Self-made *Salmonella* Pullorum agglutination antigen development and its potential practical application

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ABSTRACT Pullorum disease caused by *Salmonella* Pullorum is one of the most important infectious diseases in the poultry industry worldwide, which leads to serious economic losses in many developing countries because of its high mortality rate in young chicks. The traditional slide agglutination test with low cost, fast reaction, and on-site detection has been widely used in the diagnosis of Pullorum disease. However, in practice, the test performance is with the disadvantages of false positive results and unstable detection results. In this paper, we developed self-made agglutination antigens prepared by local isolates in the poultry farm and compare the detection performance with commercial agglutination antigens (China Institute of Veterinary Drug Control) and Group D *Salmonella* ELISA kit (BioChek UK Ltd). The results of detecting 200 serum samples indicated that the consistency of commercial agglutination antigen detecting in 2 times was only 79.5%. Using the ELISA kit as the reference method, the commercial agglutination antigen detecting results of the Kappa test were only moderately consistent (0.58 $\sim$ 0.59). Meanwhile, positive and total coincidence rates of the self-made agglutination antigen test with more reliable repeat could reach 97.4 and 88%, respectively, and the result of Kappa test was highly consistent (0.75). The Receiver Operating Characteristic curve analysis clarified that the area under the receiver-operating-characteristic curve values of self-made and commercial agglutination antigen tests could reach 0.861 and 0.804, respectively. These results were coincident when detecting known positive serum from the infected chickens. It's worth mentioning that the visible positive reaction of self-made agglutination antigen test appeared faster and stronger than commercial antigen test. In conclusion, self-made *Salmonella* Pullorum agglutination antigen developed in this study was much better than commercial agglutination antigen and is expected to be a valuable tool in the diagnosis of the epidemiology of *Salmonella* Pullorum.

Key words: *Salmonella* Pullorum, self-made agglutination antigen, agglutination test, kappa test, receiver operating characteristic curve

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INTRODUCTION

Pullorum, an acute, systemic and septic infectious disease within 2 to 3 wk old chicks and poults, is caused by *Salmonella enterica* serotype Pullorum (***S.* Pullorum**) with clinical signs such as depression, diarrhea, adherence of feces to the vent and other symptoms arising after infection in young chicks and poults (Barrow et al., 2012). This disease has a very high mortality rate, and causes serious economic losses in the poultry industry all over the world, especially developing countries (Shivaprasad, 2000; Gong et al., 2014; Dailey et al., 2016; Eriksson et al., 2018). Characteristics of *S.* Pullorum transmission go to that it can spread vertically and horizontally. Although we used to think, antibacterial drugs could effectively reduce the mortality of young chicks after infection, in consideration of bacterial multidrug resistance and carriers (post-treatment poultry), the problem is far from being solved (Barrow and Neto, 2011). In the mid-20th century, the developed poultry industries in Europe and North America started to eradicate the *S.* Pullorum by "test-and-cull", which controlled this disease effectively to a certain extent (Barrow and Neto, 2011). In order to effectively control the disease, a strict eradication procedure should be established to cull poultry with a definitely positive diagnosis to achieve eradication purposes.

The core issue of eradication is how to detect and diagnose the infection of *S.* Pullorum accurately. As we know, it takes approximately 4 to 7 d to culture and identify *Salmonella* pathogen by the current

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standard laboratory procedure and the above traditional culture methods are cumbersome and time-consuming. Besides, only the early diagnosis of the infected chickens could achieve an effective result (Parvin et al., 2012). To this end, scientists developed a series of serodiagnosis methods from the 1930s, such as the standard tube agglutination test, rapid serum agglutination test and color-stained agglutination antigen for the whole blood test (Muktaruzzaman et al., 2010). In the past few decades, the agglutination test has been widely used in the eradication of *S.* Pullorum worldwide because of its welcome advantages such as simplicity, cost-saving, reliable accuracy, and so on. However, in practice, the commercial agglutination antigen has been found that test performance was not always reliable. Johnson isolated *S.* Pullorum from cultured tissues taken from parent flocks that had earlier tested negative by commercial agglutination antigen (Johnson et al., 1992). Xiao-Xue reported that when detecting 1650 chicken serum the results of commercial agglutination antigen have a fairly high rate of missed and false detection (Xiao-Xue et al., 2018). So, the question has been raised about whether the commercial agglutination antigen prepared using strains selected several decades ago can still be suitable for the detection of the infected poultry caused by currently prevalent *S.* Pullorum strains.

