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Research Article

Production of *Salmonella* Pullorum and *Salmonella* Gallinarum Antigens for Rapid Plate Agglutination Testing in the Detection of Pullorum Disease and Fowl Typhoid in Poultry Flocks

Seyed Iman Hosseini[1], Kaveh Parvandar Asadollahi[1]*

1. Department of Clinical Sciences, College of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran.

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ABSTRACT

Fowl typhoid and pullorum disease are bacterial infections caused by *Salmonella* Gallinarum and *Salmonella* Pullorum, which can infect chickens and turkeys. These are among the most significant infectious diseases in the poultry industry, resulting in substantial economic losses in many developing countries. Serological tests are suitable for identifying the presence and estimating the prevalence of these infections within poultry flocks. The rapid agglutination test offers speed, cost-effectiveness, ease of use, and reliability over other serological tests for screening fowl typhoid and pullorum diseases. In this research, we produced a mix-stained suspension of *S. gallinarum* and *S. pullorum* from local isolates which were incubated in two different incubation times (24 and 72 hours) for the rapid plate agglutination test. To evaluate the sensitivity and specificity of the produced antigen, we compared it with another commercial rapid plate agglutination kit (SP RPAT, Lillidale®). The results obtained with this kit were considered as true negative and positive results. Comparing the mentioned commercial rapid plate agglutination kit, the prepared antigen from 24-hour cultures demonstrated a sensitivity of 100% and a specificity of 99.13%, while the antigen prepared from 72-hour cultures showed a sensitivity of 93.33% and a specificity of 98.84%. Additionally, the agglutination quality of the 72-hour culture results slightly outperformed the 24-hour culture results. In conclusion, we found that the produced antigens are similar to the commercial antigens in terms of specificity and sensitivity, making them effective for diagnosing *Salmonella* Gallinarum and *Salmonella* Pullorum infections.

1. Introduction

The causative agents of Fowl typhoid and Pullorum disease are *Salmonella* Gallinarum and *Salmonella* Pullorum, respectively (Swayne et al, 2020; Spickler, 2019). Fowl typhoid was first identified in 1888 by Klein, while Pullorum disease was recognized in 1899 by Rettger (Shivaprasad, 2000). These diseases have a global presence (Swayne et al, 2020). Fowl typhoid and Pullorum diseases are highly lethal septicemic diseases, with mortality rates can approach ranging to 100%, particularly affecting young chicks and pullets (Swayne et al, 2020; Swayne et al, 2013; Spickler, 2019; Shivaprasad, 2000). The economic impact of these two diseases on the poultry industry can be

*Corresponding authors: kaveh parvandar
Email address: kaveh_parvandar@yahoo.com

substantial, leading to significant financial losses (Swayne et al, 2020; Swayne et al, 2013; Shivaprasad, 2000).

A variety of serological tests are available to identify the presence and estimate the prevalence of infection within a flock. These tests include the macroscopic tube agglutination test (TA), the rapid serum plate agglutination test (RPA), whole blood agglutination test (commonly used in the field), the microagglutination test (MA), and the enzyme-linked immunosorbent assay (ELISA) (World Organization for Animal Health [WOAH], 2018; Markey et al., 2013; Swayne et al, 2020, Shivaprasad, 2000). Among these tests, rapid tests are of great significance for flock screening (Spickler, 2019). This test relies on the ability of specific antibodies to visibly agglutinate killed whole *Salmonella* cells, which are stained to improve visualization of agglutination reactions (Swayne et al., 2020). The rapid serum agglutination test is a fast, cost-effective, and reliable method with minimal equipment and training requirements. This test has been widely used in the diagnosis and screening of Fowl typhoid and Pullorum diseases (Yang et al., 2019; Spickler, 2019).

In this context, the preparation of suitable antigens for these two bacteria is crucial. This study involves the preparation of a suspension of inactivated antigens of *S. pullorum* and *S. gallinarum*, stained with crystal violet dye. These antigens are intended for use in the rapid plate agglutination test to detect antibodies produced against *S. pullorum* and *S. gallinarum* in chicken serum. The performance of these antigens is also compared with a similar commercial antigen.

2. Materials and Methods

2.1. Subculture and Confirmation of Bacteria

In this study, the antigen mixture of *S. pullorum* and *S. gallinarum* was prepared by utilizing the stock of these bacteria. These bacteria are revived by incubating and subculturing in the specific media. Then they were reconfirmed by various methods, including bacterial culture, serotyping, biochemical analysis, and PCR tests (Table 1) (Shivaprasad, 2000; Markey et al., 2013).

