A McAb-Based Direct Competitive ELISA to Detect O:9 Salmonella Infection in Chicken

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Salmonella enteritidis and Salmonella pullorum belonging to Group O:9 Salmonella are major causative agents of infectious diseases in chicken. O:9 antigen as a part of lipopolysaccharide (LPS) is a predominant detected target for Salmonella infection. To identify the infection, an anti-O:9 monoclonal antibody (McAb)-based direct competitive enzyme-linked assay (O:9 Dc-ELISA) was developed after constraints were optimized; the establishment and application of O:9 Dc-ELISA, compared to two commercial kits and plate agglutination test (PAT), showed that O:9 Dc-ELISA could screen out more positive samples than the PAT method could and produce the same agreement rates with commercial kits in terms of sensitivity in addition to strong specificity to clinical serum samples.

Keywords: O:9 Salmonella, McAb, O:9 Dc-ELISA, specificity, PAT

INTRODUCTION

Salmonella, an important zoonotic pathogen, is one of the major causative agents of food-borne infectious diseases worldwide (1). Consumption of foods such as egg, chicken, pork, beef, and dairy products contaminated with Salmonella can cause salmonellosis in humans (2–4). This pathogen not only brings huge economic loss in the animal industry but also impacts human health, even death (5–8). Because of these disease harms and public health hazards, efficient surveillance is very important to reduce the prevalence of Salmonella and the risk of transmission to humans.

Salmonella enteritidis and Salmonella pullorum, which are important members in group O:9 Salmonella, are the main pathogens found in modern large-scale chicken farms in China (2, 9–11). In addition to their high morbidity and mortality in young broilers, they cause non-apparent infections in adult chickens without obvious clinical symptoms. Thus, it is difficult to find Salmonella infection in adult chickens. If Salmonella-infected chicken is not found on time, it may be a source of infection causing unlimited spread in chicken, even to humans, because of horizontal transmission and vertical transmission. It is necessary to carry out a seroepidemiological survey on Salmonella for healthy breeding and food safety.
Currently, plate agglutination test (PAT) is the main detection method used during Salmonella surveillance for its easy operation and low cost, but its sensitivity and specificity are poor and can easily cause false results because of antigen detection, visual observation, and subjective judgment.

LPS is the main antigen found on the Salmonella surface and the primary target for the immune system (12). After Salmonella infection, LPS can induce and keep a high level of antibody from early stages. Serotyping using serum/antibodies to the O-antigen of Salmonella lipopolysaccharide (LPS) (13, 14) is a critical basis of current Salmonella surveillance programs. Routine serotyping helps in monitoring public health response to the global challenge of salmonellosis and the effectiveness of control measures (9, 15–17).

Therefore, the development of readily available detection systems of the Salmonella antibody in chicken is important for mass-scale laboratory diagnosis. In this study, we developed an anti-O:9 Salmonella McAb-based direct competitive ELISA method to meet the requirements of accurate Salmonella surveillance.

MATERIALS AND METHODS

Ethical Statement
The present study was conducted under the approval of Laboratory Animal Ethics Committee of Yangzhou University (Jiangsu province, China) in accordance with Laboratory Animal Ethics Committee of Yangzhou University.

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MATERIALS AND METHODS

Strain, Hybridoma Cell Line, and Animals
Salmonella enteritidis (C50041) and Salmonella pullorum (S06004) were stored by our laboratory. A 3-47-0 hybridoma cell line secreting anti-O9 McAb was developed and preserved by our laboratory. Thirty 10-week BALB/c female mice were purchased for ascites from Comparative Medical Center of Yangzhou University.

Primary Quantity of Coated LPS and HRP-Labeled O9 McAb for Competitive ELISA
Primary quantities of LPS and HRP-labeled O9 McAb were confirmed by chessboard titration to develop a direct ELISA following conventional ELISA protocol. Horizontal gradient dilution of HRP-labeled O:9 McAb and vertical gradient dilution of coating antigens were performed. The final concentration of a tested positive serum was diluted 1:10. According to the serum inhibition rate \[\text{inhibition rate} = \frac{1 \text{-detected serum OD value/blank control OD}}{\times 100\%}\], optimal balanced concentrations were selected.

Constraint Optimizations for O9 Dc-ELISA

Tested Positive Serum Dilution
Two Salmonella pullorum-positive sera, two S. enteritidis-positive sera, and two negative sera from specific pathogen-free (SPF) chickens were diluted 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256. Based on the previous direct ELISA, each tested serum dilution was used as a competitor of positive serum and subjected to a competition ELISA. The serum dilution at the highest inhibition rate was selected as the serum dilution of the competitive ELISA method.