It is the fact that with the continuous innovation of detection technology, many new methods for the detection of *S.* Pullorum and its antibody have been developed, such as ELISA (Ma et al., 2018; Li et al., 2019), sandwich immunoassay method (Sun et al., 2016), immunochromatography strip assay (Bautista et al., 2002), electrochemical immunosensor (Wang et al., 2014), immunofluorescence test (Mead et al., 1999), and so on. However, most of these new methods above are limited by the cost and high technical requirements. Therefore, at present, most of them far from being widely used like agglutination test. In an attempt to improve the agglutination test, it has been reported that the use of agglutination antigens prepared by *S.* Pullorum isolates from the local poultry farm has been found to be more reliable and effective compared with commercial agglutination antigens, moreover the visible test reaction response was stronger (Muktaruzzaman et al., 2010; Parvin et al., 2012).

In this study, we used the D-group *Salmonella* ELISA kit, commercial agglutination antigen and self-made agglutination antigen to detect 200 chicken serum samples. The Kappa test and receiver operating characteristic (**ROC**) curve were used to compare the detection performance of self-made *S.* Pullorum agglutination antigens prepared by isolated strains and commercial agglutination antigens. The Kappa test was first proposed by Cohen to evaluate the consistency of repeated results of different test methods (Cohen, 1960). The ROC curve is a 2-dimensional image drawn with sensitivity and specificity. The area under the ROC curve is **AUC** value, which is widely used and considered as the best evaluation index in evaluating the performance of medical diagnostic tests (Goodenough et al., 1974; Metz, 1978; Zweig and Campbell, 1993; Vanagas, 2004). The objective of this study was to assess the current effectiveness of the traditional *S.* Pullorum agglutination antigen and demonstrate the applied potential of self-made agglutination antigen as an alternative optimization.

MATERIALS AND METHODS

Preparation of S. Pullorum Isolated Strains and Reference Strains

We screened and isolated 168 *S.* Pullorum strains by the conventional method of isolation and identification from death eggs (at later hatching stage) of a couple of large-scale poultry farms in Guangxi Autonomous Region, China, from January to August 2018 (Roy et al., 2002; Sodagari et al., 2015; Alzwghaibi et al., 2018). In the preliminary experiment, we randomly selected 16 isolates from different batches of the isolates above as candidate strains for preparing self-made agglutination antigens. Then these isolates candidates were carried out for the self-agglutination test, non-specific agglutination with Phosphate Buffer Saline (**PBS**), Specific Pathogen Free (**SPF**) chicken serum and *S.* Pullorum negative chicken serum. The positive serum agglutination titer against *S.* Pullorum was compared and analyzed. Finally, 2 isolates (S.P 2–1 and S.P 3–1) were selected as self-made agglutination antigens candidates. Four reference strains in this study were from China Veterinary Culture collection Center (**CVCC**), which were CVCC523, CVCC526, CVCC535, and CVCC540.

Commercial S. Pullorum Antibody Detection Reagent

Commercial *Salmonella* Pullorum/Gallinarum colored agglutination antigen A was purchased from China Institute of Veterinary Drug Control (**CIVDC**, Beijing, China, Serial No. 201,601). Group D *Salmonella* ELISA kit was purchased from BioChek UK Ltd (Ascot, Berkshire, United Kingdom, Serial No. FS7043).

Preparation of Self-Made Agglutinating Antigens

An optimal protocol was developed for the preparation of self-made agglutinating antigens using the isolated. A single colony of *S.* Pullorum was inoculated into the Luria-Bertani (**LB**) culture. Then the LB culture was placed in the 37°C incubation for 24 h. After purity test, the bacterial culture solution was centrifuged at 4000 rpm for 10 min, the supernatant was decanted and cells were suspended in 0.4% formaldehyde saline and then inactivated at 4°C for 24 h.

Afterward, the cells were centrifuged at 4000 rpm for 30 min at 4°C, and the cells were suspended in precooled PBS, washed and suspended twice after centrifugation, and the antigen was suspended to working concentration with 0.25% formaldehyde saline at the third time centrifugation. The agglutination antigen was stained thrice with 1% crystal violet in 10% volume and placed at 37°C overnight. Finally, the staining agglutination antigen was stored at 4°C.

