Isolation, Identification and Production of Salmonella Pullorum Coloured Antigen in Bangladesh for the Rapid Whole Blood Test

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ABSTRACT : Postmortem examination was conducted on 350 (three hundred and fifty) chickens. Related samples (Liver, heart, ovary, spleen, bone-marrow, and caecal junction) were collected. The appropriate materials from the samples were cultured into different media. A total 40 (forty) isolates of *Salmonella pullorum* and *S. gallinarum* were identified and preserved. Characterization of the isolates were done by cultural, morphological, biochemical, and serological tests. *Salmonella pullorum* antigen was prepared from the local isolate, standardized and tested. This antigen was used in the field for the detection of pullorum or fowl typhoid infection or carrier birds. The antigen consisted of suspension of Salmonella pullorum in 0.50 percent sodium chloride plus 1.5 percent sodium sulfate and inactivated with 1% formalin U.S.P. and standardized with McFarland scale iv or by pour plate method containing 800 million organisms per milliliter and stained by the addition of alcoholic crystal violet. Sterility, safety and potency were tested and found as good as other international antigens. The antigen was found to retain its quality for six months when preserved at room temperatures. The test was made by mixing one drop of the antigen with a drop of blood or a drop of serum, on a glass plate or white tile. The locally produced antigen was as good as antigens from Japan, Hungary, Holland and India. A serological study was conducted with the locally prepared antigen in different farms, and the incidence was 0-4% in government farms, 5-10% in commercial imported breeds and 0-3% in cross breed local farms respectively.

(**Key Words** : *Salmonella pullorum*, Antigen, Plate Test, Fowl Typhoid, Bangladesh)

INTRODUCTION

Pullorum disease and fowl typhoid are diseases of chickens caused by *Salmonella pullorum* and *S. gallinarum* of world wide significance. The economic importance of the diseases are more important in parts of the countries where poultry, are beginning to intensify their industry, such as countries in South and Central America, the Middle East, the Indian subcontinent, and parts of Africa (Pomeroy, 1984). Domestic poultry constitute the largest single reservoir of salmonella organism existing in nature. Among all animal species the salmonella are most frequently reported from poultry and poultry products (Barrow, 1993). Several measures are available for control. Immunization breeding stock with live attenuated vaccine can prevent infection and reduce the incidence of vertical transmission. However, the most widely used vaccine, the rough 9R strain (Gordon et al., 1959; Gordon and Luke, 1959; Harbourne et al., 1963; Silva et al., 1981; Smith, 1969 and Smith et al., 1981) still posses some virulence for some breeds of chicken (Gordon et al., 1959; Gorden and Luke, 1959; Silva et al., 1981 and Bouzouboaa et al., 1989) and protection is adequate only for 6 months. Such vaccines are not permitted in many countries such as the United States. Incorporation of antibiotics, such as furazolidone in the feed can prevent mortality and reduce the carrier rate but its extensive use over many years has led to the emergence of strains of *S. gallinarum* showing increased resistance to the drug (Smith et al., 1981; Stuart et al., 1967; Pomeroy, 1984 and Hall and Cartrite, 1961).

The most effective means of control of pullorum disease and fowl typhoid are a combination of stringent management procedures and eradication (Snoeyenbos, 1991). Many countries have eradicated the diseases by adopting national policy that have had a poultry industry for many years (Barrow et al., 1992). Eradication is normally done by identifying infected flocks and eliminating individual reactor birds by using a serological test, the control of salmonella infection in the hatchery and keeping of commercial and breeder stock free from infection (Barrow, 1993; Fahervari, 1994 and Tanaka, 1975).

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Serology is used because *S. gallinarum* and *S. pullorum* are not excreted extensively in the faeces (Jones, 1913). Agglutinations of *S. pullorum* cells by sera from chickens infected with this organism was demonstrated by Jones (1913) using a tube agglutination test. This was rapidly developed for large scale use and modified to provide a rapid slide test using serum (Runnels et al., 1927) and a stained antigen whole blood test (Schaffer et al., 1931). The test is easy to use and has largely eliminated these infections from many countries when used as a flock test. However, the test can yield erratic results which can be dependent on antigen quality (Barrow, 1992 and Tanaka, 1975).