2.2. Stained antigen preparation

To generate a substantial bacterial population, the confirmed bacteria, were incubated in two different incubation times of 24 and 72 hours at 37 °C in a liquid TSB medium. To produce an inactivated antigen suspension, first, the liquid cultured media (TSB) was centrifuged at 2000 g for 10 minutes, then the supernatant was discarded, and a sterile formal saline buffer with a pH of 6.5 was added to the sediment and the suspension was left at room temperature. At this step, the morphology and purity of the suspensions were checked by the Gram-stain method. 30 minutes after adding formal saline, 200 ml of absolute alcohol (96% ethanol) was added to each 100 ml of suspension then gently agitated and left at room temperature for 48 hours. After that, the suspension was centrifuged at 2000 g for 10 minutes, and the supernatant was discarded. Then, to reach the 50× No.1 tube McFarland standard suspension, the sediment was diluted with a PBS solution containing 10% v/v sterile glycerol. For staining, 3% w/v alcoholic crystal violet dye solution was added to the final suspension to reach 1% v/v dye concentration. To absorb the stain, the suspension was left at room temperature for 48 hours. All of the mentioned steps were done separately for two different incubation times (24 and 72 hours).

2.3. Rapid serum plate agglutination test

The rapid serum plate agglutination tests were conducted on 360 blood serum samples of two broiler breeder farms by the prepared stained antigens. In accordance with the SP-MS-MG detection method of the Iran Veterinary Organization (Iran Veterinary Organization [IVO], 2017), the Rapid Serum Plate Agglutination test was conducted as follows:

- Before each test, the antigen suspension was thoroughly mixed and let it come to room temperature.

1. 25 µl of blood serum from the samples was placed on a slide.
2. An equal amount of stained antigen suspension was added to the serum drop on the slide.
3. Both drops were thoroughly mixed.
4. If agglutination was not observed within two minutes, the result was considered negative.

5. If agglutination was observed, the serum was diluted to 1:16 (one sixteenth) using either physiological normal saline or phosphate-buffered saline (PBS), and the test was repeated.
6. If agglutination was observed in the repeat test on diluted serum, the result was considered positive.

2.4. Comparison with another commercial kit

The stained antigens prepared in this research were compared with a similar commercial *S. pullorum* antigen (SP RPAT Antigen, Lillidale® Diagnostics, Wimborne, United Kingdom, Cat. # V2025, Lot # SP022201).

2.5. Statistical Analysis

To evaluate the produced antigen, SP RPAT Antigen (Lillidale®, United Kingdom) was considered as the primary reference for calculating the sensitivity and specificity of the produced antigen. Therefore, the results of the rapid serum agglutination test from this antigen were assumed as a true result (true positive or negative). The sensitivity and specificity were calculated using the following formulas (Petrie & Allen, 2013).

Sensitivity= True positive/ (True positive + False negative)

Specificity= True negative/ (True negative + False positive)

Table 1. Primers used for identification of *S. pullorum* and *S. gallinarum*. SP, *S. pullorum*; SG, *S. gallinarum*; bp, base pair. (Xiong et al., 2018).

Primers	Primer sequence (5'→3')	Amplicon Size (bp)	*Salmonella* serovars	
			SP	SG
I137_08605 F	CACTGGAGACTCTGAGGACA	290	+	+
I137_08605 R	GGGCAGGGAGTCTTGAGATT			
ratA ROD F	ATTGCTCTCGTCCTGGGTAC	571	-	+
ratA ROD R	TACCGATACGCCCAACTACC			

3. Results

The results obtained from the rapid serum plate agglutination test of 360 serum samples using our stained antigen with two different incubation times were very similar to the commercial kit (SP RPA test, Lillidale®, United Kingdom). Comparing the commercial kit, the results from the suspension prepared with 24 hours of incubation, exhibited 100% sensitivity and 99.13% specificity, and the suspension prepared with 72 hours of incubation demonstrated 93.33% sensitivity and 98.84% specificity. However, the antigen derived from 72-hour incubation time showed slightly superior visible agglutination compared to the 24-hour incubation time (Table 2) (Figures 1&2).

Table 2. The sensitivity and specificity percentage of produced antigens in this study by considering the results of the SP RPAT Antigen kit (Lillidale®, United Kingdom) in the RPA test as reference results by conducting the test on 360 Chicken serum samples.