Specificity Test

In order to verify the detection accuracy of the self-made agglutination antigen, we performed agglutination test to detect antisera of chickens infected with *Salmonella* Enteritidis (***S.* Enteritidis**), *Salmonella* Typhimurium (***S.* Typhimurium**), and *Escherichia coli* (***E. coli***) O_1, O_2, and O_{78} serogroups.

Preparation of Samples Tested

The clinical serum samples were taken from the same poultry farms in Guangxi Autonomous Region. We randomly selected 200 samples, numbered 1 to 200. The quality control serum such as SPF chicken serum, *S.* Pullorum positive and negative serum was purchased from CIVDC. The antisera of chickens of *S.* Enteritidis, *S.* Typhimurium, and *E. coli* O_1, O_2, and O_{78} serogroups were preserved by our laboratory.

Operation of Agglutination and ELISA Test

The agglutination antigen was mixed uniformly before each test. For the prepared agglutination antigen, after inactivating *S.* Pullorum, considering the bacterial contamination, self-agglutination test and nonspecific agglutination should be carried out with PBS, SPF chicken serum and non-*Salmonella* serum. In order to test the validity, before clinical samples were tested, *S.* Pullorum positive serum should be tested by self-made agglutinating antigens. Then, the slide agglutination test was performed with clinical serum samples.

The result was recognized and judged within 2 minutes: The performance of "+++" indicates that the agglomerated particles were very conspicuous, and they were dark blue-violet granules. It could be defined "++" when the agglomerated particles were obvious, mainly in the form of dark blue-violet fine sand, and it was "+" when the fine sand like aggregates was observed to slide with the droplets. When the performance was "+" and above, it could be judged as "positive", otherwise "negative". It was necessary to keep the same experimental condition to judge the agglutination performance between different sets. Except for the field test, the detection environment and experimenters of the other groups remained unchanged. Each serum sample should be tested at least twice. If the results of the 2 tests were inconsistent, the third test should be carried out, and the test results should be subject to the third test.

When using the ELISA kit, the operation and results were determined strictly according to its manual instructions.

Grouping Sets

A total of 5 groups were set up, which were ELISA set (*Salmonella* Group D ELISA kit), antigen A set one (Colored agglutination antigen A, field test), antigen A set 2 (Colored agglutination antigen A, laboratory test), reference strain set (self-made agglutination antigen prepared by CVCC 523, CVCC 526, CVCC 535, and CVCC540), and isolated strain set 1 (self-made agglutination antigen prepared by S.P2–1 and S.P 3–1).

Preparation of Known Positive S. Pullorum Chicken Serum

In order to confirm the detection results from agglutination antigens, the forty 70-day-old laying hens were randomly selected from the same poultry farms. The sera collected from these forty chickens were detected by performing agglutination test, used commercial and self-made antigens, respectively, to sequentially double-check whether *S.* Pullorum strains could be isolated and identified from these chickens with actual infection status by traditional cultural methods (Roy et al., 2002; Aragaw et al., 2011; Lee et al., 2015; Sodagari et al., 2015; Alzwghaibi et al., 2018), We used a variety of S*almonella* selective cultural methods, which were Selenite Cystine (**SC**) Broth, Xylose Lysine Desoxycholate Medium and Maconkey Agar, to screen *Salmonella* strains from tissue slurry and picked individual bacterial colonies from the selective agar mediums. Subsequently, the suspect positive colonies were screened according to their morphology and then identified by Polymerase Chain Reaction (**PCR**) assay using five pairs of primers for targeted genes (de Freitas et al., 2010; Kang et al., 2011; Zhang et al., 2014) (Table 1), bacterial biochemical reaction and serotype test using slide agglutination with different O and H anti-sera. The sera of chickens positive for the isolation of *S.* Pullorum were considered as known positive clinic sera.

Statistical Analysis

The positive coincidence rate, the negative coincidence rate, and the total coincidence rate were analyzed when using the results of ELISA set or the isolation results of the bacteria as the reference standard. The Kappa test was used to determine the consistency of the ELISA and the four sets of agglutination reagents. The test results of each group were imported into IBM SPSS 22.0 software to generate ROC curve, and then the AUC value was obtained.

