incubated at 37°C for 30, 45, 60, and 90 min with rabbit anti-chicken antibody labeled with HRP, which was diluted to 1:10,000, 1:20,000, 1:30,000, 1:40,000, 1:50,000, 1:60,000, 1:70,000, and 1:80,000 in PBST (100 μL/well) with 5% rabbit serum. After washing for 6 times, color development was performed using TMB Single-Component Substrate Solution (100 μL/well) in dark for 5, 10, 15, 20, 25, and 30 min, and the reaction was stopped by addition of 50 μL of 2 M H_2SO_4. The absorbance values at 450 nm (**OD_{450}**) were measured using an ELISA reader. The optimal condition was determined according to the evaluating of OD value and the positive/negative ratio (**P/N**) of the samples. The cut-off of the ELISA was calculated by the average value (X) of OD_{450} and standard deviation (**SD**) of 77 sera of SPF chickens.

Detection of Serum Samples by IDEXX ELISA Kit

A total of 56 serum samples were randomly selected from the 100 serum samples obtained from the chicken flock vaccinated with CIAV for detection of antibody against CIAV by using IDEXX ELISA Kit. According to the evaluation standard, the OD_{650} value of the sample was measured. If the S/N was more than 0.6, the sample was judged as negative, otherwise it was positive.

RESULTS AND DISCUSSION

To develop a rapid and convenient diagnostics tool for the detection of antibody against CIAV, the VP1 protein of the classical CIAV strain CUX-1 (GenBank: M55918) was first analyzed by using the PROTEAN software, and 3 peptides (P1, P2, and P3) derived from

VP1 with potential antigenicity and hydrophilicity were selected and synthesized. The sequences of the 3 peptides are listed in Table 1. To evaluate which peptide is suitable as an antigen for detecting the antibody against CIAV, the CIAV positive and negative sera were first tested by the peptide-based indirect ELISA. As shown in Figure 1A, P1 with or without KLH showed more efficient reaction with CIAV positive sera with an OD_{450} value higher than the P2 and P3 peptides. Moreover, the different sera collected from the chickens experimentally immunized with CIAV strain CIAV-HA at different time points were tested. As shown in Figure 1B, the P1 peptide used in the indirect ELISA showed efficient reaction with the sera collected from the CIAV-HA immunized chickens after d 35, whereas P2 and P3 peptides did not react well with these sera (Data not shown). Notably, the OD_{450} values of sera in the P1-based ELISA were increased by the days of the immunized chickens. The average OD_{450} value of sera at 21 and 105 d of the immunized chickens in this ELISA was 0.3 and 0.9, respectively. These results demonstrate that the P1 peptide carries efficient B cell epitopes and can be used as a coating peptide in ELISA to screen for antibody against CIAV.

To establish an efficient pELISA for detection of antibody reactive with CIAV, the pELISA was first optimized by using CIAV positive sera and CIAV negative sera for individual factors in pELISA. Based on the best ratio of the OD_{450} value of CIAV positive sera and CIAV negative sera (P/N), 2 μg/mL of P1 peptide in 0.1 M carbonate/bicarbonate buffer (pH 9.6) was coated into 96-well plates (300 μL/well) for 12 h at 4°C (Figure 1C) and plates were then blocked with the blocking buffer (5% rabbit serum in PBST) for 2 h at 37°C (Figure 1D). After the plates were washed 3 times with PBST, 100 uL/well of serum samples (1:100 diluted with 5% rabbit serum in PBST) were added into plates (Figure 1E) and incubated at 37°C for 1 h, followed by 5 washes with PBST. Then, 100 μL/well of rabbit anti-chicken antibody labeled with HRP (diluted 1:30,000 with 5% rabbit serum in PBST) were added into plates (Figure 1F) and incubated at 37°C for 1 h, followed by 6 washes with PBST. After then, the color development was performed using TMB Single-Component Substrate Solution (100 μL/well) in dark for 15 min (Figure 1G) and the reaction was stopped by addition of 50 μL of 2 M H_2SO_4. The absorbance values at 450 nm (OD_{450}) were measured using an ELISA reader. The cut-off of the ELISA was calculated as OD_{450} of 0.5 by the average value (X) of OD_{450} plus 3-fold of standard deviation (SD) of the 77 sera from SPF chickens (Data not shown). The sample with OD_{450} of more than 0.5 in this pELISA was regarded as positive sample, otherwise as negative sample.