Quantity of Coated LPS and HRP-Labeled O:9 McAb
Based on the previous ELISA, LPS were divided into four groups, 480, 320, 190, and 160 ng/mL. By comparing the N/P values (negative serum OD value/positive serum OD value), the coating concentration at which the N/P value was the largest was chosen out as the optimal concentration.

Time of LPS Being Coated Onto a Plate
Three ELISA plates were coated at 100 µL/well at an optimized coating concentration. The coating time of the three ELISA plates was 16, 24, and 36 h, respectively.

Time of HRP-labeled McAb Binding With LPS and Reacting With TMB
Competitive ELISA was performed with the LPS coating concentration and HRP-labeled McAb and serum dilution, which were optimized in the previous steps. To ensure McAb to bind with coated LPS as possible, the incubation time of the HRP-labeled McAb was set to 1.0, 1.5, 2.0, 2.5, and 3.0 h, respectively, for analysis based on the N/P value.

Setting Up of the Cutoff Value and Comparison With Commercial Kits and PAT
One hundred serum samples from artificially infected chickens at different time points as positive control and 100 serum samples from SPF chickens as negative control were detected using the France ID.vet Salmonella kit, and these 200 serum samples were detected by O9 Dc-ELISA; the receiver operating characteristic (ROC) curve was made according to the inhibition rate. Based on these results, the cutoff value which was the value of negative samples + 3SD as a negative/positive judgment boundary was set up.

Fifty random clinical serum samples were tested using a double blind test by O9 Dc-ELISA and compared to IDEXX ELISA kit (IDEXX USA, 99-0002040) and ID.vet ELISA kit (ID.vet France, SALSGPD-5P) to judge the accuracy of O9 Dc-ELISA in clinical application.

The coincidence rate was calculated by the following formula: number of \([(+,+) + (,-,)]/\text{total number} \%\).
Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, USA). One-way ANOVA followed by Dunnett's multiple-comparison tests was used to determine the statistical differences between multiple experimental groups. All data are expressed as mean ± standard error of the mean (SEM) unless otherwise specified. \( P < 0.05 \) was considered statistically significant. \( * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 \).

RESULTS

Preparation of Coated LPS and HRP-Labeled O\(_9\) McAb

In this study, LPS were purified by the hot phenol–water method (18) and its concentration was calculated according to the standard sugar curve made by the anthracene ketone method. According to the measured OD\(_{620}\)nm value and standard curve (Figure 1), the final concentration of LPS was 484.31 µg/mL. McAb against O\(_9\) LPS (O\(_9\) McAb) was purified from hybridoma supernatants by caprylic/ammonium sulfate precipitation (19) and labeled with horseradish peroxidase (HRP) (20). The titer of HRP-labeled O\(_9\) McAb (HRP-O\(_9\) McAb) was up to 51,200 by indirect ELISA.

Constraint Determination of Competitive ELISA

A series of dilutions of LPS, HRP-labeled O\(_9\) McAb, and positive sera were prepared for chessboard titration and optimization (Figure 2). After optimization assay, 320 ng/ml LPS for coating (Figure 3), 41.6 ng/ml HRP-labeled O\(_9\) McAb (Figure 4), and positive serum dilution of 1:4 (Figure 5) were selected for developing O\(_9\) Dc-ELISA. The inhibition rate of positive serum by Salmonella pullorum and Salmonella enteritidis was up to 94 and 89%, respectively. On this basis, a standard operating procedure was formulated, after 96-well plates (Biofil company, Canada, FEP101896) were coated with ~100 µL purified LPS (320 ng/ml) in carbonate bicarbonate buffer (CBS, pH 9.4) at 4°C for 24 h (Figure 6) and washed with PBST (0.05% Tween 20 in phosphate-buffered saline) two times; 200 µL/well 2% BSA PBS solution was added again for blocking for 3 h at 37°C, then 50 µL 1:2 diluted chicken serum (PBS for blank control) and 50 µL HRP-labeled O\(_9\) McAb of 41.6 ng/ml were added at the same time. After incubation at 37°C for 2 h (Figure 7), all unbound materials were removed by washing with PBST six times. 100 µL of TMB chromogenic substrate was added to each well and incubated at 37°C for 3 min (Figure 8). After the color development was completed, 50 µL of 2 M H\(_2\)SO\(_4\) was added to each well to terminate the color development, and the OD\(_{450}\)nm absorption value was read.