Table 1. Primers used for identification of isolates.

Gene	*Bacteria*	*Primers (5′-3′)*	*Amplicon size (bp)*
fimW	*Salmonella* spp.	F:AACAGTCACTTTGAGCATGGGTT R:GAGTGACTTTGTCTGCTCTTCA	477
glgC	*S.* Pullorum/Gallinarum	F: CGGTGTACTGCCCGCTAT R: CTGGGCATTGACGCAAA	252
speC	*S.* Gallinarum	F:ATCTGCTGCCAGCTCAA R: GCGCCCTTTTCAAAACATA	174
SdfI	*S.* Enteritidis	F: TGTGTTTTATCTGATGCAAGAGG R: TGAACTACGTTCGTTCTTCTGG	304
Spy	*S.*Typhimurium	F: TTGTTCACTTTTTACCCCTGAA R: CCCTGACAGCCGTTAGATATT	401

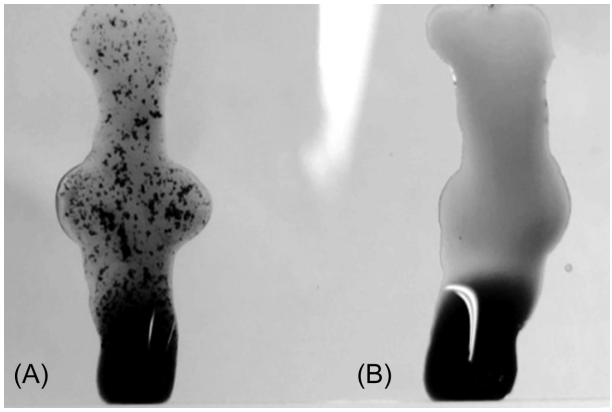

Figure 1. The agglutination performance of self-made *S.* Pullorum antigens, A. Positive agglutination performance with known *S.* Pullorum positive serum, B. Negative agglutination performance with SPF chicken serum.

Ethics statement

All experimental work involving live animals was conducted in compliance with the guidelines of the Animal Welfare and Ethics Committee of Yangzhou University.

RESULTS

The Performance of Self-Made S. Pullorum Antigens

When detected by self-made agglutination antigens, there was no self-agglutination, non-specific agglutination performance, and cross-reaction. The positive agglutination reaction of *S.* Pullorum positive serum showed visually clear and strong (Figure 1).

Comparison of Different Commercial Detection Reagents

Comparison of Different Types of Methods The antibody positive rate by ELISA was 58.5%, and the positive results of 2 sets of antigen A were 50 and 55%, respectively. Compared with ELISA, the total coincidence rates of 2 sets of antigen A was consistent, but the positive coincidence rate of antigen A set 2 was higher, while the negative coincidence rate was lower than antigen A set 1. The Kappa test results of two sets of antigen A were only 0.58 to 0.59, which indicated the consistency was moderate rather than substantial (0.61 to 0.80) or almost perfect (0.81 to 1).

Comparison of the Same Commercial Antigens When the same batch of 200 serum samples were tested using the same commercial antigens at different times. In the consistency comparison between antigen A set 1 and antigen A set 2, we found that the total coincidence rate of the two was only 81%, and the consistency did not reach "almost perfect" (the Kappa coefficient was only 0.62).

Comparison of Self-Made Agglutination Antigens

Even though prepared by the same technical route, the coincidence rate of the reference strain set was lower than that of the isolated strain set. Compared with commercial antigens, the isolated strain set was significantly better than 2 antigen A set, the sensitivity was higher, the total coincidence rate was higher, and the kappa coefficient increased from "moderate" to "substantial"(Table 2).

Using ROC Curves to Compare the Detection Accuracy of Different Sets of Agglutination Antigens

The positive and negative result data of each set was introduced into SPSS 22.0. After ROC curve analysis, among the 4 sets of detection antigens, the isolated strain set with the best detection accuracy showed its AUC value was 0.861 (95% confidence interval = 0.801 $\sim$ 0.920, $P < 0.05$) (Table 2).

