IN PROCESS QUALITY CONTROL FACTORS AFFECTING THE QUALITY OF LOCALLY PREPARED S*ALMONELLA GALLINARUM* ANTIGEN

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ABSTRACT

Seroserveillance of fowl typhoid using imported antigen is not cost effective, more over import of meager quantity of antigen is always not possible. Therefore, Fowl typhoid antigen was locally prepared and evaluated for sensitivity and specificity. Effect of preservatives and temperature on sensitivity of local antigen was also studied. Of three test antigens (6, 8 & 10 million cells/ml), one with 10 million cells showed visible agglutination with known antisera upto log 10^{-3} dilution (3000 cells/0.3 ml) as compared to other two antigens (8, 6 million cells/ml) that showed agglutination upto 10^{-2} dilution (24000cells/0.3ml, 18000cells/0.3ml respectively). Antigens stored at -20°C and 25°C showed visible agglutination up to 90 and 30 days post preservation respectively in presence of both preservatives thimerosal sodium and sodium azide, separately, while no change in agglutination reaction was observed for antigen stored at 4°C throughout the whole storage period of 6 months. No cross reactivity of local antigen with antisera against *Mycoplasma gallisepticum*, *Mycoplasma synoviae* and negative control serum was observed.

On comparison, local antigen proved equally efficient as imported antigen with known positive and negative control sera and may be used for screening of *Salmonella gallinarum* infection in poultry.

Key words:*Salmonella gallinarum*, Fowl typhoid, Diagnosis, Antigen, Pakistan

INTRODUCTION

Fowl typhoid caused by *S.gallinarum* biovar*gallinarum* has major economic significance in many parts of the world including Pakistan. It is an acute or chronic septicaemic disease that usually affects the adult birds (Chappell *et al.* 2009). With the expansion of poultry industry in Pakistan, incidence of Fowl typhoid (FT)has been increased tremendously resulting in heavy economic losses in the form of morbidity, mortality, reduced production and above all, vertical transmission to subsequent generation (Khan *et al.* 2014). Strategies to combate this problem in commercial poultry include surveillance, control and eradication programs along with use of antimicrobials. Use of antimicrobial is not preferable because the excessive use may lead to resistance in food borne pathogens (Zhang-Barber *et al.* 1999). It is only possible to control the problem by establishing breeding flocks free of S. *gallinarum*, and to hatch *Salmonella* free chicks along with rearing of flocks in environment where there is no direct and indirect contact with infected birds.Eradication is normally done by identifying the infected flocks and eliminating the reactor birds by using serological tests. Slide agglutination test is most suitable and cost effective test for this purpose and is being used in diagnostic labs(Barrow *et al.* 1992).In Pakistan imported antigens are used for screening the reactors through agglutination test. Cost of one test varies from Rs. 100-200. The main problem, the labs are facing is availability of antigen. Supplier hasitate to import few vials of antigens due to fright charges leaving lab with option to postponed secreening testing until they get some big supply order. Keeping in vew this situation efforts were made to isolate *Salmonella gallinarum* from local poultry farmsand was used to prepare antigen. Sensitivity and specificity of locally prepared antigen as well as effect of preservatives and temperature on sensitivity of local antigen was also evaluated in this study.

MATERIALS AND METHODS

Identification and confirmation of *Salmonella gallinarum:* Local isolate of *Salmonella gallinarum* taken from Poultry Research Institute (PRI) were revived in selenite cystine broth tubes following by subculturing on Salmonella shigella agar. Black colored colnoies were subjected for confirmation of *Salmonella gallinarum* through Polymerase chain reaction (PCR).

Polymerase Chain Reaction: The PCR amplification was performed in a total volume of 25µl by adding 4 µl of extracted DNA,1 µl each of previously described primer pair i.e.5'-GACGTCGCTGCCGTCGTACC-3'(F),5'-TACAGCGAACATGCGGGCGG-3'(R)(Batista *et al.* 2013), 12µl of PCR master mix and 7µl of PCR grade water. Cycling conditions used were initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 63°C for 1 min and 72°C for 1 min with a final step at 72°C for 5 minutes. PCR amplicons were analyzed by gel electrophoresis at 80 V and 100 mA current for 45 min in a 1.7% (w/v) agarose gel stained with ethidium bromide followed by imaging.