To test the feasibility of the pELISA, the specificity and repeatability analysis were performed. As described in Figure 1H, the pELISA only reacted with the sera against CIAV, not with sera against other pathogens tested including REV, ILTV, IBV, NDV, AIV-H5/H9, and ALV-A/J/K with the OD_{450} value less than the cut-off. The variability of the intra/inter-assay for the pELISA revealed that the CV of the intra/inter-assay was 6.8 to 9.22% (Data not shown). These data demonstrate that the pELISA has great specificity for CIAV and high stability.

Moreover, the pELISA was compared with a commercial ELISA kit (IDEXX) for clinical sera from chickens immunized with the live attenuated CIAV vaccine. Among the 100 sera of chickens tested in the pELISA, 18 were negative and 82 were positive. Among the 56 sera tested in the IDEXX ELISA Kit, 11 were negative and 45 were positive. The positive rate in the 100 sera in the pELISA was 82% and that in the 56 sera in the IDEXX ELISA Kit was 80.4%. Although the positive rate in the 56 sera tested by IDEXX ELISA Kit in the pELISA was 48/56 = 85.7%, the positive and negative coincidence rate of the pELISA and IDEXX Kit was not high. This comparison assay revealed that the 2 approaches showed similar positive rate for the same flock, but not high compatibility for individual sample

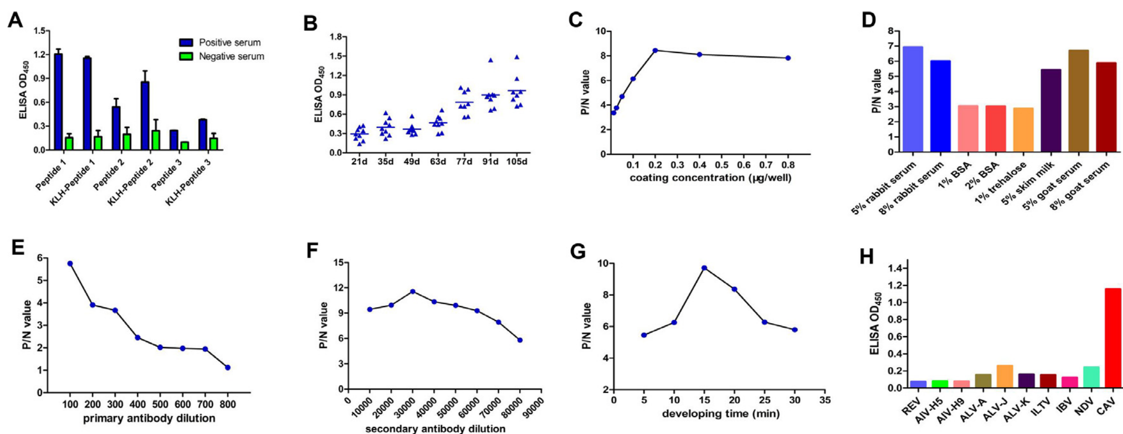

Figure 1. Characteristics of the pELISA. (A) The best peptide was selected in the ELISA for detection of antibody against CIAV. (B) P1 peptide-based ELISA showed efficient reaction with the sera collected from the CIAV-HA immunized chickens after d 35. (C) The concentration of the coating antigen in the pELISA was optimized. (D) The efficient blocking solution was selected in the pELISA. (E) The optimal dilution of the sera samples in the pELISA was determined. (F) The optimal dilution of the secondary antibody in the pELISA was determined. (G) The best time of the color development in the pELISA was determined. (H) The specificity of the pELISA was tested.

possibly due to the different sensitivity and different antigenic epitopes used in the 2 methods.

The commercial IDEXX ELISA Kit used in this study is a monoclonal antibody (**mAb**)-based blocking ELISA. In the blocking ELISA, the purified CIAV was coated in the plate, and HRP-labeled mAb was used as secondary antibody. Therefore, the antibody detected in the IDEXX ELISA Kit was specific to the epitope recognized by the mAb used in the IDEXX ELISA Kit. The antibody detected in the pELISA developed here was specific to the peptide used in the pELISA. Since the different sequences between the peptide in the pELISA and the epitope recognized by the mAb in the IDEXX ELISA Kit, the antibodies against CIAV detected in the 2 ELISA were totally different, which might contribute to the incompatibility for individual samples in the 2 ELISA. This analysis and our data indicate that the level of the kinds of antibodies detected in the 2 ELISAs in individual chickens might be highly variable in the vaccinated chicken flock. All these also indicate that although both ELISA can be used for detection of antibody against CIAV to monitor the vaccinated chicken flock, the false negative data may be present in both ELISAs. Therefore, the multiple detection of antibody against CIAV using these ELISAs at different time points is critical to completely exclude the false negative chickens.