Kit		True Positive	False Positive	True Negative	False Negative	Sensitivity (%)	Specificity (%)
SP RPAT Antigen (Lillidale®)		15	-	345	-	100	100
Prepared stained antigen	24hours incubation	15	3	342	0	100	99.13
	72hours incubation	14	4	341	1	93.33	98.84

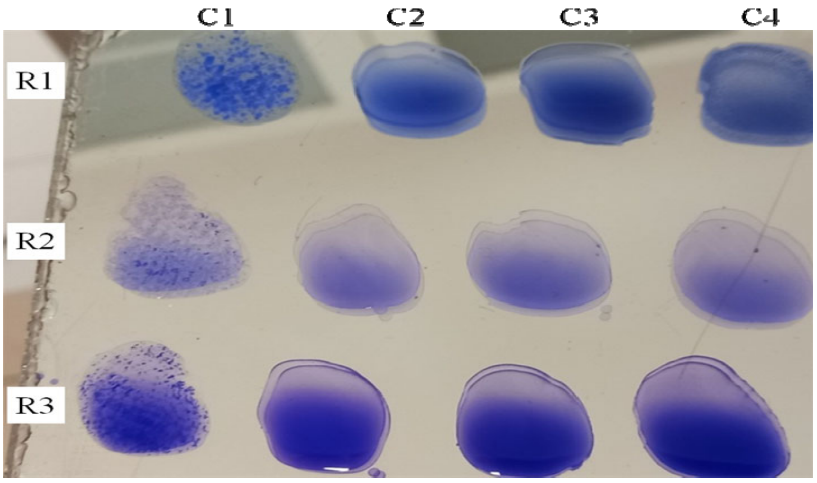

Figure 1. Rapid agglutination test on the plate. R1 is the SP RPAT Antigen of Lillidale®. R2 and R3 are stained antigens obtained from 24 and 72 hours incubation time respectively. C1 is positive and C2, C3 and C4 are negative. (R= Row, C= Column)

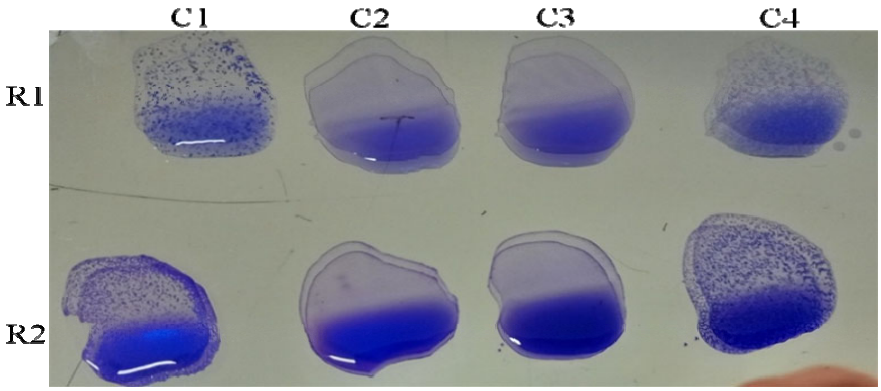

Figure 2. Rapid agglutination test on the plate with the stained antigens. R1 and R2 are 24 hours and 72 hours incubation time respectively. C2 and C3 are negative and C1 and C4 are positive. (R= Row, C= Column).

Discussion

Serological tests serve as valuable tools for identifying and estimating the prevalence of fowl typhoid and pullorum diseases within commercial flocks. The rapid serum plate agglutination test with stained antigens stands out as a rapid, cost-effective, and reliable method for screening these diseases.

In a study by Bidhendi et al. (2008), stained antigens of *Salmonella* Pullorum were produced and compared to a similar commercial product. Bidhendi found that a higher antigen titer could be achieved by culturing in Tryptic Soy Agar (TSA) medium compared to McConkey medium and Veal infusion agar. The results of the commercial sample were used as the gold standard for sensitivity and specificity calculations, yielding values of 93.55% for sensitivity and 99.66% for specificity. It's noteworthy that our results exceeded those of Bidhendi et al., suggesting the effectiveness of our stained antigen preparation.

Williams and Whittemore's (1971) research on the preparation of stained *S. pullorum* antigens involved the use of Veal Infusion agar and Veal Infusion Broth, along with neotetrazolium chloride dye for staining. In a subsequent study, Williams and Whittemore (1973) explored the production of *S. pullorum* antigen on a solid medium, comparing it to a liquid medium. Their findings indicate that antigen production in solid media is not only easier but also leads to a higher titer compared to liquid medium.

In our study, we employed crystal violet for staining the antigens, which offers practical advantages such as ease of storage at room

temperature. This contrasts with neotetrazolium chloride, which requires specific temperature control (2 to 6°C). Additionally, we used Tryptic Soy Broth (TSB) as the liquid medium for high titer bacterial culture, making the extraction of antigens more straightforward compared to the solid agar culture medium. However, it's important to note that TSB may yield a smaller bacterial quantity compared to solid media, such as TSA medium when considering an equal weight of the culture medium.

In a study by Yang et al. (2019) on *S. pullorum*, They prepared a suspension of *S. pullorum* antigen isolated from China and compared it with a similar commercial product from the China Institute of Veterinary Drug Control. They used a Luria Bertani medium in their bacterial culture. Using experimentally infected chickens, they evaluated the sensitivity of antigens in the rapid plate agglutination test, reporting an antigen sensitivity of 77.8% compared to the commercial sample's 66.7%.