Antigen Production: The antigen was prepared from *Salmonella gallinarum* colonies which showed agglutination with specific sera(Charles River, USA) but didn't agglutinate with acriflavin(Sigma, Aldrich), normal saline or negative control sera The antigen was prepared using confirmed isolates of *S. gallinarum*as per method described in OIE manual with partial modification (Hoque *et al.* 1997). The typical colonies of local isolates were seeded into nutrient agar slopes and incubated at 37ºC for 24hrs. The growth was emulsified in sterile normal saline which provided 2ml seed inoculum for each roux flask. Each roux flask containing 200 ml nutrient agar, was inoculated with the 2ml seed inoculum and incubated at 37ºC for 60 hours.To harvest the antigen 10 ml of sterile normal saline was added to each flask, flasks were rocked after the additon of sterile glass beeds to get the even suspension, suspension was collected in a separate flask and was treated with absoulte alcohal at 2:1 by volume and allowed to stand for 36 hours for complete precipitation. The clear supernatent was disposed off after centifugation to remove the alcohal and cells were stored at 4°C. Using McFarland scalethree different cell concenterations were adjusted i.e. 6 million cells/ml, 8 million cells/ml and 10 million cells/ml (Normal saline was used for dilution purpose). For the staining of antigen all three antigens, 10% (V/V) glycerol and 1% of 3% alcohalic crystal violet solution were added.

Sensitivity of local antigen: Serial tenfold dilutions (Undiluted, 10^{-1} to $10^{-6)}$ of all the three antigens were prepared in Normal saline and each dilution was subjected to agglutination test with known positive and negative antisera to find out the minimum number of bacterial cells (best dilution) required to be used for visible agglutination.

Specificity of local antigen: The best working dilution of each antigen were also tested to check the specificity of local antigens using known antisera of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*(Charles River, USA).

Effect of Preservativesand Temperature on antigen: Three vials of each antigen (total 9 vials) using best working dilution were prepared. Two preservatives i.e. sodium azide (0.01% W/V) and thiomersal sodium (0.1% W/V) were added to the antigens as described by Hromatka and Adler, 1969. After addition of preservatives, one vial of each of three antigens were stored at 4°C, -20°C and 25°C and tested by agglutination test using *Salmonella gallinarum* positive and negative control sera (Charles River, USA) after every 15 days post storage till 6 months.

Comparison of antigen: Locally prepared antigen of *Salmonella gallinarum* was also compared with the imported antigen (Charles, River, USA). Imported antigen was also stored at 4°C, -20°C and 25°C and tested by agglutination test using *Salmonella gallinarum*positive and negative control sera after every 15 days post storage till 6 months.

RESULTS

Of 15, 2 isolates that showed typical grayish black colonies on SS agar media were accepted as *Salmonella gallinarum* through PCR. A 1047 bp sized PCR product (specific for *S. gallinarum)* was visualized in 1.7% agarose gel under UV illuminator (Fig: 1).

Sensitivity of antigen: Out of three test antigens (6, 8 & 10 million cells/ml), one with 10 million cells showed visible agglutination with known antisera upto log 10^{-3} dilution (3000 cells/0.3 ml) as compared to other two antigens (8, 6 million cells/ml) that showed agglutination upto 10^{-2} dilution (24000cells/0.3ml, 18000cells/0.3ml respectively). This study depicted that a minimum of 3000 cells/0.3ml are required to show agglutination(Table 1).

Specificity of local antigen: All the three antigens were found specific when tested against available antisera in the lab. No cross reactivity of local antigen with sera against *Mycoplasma gallisepticum*, *Mycoplasma synoviae* and negative control serum was observed.

Effect of Preservativesand Temperature on antigen: Both preservatives thimerosal sodium and sodium azide were accepted equally effective (p=1) in our hands however variation in storage temperature (-20°C, 25°C, 4°C) affected the sensitivity of antigen. Antigens stored at -20°C and 25°C showed visible agglutination up to 90 and 30 days post preservation respectively, while no change in agglutination reaction was observed for antigen stored at 4°C throughout the whole storage period of 6 months.

Comparison of local antigen with impoted antigen: On comparison, local antigen stored at 4°C proved equally efficient as imported antigen with known positive and

negative control sera. No change in agglutination reaction was observed throughout the whole storage period of 6 months. Imported antigens stored at other two temperature (-20°C, 25°C) showed similar results as those of local antigens.