It should be also noted that the P1 peptide used in the pELISA was located in the N-terminus of VP1 protein and was highly arginine-rich with positive charge. Previous studies reported that the N-terminal forty amino acids of VP1 could affect the expression of VP1 in *E.coli* expression system (Lee et al., 2011). Recently, Wanganurakkul et al. 2020 generated a highly expressed and truncated VP1 protein (**Δ60VP1**) without N-terminal 60 amino acids in *E.coli*, and developed an indirect ELISA. Although the Δ60VP1 indirect ELISA showed the 90.79% compatibility with the IDEXX ELISA Kit, the sensitivity and specificity of the Δ60VP1 indirect ELISA were somewhat lower than those in the whole VP1-based ELISA (Wanganurakkul et al., 2020), highlighting the deleted 60 amino acids from the N-terminal region of VP1 may contain crucial viral epitopes. Our data confirmed that N-terminal 25 to 45aa of VP1 carried efficient B cell epitopes, which could be used as an antigenic target for serological diagnostics for CIAV.

Notably, the commercial IDEXX ELISA Kit for detection of antibody against CIAV is very expensive due to its difficulty to prepare the purified CIAV as the coating antigen. In contrast, the peptide used in the pELISA is synthesized with more than 95% purity. Therefore, the preparation of the antigen in the pELISA is very simple and convenient. In addition, the detection of neutralization antibody against CIAV is tedious and time-consuming, and the IFA approach also has the problems of non-specific reaction. In conclusion, in terms of the specificity, speed and cost, the pELISA developed here is well suited for serological detection of antibody against CIAV for both SPF and commercial chicken flocks.

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Ethical approval and consent to participate: The animal study was reviewed and approved by the Animal Care Committee of Yangzhou University.

DISCLOSURES

The authors declare no conflicts of interest.

REFERENCES

Adair, B. M. 2000. Immunopathogenesis of chicken anemia virus infection. Dev. Comp. Immunol. 24:247–255.

Eltahir, Y. M., K. Qian, W. Jin, P. Wang, and A. Qin. 2011. Molecular epidemiology of chicken anemia virus in commercial farms in China. Virol. J. 8:145.

Lee, M. S., Y. C. Hseu, G. H. Lai, W. T. Chang, H. J. Chen, C. H. Huang, M. S. Lee, M. Y. Wang, J. Y. Kao, B. J. You, W. Lin, Y. Y. Lien, and M. K. Lin. 2011. High yield expression in a recombinant E. coli of a codon optimized chicken anemia virus capsid protein VP1 useful for vaccine development. Microb. Cell. Fact. 10:56.

Noteborn, M. H., and G. Koch. 1995. Chicken anaemia virus infection: molecular basis of pathogenicity. Avian Pathol. 24:11–31.

Rosario, K., M. Breitbart, B. Harrach, J. Segales, E. Delwart, P. Biagini, and A. Varsani. 2017. Revisiting the taxonomy of the family Circoviridae: establishment of the genus Cyclovirus and removal of the genus Gyrovirus. Arch. Virol. 162:1447–1463.

Todd, D., J. L. Creelan, D. P. Mackie, F. Rixon, and M. S. McNulty. 1990a. Purification and biochemical characterization of chicken anaemia agent. J. Gen. Virol. 71(Pt 4):819–823.

Todd, D., D. P. Mackie, K. A. Mawhinney, T. J. Connor, F. McNeilly, and M. S. McNulty. 1990b. Development of an enzyme-linked immunosorbent assay to detect serum antibody to chicken anemia agent. Avian Dis. 34:359–363.

Todd, D., K. A. Mawhinney, D. A. Graham, and A. N. Scott. 1999. Development of a blocking enzyme-linked immunosorbent assay for the serological diagnosis of chicken anaemia virus. J. Virol. Methods 82:177–184.

von Bulow, V. 1988. Unsatisfactory sensitivity and specificity of indirect immunofluorescence tests for the presence or absence of antibodies to chicken anaemia agent (CAA) in sera of SPF and broiler breeder chickens. Zentralbl. Veterinarmed. B. 35:594–600.

Wanganurakkul, S., D. R. Smith, L. Chintapitaksakul, and W. Assavalapsakul. 2020. Effective production of recombinant Delta60VP1 chicken anemia virus protein in Escherichia coli and its application to a serodiagnostic indirect ELISA. J. Virol. Methods 282:113887.