Comparison of Reaction Speed and Strength of Different Agglutination Antigens

For true positive specimens of each antigen set, which was agglutination positive consistent with the ELISA results, the positive performance of the test within 30 s was defined as the "fast response performance", and the positive performance which showed "++" or stronger within 60 s was defined as the "strong response performance". Figure 2 clarified the proportion of the

Table 2. Analysis of 4 detection antigens for *S.* Pullorum.

	Kappa Test				ROC Curve		
	SEN[a] (%)	SPE [b](%)	TCR[c](%)	Kappa Coefficient	PPV[d](%)	NPV[e] (%)	AUC Value
Antigen A set one	75.2	85.5	79.5	0.59 (moderate)	88.0	71.0	0.804 ± 0.064
Antigen A set two	79.5	79.5	79.5	0.58 (moderate)	84.5	73.3	0.795 ± 0.066
Reference strain set	82.1	66.3	75.5	0.49 (moderate)	77.4	72.4	0.742 ± 0.073
Isolated strain set	97.4	74.7	88.0	0.75 (substantial)	84.4	95.4	0.861 ± 0.060

Using the *Salmonella* Group D ELISA kit as a reference standard method, the results of four sets detecting 200 sera were statistically analyzed separately.

[a]Sensitivity (SEN); [b]Specificity (SPE); [c]Total Coincidence Rate (TCR); [d]Positive Predictive Value (PPV); [e]Negative Predictive Value (NPV).

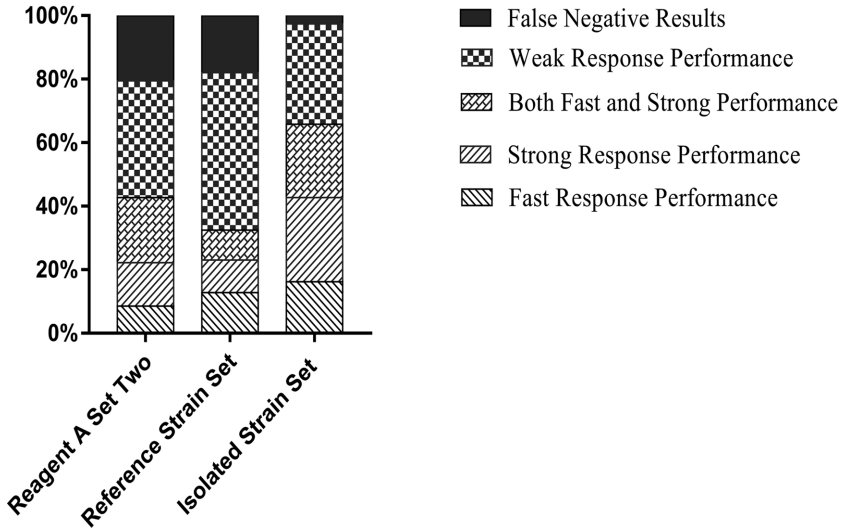

Figure 2. The comparison of the proportion of the samples from different sets with different agglutination performance in all 118 ELISA positive results.

specimens from different sets with fast, strong and other response performance in all 118 ELISA positive results. The result indicated that different agglutination antigens showed significant differences in the visible strength and speed of the positive reaction, the proportion of positive reaction of isolated strain set were higher, which was the best for the proportion of strong response performance (Figure 2).

Comparison of Detection of Known Infection Serum

The results of isolation and identification of *Salmonella* indicated that there were 9 *Salmonella*-positive chickens. The serotypes of the isolated *Salmonella* strains were confirmed by PCR and serotype test, which were all *S.* Pullorum. We used commercial and self-made agglutination antigens to detect 9 positive chicken serum. The sensitivity of commercial agglutination antigens was 66.7% (6/9), the fast (or strong) response performance accounted for 33.33% (2/6); correspondingly, the sensitivity and the rate of self-made agglutination antigens were 77.8% (7/9) and 57.1% (4/7).