With the great expansion of the poultry rearing and farming, pullorum and fowl typhoid has become wide spread problem in Bangladesh like other areas of the world (Amin 1969, Sarkar, 1976 and Rahman 1979). The incidence rate was 0-10%, mortality 40-50%, egg production reduced 20-30%, hatchability reduced 20-30% (Fehervari, 1994). In recent years, with the advent of mass poultry raising in this country, particularly when broilers are raised, the disease has become one of great economic importance. Heavy losses occur not only in broiler flock but also in laying birds due to morbidity, mortality, reduced production and poor chick quality. Mortality may vary from negligible to 10 to 80% or higher in severe outbreaks (Williams et al., 1990; Kumar and Kaushik, 1988; Kaura et al., 1990). Vaccine and antigens are imported from abroad and only the government farms are using them. On the other hand, to monitor diseases antigens are imported from abroad which are costly and could not reach on time to the farmers. So attempt was undertaken to prepare antigen from the local isolates for whole blood agglutination test in order to find out the incidence of the disease in different farms and breeds.

MATERIALS AND METHODS

(a) Isolation and identification of organisms :

Postmortem examination was conducted on 350 (three hundred and fifty) chickens in Central Diseases (Veterinary) Investigation Laboratory, Dhaka. The whole work was conducted during the period of 1994-1995. Related samples (liver, heart, ovary, spleen, bone marrow and caecal junction) were collected and inoculated in nonselective and selective media, such as Nutrient broth, Selenite broth, Tetrathionate broth, nutrient agar, MacConkey agar, Brilliant green agar, Desoxycholate citrate agar, Salmonella shigella agar. A total of 40 (forty) isolates of *S. pullorum* and *S. gallinarum* were isolated and identified on the basis of their cultural characteristics, morphological, serological and biochemical tests (OIE Manual, 1992, Carter and Cole, 1990, Malik, B. S. 1988, Cappuccino and Shermen, 1987). Biochemical tests were also conducted with API (Analytical Profile Index) system Kit from France and followed the manufacturing Companies Instruction.

(b) Production of antigen :

The antigen was prepared from smooth Salmonella pullorum colonies which have shown to be agglutinated by specific antisera and not by acriflavine or normal saline or normal serum but pathogenic for day old chicks. The method followed with the partial modification of OIE Manual, 1992. Typical colonies were seeded into Nutrient agar slopes, incubated at 37℃ for 24 hours, and the growth was emulsified in sterile normal saline which provided about 2 ml seed inoculum for each Roux flask. Instead of agar slope nutrient broth culture was also practiced. The flasks were then seeded with inoculum and evenly distributed by gently rocking. Adequate growth occurred within 48 hours.

The antigen was harvested by the addition of sterile glass beads and inactivated with 1% sterile formalsaline of 10 ml to each Roux flask. The flask was rocked until the growth was in suspension and was left in vertical position for 15 minutes. Then the suspension was transferred in a large flasks and treated with absolute alcohol at the ratio of 2:1 by volume. It was mixed and allowed to stand until the precipitation was completed. It was taken 5 days.

The clear supernatant fluid was drawn off and the cells stored at 0-4℃ for standardization. A small quantity of buffered formalsaline was added to make an even suspensions. 1 ml volume was diluted in saline to determine the concentration of cells present. The McFarland standard scale of Biomeurex France was used having number 70900 of 30331-FA/03/93 of tube number iv where concentration was 800 million organisms. The standardization was also done by plate method (Cappuccino and Sherman, 1987) by counting the colonies. The bacterial concentration was 800 millions. The method followed by partial modification of the process is described in OIE Manual, 1992 and Schaffer, 1931. For the preparation of stained antigen, the suspension was diluted in formalsaline (pH 6.5) to give in the final product, then 10% (V/V) glycerol and 1% (V/V) of 3% alcoholic crystal violet solution were added.

The comparative studies of different antigens were conducted on different antigens with standard reference sera with the locally produced antigen and sera in white tile and the procedure followed the OIE Manual, 1992.

(c) Control method of salmonella antigen :

For control methods of Salmonella antigen, the procedure followed by the OIE Manual, 1992 and Fahervari, 1994. The purity, safety and potency tests were done as follows :

(i) Purity test :

Before harvesting the *Salmonella pullorum* used for antigen production, cultures were examined under microscope for uniformity of bacteria. Gram staining was performed.

Evaluation :

No other form than Salmonella bacteria was allowed in the culture. After harvesting but before formalsaline treatment, nutrient agar, blood agar, MacConkey agar were inoculated and cultured for 24 hours. Colonies were disseminated over the surfaces allowing researcher to examine separate colonies.

Evaluation :

No other then typical Salmonella colonies were grown on the agar in good numbers.