Specificity Analysis of O\(_9\) Dc-ELISA

We prepared the tested sera from chicken infected by Escherichia coli, Proteus mirabilis, non-O\(_9\) Salmonella [Salmonella typhimurium (O:4)], the negative sera from SPF chickens, and the positive sera from chickens infected with Salmonella pullorum and Salmonella enteritidis; the results showed that O\(_9\) Dc-ELISA could not check out the sera against non-Salmonella and non-O\(_9\) Salmonella. The value of negative sera was more than 1.0 whereas the OD value of positive sera was less than 0.25 as a control (Figure 9) based on P/N\(\geq 2.1\).
Setting Up of the Cutoff Value

According to the results using the France ID.vet Salmonella kit as a reference of positive and negative chicken sera, and the percent inhibition (PI) values by O9 Dc-ELISA which were calculated using the formula PI (%) = (1 – OD_{450} of test serum/OD_{450} of blank control) × 100%, the cutoff based on the ROC curve was 38% (Figure 10). Under PI of 38%, the specificity of O9 Dc-ELISA reached up to 99.7% and the sensitivity reached up to 96.2% in ROC. The distribution of 100 positive serum samples and the 100 negative serum samples determined by O9 Dc-ELISA showed that 38% of inhibiting rate was indeed a threshold which could distinguish positive serum and negative serum (Figure 11).

Comparison Among O9 Dc-ELISA, Three Commercial Kits, and PAT

To validate the test ability of O9 Dc-ELISA, we randomly collected 50 serum samples for comparison to the results using O9 Dc-ELISA, PAT, IDEXX ELISA kit, and ID.vet ELISA kit; the results revealed that their coincidence rates were 88% (44/50, Table 1), 98% (49/50, Table 2), and 98% (49/50, Table 3), respectively. Although four samples negative with PAT were positive with O9 Dc-ELISA and two commercial ELISA kits, and 1 sample negative with O9 Dc-ELISA, IDEXX, and ID.vet ELISA kit was positive with PAT, there was no statistical difference among 4 methods. The results showed that O9 Dc-ELISA could screen out more positive samples than the PAT method could and produced the same agreement rates with two commercial kits in terms of sensitivity in addition to strong specificity.

DISCUSSION

Salmonella enteritidis and Salmonella pullorum are two of the most important Salmonella spp. that threaten the poultry industry, and humans are infected by directly or indirectly...
eating contaminated water and food, which causes great hazard to human public health security (21, 22). In our study, a McAb-based competitive ELISA was established to detect O:9 Salmonella infection in chicken. In order to achieve a better reaction system, we explored various conditions, including concentration of LPS coating and HRP-labeled O:9 McAb, serum dilution, LPS coating time, and reaction time of HRP-labeled McAb.

In order to confirm that the established O:9 Dc-ELISA did not cause a cross-reaction, we used O:9 Dc-ELISA to test Escherichia coli, Proteus mirabilis, Salmonella typhimurium (O:4), and negative sera from SPF chicken, Salmonella pullorum, and Salmonella enteritidis. The tests showed that only sera from Salmonella pullorum and Salmonella enteritidis could cause significant inhibition.

By testing 100 artificial positive samples and 100 negative serum samples from SPF chickens and 50 random clinical serum samples, the sensitivity and specificity at different thresholds were compared, and the final selected inhibition rate was 38% as the critical value of the competition ELISA kit. According to ROC, the specificity of O:9 Dc-ELISA was 99.7%, and the sensitivity was 96.2%. This O:9 Dc-ELISA was compared with PAT, IDEXX ELISA kit, and ID.vet ELISA kit, respectively. The results showed that the coincidence rate of the O:9 Dc-ELISA kit and ID.vet ELISA kit was 98%; the coincidence rate with the Salmonella enteritidis test kit was 98%; and the coincidence
rate with PAT was 88%. The above results indicated that this O9 Dc-ELISA has a good detection effect on the O9 antibody and had better performance than the PAT method based on more positive samples being checked out and the same agreement rates with commercial kits in terms of sensitivity in addition to strong specificity in the detection of clinical samples. This kit offered a good base as a first-generation product; it will be further evaluated and optimized according to clinical detection performance based on more serum samples to develop a second-generation kit in the future.

CONCLUSION

O9 Dc-ELISA has good ability in O9 antibody detection and had better performance than the PAT method and agreement rates with commercial kits in terms of sensitivity during the detection of clinical chicken serum samples. It must play an important role in O:9 Salmonella detection for Salmonella clearance in China in the future.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Welfare and Ethics Committees of Yangzhou University.

AUTHOR CONTRIBUTIONS

SG, XJ, and HG designed the paper. HG, ZX, and DS performed the experiments. YC, JZ, and YW provided help during experiments. ZP and XJ made critical revisions to the paper and contributed to paper writing. All authors contributed to the article and approved the submitted version.

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