DISCUSSION

Since *S.* Pullorum was first reported by Rettger in 1889 (Blaxland et al., 1956), this pathogen is very common and remains one of the most serious threats in poultry breed industry all over the world in recent years (Gong et al., 2014; Dailey et al., 2016; Eriksson et al., 2018). In order to control and eradicate this pathogen, the whole blood agglutination antigen is widely used to detect the serum of infected breeding flocks to screen for specific serum antibodies in China (China National Food Safety Standard GB/T 17,999.8–2008). In the present study, the total coincidence rate of two commercial agglutination antigen sets was only 81%, indicating that the detection results of the commercial antigen remained to be unstable, which led to a relatively high rate of error results, as had been reported earlier (Johnson et al., 1992; Xiao-Xue et al., 2018). One of the possible reasons is the commercial agglutination antigens are limited to accurately detecting serum antibodies infected by newly-occurring variant strain due to bacterial variation (Gast, 1997). It has been reported that the antigenic forms of *S.* Pullorum are somewhat unstable, which include designated standard, variant, and intermediate. Different

S. Pullorum types express different proportions of somatic (O) antigens 12_2 and 12_3 (Edwards and Bruner, 1946, 1948; Snoeyenbos et al., 1950). Although the commercial agglutination antigens used in this study combined both standard and variant strains (Gast, 1997), considering geographical factors, the use of *S.* Pullorum isolates to prepare self-made antigens theoretically has the advantage of improved detection sensitivity and specificity (Muktaruzzaman et al., 2010; Parvin et al., 2012). In order to verify it, in this research, self-made agglutination antigens prepared by isolated strains were compared with commercial agglutination antigens used Kappa test and ROC curve. The results indicated that the self-made antigen prepared in our study has the following advantages: The sensitivity and consistency were significantly improved, and the positive reaction appeared faster and stronger. This finding confirmed the previous reports (Muktaruzzaman et al., 2010; Parvin et al., 2012).

For the self-made antigens prepared by isolated strains and reference strains, the detection performance of the isolated strain set was significantly more accurate than that of the reference strain set, which were prepared by the same method. The results indicated that, in this study, the difference in detection performance of agglutination antigens was mainly due to the strains self instead of the production method. Based on the results we got for self-made agglutination antigens effects, we are currently characterizing the two strains (S.P 2–1, S.P 3–1) to further improve the self-made agglutination antigen.

The *Salmonella* ELISA kit was used in this study as a reference standard method, in which inactivated Group D LPS antigen has been pre-coated (O antigen) to measure the amount of antibody to *Salmonella* Group D such as *S.* Gallinarum, *S.* Typhimurium, *S.* Enteritidis, and *S.* Pullorum (Minga and Wray, 1992; Guo et al., 2017). In our previous study, we investigated the prevalent serotype of *Salmonella* infections, and in the epidemiological investigation of the poultry farm 442 death eggs at later hatching stage and 40 chickens, we found that the prevalent serotype of *Salmonella* in the poultry farm was *S.* Pullorum. Considering the high accuracy of the D group commercial ELISA kit, we considered it could be used as a reference reagent in this study when no other D group *Salmonella*.

Some scholars believe that the bacterial pathogen isolation can also be regarded as the gold standard of *Salmonella* infection (Aragaw et al., 2011). In this study, we used traditional culture methods to isolate and identify *S.* Pullorum in forty chickens to investigate the actual infection. Then, we compared the detection ability of commercial and self-made agglutination antigens by testing known infection serum. The test results verified that the self-made agglutination antigens are better, and the response is faster and stronger one more time.

This study indicated the traditional method taken to detect and diagnose the infection of *S.* Pullorum now remains to be improved urgently. A self-made agglutination antigens-based new approach suitable for field test is expected to be developed, which can effectively improve the detection accuracy while maintaining the advantages such as operational simplicity and cost saving (Proux et al., 2002). Preparation of agglutination antigens by local isolates had a lower cost than commercially available products and could much effectively improve detection sensitivity and accuracy, and might be a better choice in countries and regions which are relying on commercial agglutination antigens in the process of eradicating Pullorum infection. Considering these serological tests detecting antibodies in serum depend on *S.* Pullorum whole bacterial cells as antigens, the results might give rise to false positive reactions because of cross-reactivity with other Gram-negative bacteria (Ismael et al., 2016). Therefore, in the long run, it requires further exploration to improve the accuracy of the detection methods of *S.* Pullorum serum antibody.

CONCLUSION

In this research, the Kappa test and ROC curve were used to analyze the detection performance of self-made *S.* Pullorum antigens. The results showed that the detection performance of self-made agglutination antigens prepared by *S.* Pullorum local isolated strains was much better than commercial agglutination antigens, mainly in the superior consistency, higher AUC value, faster and stronger positive reaction. We are looking forward to applying to the clinical field.

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