(ii) Potency test :

Control of working capacity : The ready made antigen was tested with positive and negative *S. pullorum* sera and with saline as well as distilled water for agglutination. Slide agglutination test was tested. At the meantime known antigen (previous batch or proved product from abroad) was also submitted to the same agglutination as positive control. In case of emergence Salmonella serum other than pullorum (e.g. typhimurium, enteritis) were also added to the test.

Evaluation : Agglutination was allowed only with positive *S. pullorum* serum in both testing and known antigens. No agglutination or any slight precipitations were allowed with other materials than specific sera. The reaction appeared within one minute, optimally about 30 seconds but at least in the same time than the known antigen reacted.

Adjusting working dilution : For an optimal efficacy, the antigen was diluted with buffered formal saline up to the last dilution before the end dilution. Tenfold dilutions were prepared from the antigen and equivalent amount of positive serum was added to each dilution.

End dilution : The last dilution where the agglutination was complete, no little bacterial bottom was seen.

Working dilution : One dilution before the end dilution. A good quality of antigen was diluted up to 1:20 to 1:40.

(iii) Sterility test :

Ready made, formal saline treated antigen before adjusting working dilution and testing working capacity was submitted to sterility test. Antigen to be tested was inoculated on and into suitable culture media.

Media used : Solid nutrient agar, blood agar, MacConkey agar.

Liquid : Thioglycolate broth, 10 ml. per tube (reheated to 100 grade C for 10 minutes before use). Trypticase soya broth, 10 ml. per test tube.

Solid media : Incubated 48 hours after inoculation and evaluated every 6 hours for growth of germ.

Liquid media : Materia was taken in 0.5 ml. from the original antigen solution and inoculated into each of a least five culture media of the two type of broth mentioned above.

Culture condition : Thioglycolate broth was incubated at 30-35 grade C and observaed for 14 days. Tryptocase soya broth was also incubated in 5 tubes at 30-35 grade C, additional 5 tubes at 20-35 grade C, was observated for 14 days. When turbidity was seen subculture was prepared and 1 ml. of the turbid broth of a tube transferred to a fresh culture, and Incubated the subculture for seven further days.

Evaluation : The material passed the test if no growth of micro-organisms was observed in any of the media during the incubation period. The material failed the test if growth was observed in any of the media and product was not allowed to be used as antigen but it has to be discrete.

(d) Blood agglutination test in different government farms and non-government farms and commercials farms :

Blood samples were collected from the wing vein with a disposable syringe. A drop of crystal violet stained antigen was put on a white tile and a similar drop of fresh whole blood was added to it. The plate was gently rocked and the results read within 2 minutes. Visible clumping of antigen within one minute was a positive reaction, but if there was no clumping within 2 minutes the sample negative. The antigen was tested against known positive and negative control sera. The samples which were shown positive were further tested using tube agglutination test.

(e) Preservation of antigen :

The prepared antigen was preserved in different temperature for six months i.e. 0-4 C, 10 C and room temperature. The antigens were carried in the field both cool chain system and at room temperature.

RESULTS AND DISCUSSION

Isolation and Identification of *Salmonella pullorum* :

A total forty isolates were identified, of which 18 were *S. pullorum* and 12 were *S. gallinarum*. *S. pullorum* was mainly identified in young chicks and *S. gallinarum* was mostly found in older ages. *Salmonella pullorum* isolate was identified which was gram negative coccobacillary tappary rod. It was small, smooth, transparent colourless colonies in MacConkey agar media, produced very small colonies in DCA produced minute strain pale red and smooth colonies in BG agar. This isolate agglutinated with polyvalent O antisera (A-G) and also with group specific antisera of 0:9 in Group D. Biochemically it produced acid with glucose, fermented mannitol and rhamnose and decarboxylase ornithine. It did not ferment lactose, sucrose, salicin, maltose and dulcitol. There was no reaction with indole and urease. It was nonmotile in semisolid media. Besides the cultural characteristics and serology, *S. pullorum* was distinguished from *S. gallinarum*, mainly by biochemical tests, i.e., *S. pullorum* decarboxylased ornithine, whereas *S. gallinarum* fermented maltose and dulcitol. This findings agreed with the findings of Snoeyenbos, G. H., 1992 and OIE Manual, 1992.

Comparative studies of different antigen :

A comparative study was conducted with the local antigen and imported ones from Japan, Hungary, Holland and India. Positive agglutination was found 30 seconds to 60 seconds with different reference sera such as Polyvalent O from Murex, *Salmonella pullorum* antisera from Hungary and known sera prepared in our laboratory. It was revealed from the findings that the locally produced antigen was as good as the imported ones.