Yang et al, just worked on *S. pullorum*, whereas our research included a mix of *S. pullorum* and *S. gallinarum*, also we utilized naturally infected samples, which may better represent real-world conditions. TSB medium was chosen over Luria Bertani, as it is considered a richer medium (Jaradat, 2002). Furthermore, we applied two different incubation times (24 and 72 hours) and observed that the sensitivity of both antigens exceeded that of Yang's antigen.

Parvin et al. (2012), made stained antigen from *S. pullorum* with neotetrazolium dye and they incubated bacteria for 48 hours in Luria Bertani as a liquid culture media then they added dye into the liquid culture and incubated it for the next 2 hours. Also, they applied 0.5% phenolized saline, 0.5% formal saline and 0.09% sodium azide as preservatives. Parvin et al evaluated their antigen by comparing results with a commercial ELISA kit (GUILDHAY, UK) in different serum dilutions, and they found that the stained antigen was more sensitive than the ELISA kit. In our study, we used a mix of *S. pullorum* and *S. gallinarum*. We didn't apply a preservative and we added dye in the final step (not in liquid culture media) to absorb the stain, the suspension was left at room temperature for 48 hours. We employed crystal violet for staining the antigens, which offers practical advantages such as ease of storage at room temperature. This contrasts with neotetrazolium chloride, which requires specific temperature control (2 to 6°C). We applied TSB medium and two different incubation times (24 and 72 hours). TSB medium was chosen over Luria Bertani, as it is considered a richer medium (Jaradat, 2002). Also, we compared our produced antigens with a commercial rapid serum plate agglutination kit.

Raduta et al. (2014), produced stained antigens from *Salmonella* Enteritidis for the diagnosis of its antibodies in the chicken's serum. They incubated bacteria in a solid agar medium for 48 hours. The bacterial *S.* Enteritidis suspension was then inactivated with formalin. They stained the *S.* Enteritidis antigens with 1% crystal violet solution and kept them at 37ºC for 24 hours. Also, they prepared *S*. Enteritidis and *S. pullorum* polyclonal antiserum as a Positive Control and SPF chicken's serum as a negative serum. Their stained antigen was positive in the rapid serum plate agglutination test with *S. pullorum* polyclonal antiserum up to 1/20 serum dilatation and with *S.* Enteritidis polyclonal antiserum up to 1/320 serum dilution. Also, all negative controls were negative with their stained antigen. In our study, we used a mix of *S. pullorum* and *S.* Gallinarum, and we used naturally infected samples. We employed 3% crystal violet for staining the antigens to absorb the stain, the suspension was left at room temperature for 48 hours. We applied TSB medium and two different incubation times (24 and 72 hours). Also, we compared our produced antigens with a commercial rapid serum plate agglutination kit,

Muktaruzzaman et al. (2010) prepared a stained *S. pullorum* antigen with neotetrazolium dye. They produced 8 groups of stained antigens with two different incubation times (24 & 48 hours), two different staining times (2 & 24 hours), and two different inactivator agents (phenol or thiomerasol). The best results were obtained from the group with 48 hours incubation time, 24 hours staining time, and thiomerasol as an inactivator. The bacterial culture media was Difco^{TM} vial infusion agar. Also, they applied different preservatives (0.5% phenolized saline, 0.5% formalized saline, and 0.09% sodium azide) to maintain the shelf life of the prepared antigen, and all the preservatives showed similar results for up to six months. They recommend 0.5% phenolized saline as a

preservative because it is cost-effective. They tested their produced antigens with known positive and known negative serums and whole blood samples. Their produced antigens in rapid serum plate agglutination test were positive with known positive and negative with known negative samples. In our study, we used a TSB medium which is better than Difco^{TM} vial infusion agar for *Salmonella,* as it is considered a richer medium (Jaradat, 2002). We employed crystal violet for staining the antigens, which offers practical advantages such as ease of storage at room temperature. We added dye in the final step (not in liquid culture media) and to absorb the stain, the suspension was left at room temperature for 48 hours. We used a mix of *S. pullorum* and *S. gallinarum*. We didn't apply a preservative. We added dye in the final step. Also, we used formal saline as an inactivating agent.

Based on the findings from this research, the following conclusions can be drawn:

1. The optimal duration for cultivating the combination of *S. gallinarum* and *S. pullorum* in TSB medium at 37°C, considering visual agglutination, is 72 hours. However, in terms of sensitivity and specificity, 24 hours of cultivation is preferred.
2. Given the high levels of sensitivity and specificity, along with the alignment with the commercial product used in this study, our method stands as a suitable approach for producing stained antigen suspensions of *S. gallinarum* and *S. pullorum* for use in rapid serum plate agglutination tests.

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Refereces

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