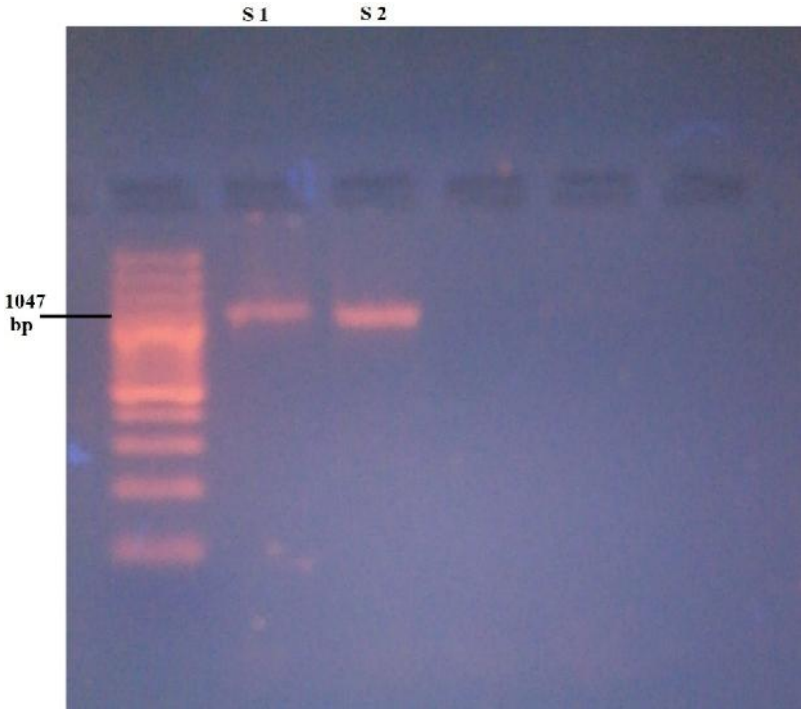

Figure 1: Gel electrophoresis: bands of 1047 bp, specific of *S. gallinarum*

Table 01. Comparison of *Salmonella gallinarum* slide agglutination with various dilutions of antigen.

Antigen ID	Agglutination with various Dilutions of antigen						
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
SAg1	+	+	-	-	-	-	-
SAg2	+	+	+	-	-	-	-
SAg3	+	+	+	+	-	-	-

Antigen 1(SAg1) = 6 million bacterial cells/ml, Antigen 2 (SAg2) = 8 million bacterial cells/ml,
Antigen 3 (SAg3) = 10million bacterial cells/ml, + visible agglutination, - no visible agglutination

DISCUSSION

In Pakistan, Poultry sector is one of the most organized and vibrant segments of agriculture industry.With the expansion of poultry industry in Pakistan, the problem of fowl typhoid (FT) has been emerged as one of the great economic threat.It is matter of concern for developing poultry industry and results from infection by *Salmonella gallinarum* (*Salmonella enteric* subsp. *Enterica* sero var *Gallinarum* biovar *Gallinarum*). Confirmed diagnosis is made through isolation of the causative agent ,however, serological tests are also suitable forestimating the prevalence of infection within a flock (OIE, 2008). The most readily applied tests include serum plate agglutination (SPA) test. In Pakistan, forSPA test antigen is being imported by spending lot of revenue. In Pakistan, as there are few labs that offer screening test for fowl typhoid, most of the times, supplier delayed import of antigen until they get some huge supply order to minimize freight charges.

This study was therefore designed to prepare*Salmonella gallinarum* antigenusing local isolates as per already described protocol with some modification (OIE 2008).

Three antigens were prepared having different number of cells. Of three test antigens (6, 8 & 10 million cells/ml), one with 10 million cells showed visible agglutination with known antisera upto log 10^{-3} dilution (3000 cells/0.3 ml) as compared to other two antigens (8, 6 million cells/ml) that showed agglutination upto 10^{-2} dilution (24000cells/0.3ml, 18000cells/0.3ml respectively). This study depicted that a minimum of 3000 cells/0.3ml are required to show agglutination. In this study, stability of antigen was also checked with

different preservatives and at different storage temperatures. Regular study revealed that antigens stored at 4°C showed the best results as they showed visible agglutination reaction throughout the duration of six months, while antigens stored at 25°C and -20°C were found effective up to one and three months respectively. These results are in agreement with the findings of Ker *et al.* (2013) who reported that thiomersal sodium containing antigen remained effective for more than 6 months if stored at 4°C. These findings are also supported by Hoque *et al.* (1997) who reported that antigen stored at 0-4°C showed best activity.Contrary to this, our study results do not match with the findings of Hromatka and Adler (1969) where scientists were of the point of view that thiomersal sodium or sodium azide did not prevent contamination if they are used alone but in combinations. The possible reason of this disagreement may be that antigen storage temperature has not been mentioned, storage at high temperature might be the cause of this disagreement.

On comparison, local antigen stored at 4°C proved equally efficient as imported antigen with known positive and negative control sera. No change in agglutination reaction was observed throughout the whole storage period of 6 months. Imported antigens stored at other two temperature (-20°C, 25°C) showed similar results as those of local antigens.

In our study, it was found that the locally prepared antigen of *Salmonella gallinarum* and imported antigen were equally effective to be used for screening of reactor poultry flocks. It was also concluded that antigen activity remained best at 4°C and thiomersal sodium or sodium azide are equally good to be used as preservative. This newly developed slide agglutination antigen was found to be sensitive, reliable, and cost effective. This technique can be used for the screening of Fowl typhoid.

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