From the table 1, it was found that with the Hungarian *Salmonella pullorum* sera, the antigens from India, Hungary, Japan, Local type (Bangladesh) and Netherlands was agglutinated within 6, 6, 3, 7 and 7 seconds respectively. With the Murex Polyvalent O antisera the agglutination was occurred within 17, 35, 15, 15 and 17 seconds respectively. Known sera collected from infected birds showed agglutination within 20, 75, 22, 22, and 20 seconds respectively. In addition, agglutination reactions were not seen with the control or negative sera within 2 minutes.

The rapid whole blood agglutination test was very reliable for identifying infected chickens when it was employed 20 or more days after infection (Smith, 1956, Bourhy et al., 1988) which agreed with our findings.

Moreover rapid whole blood test was the most widely applied procedure for detecting *S. pullorum* and *S. gallinarum* infections in chickens because it could be used under field conditions and the birds had to be handled only once (Williams, et al., 1990). The deep staining of the bacteria appeared to be a distinct advantages. It permitted the use of the white background as a contrast to

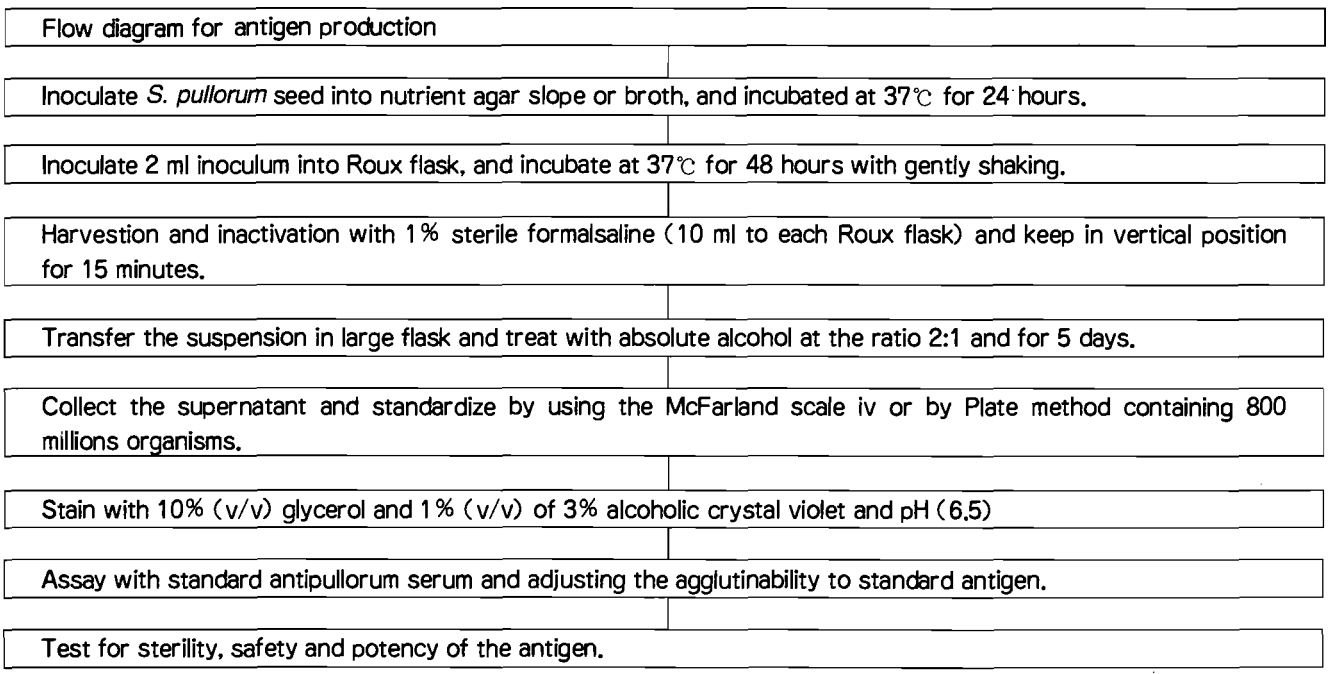

Figure 1. Procedure for preparation of the pullorum antigen for whole blood test.

the strain on the bacteria. The reflecting power of the white background made the reading of the test less difficult and the violet coloured clumps of the agglutinated bacteria stand out in sharp contrast to the red blood. The crystal violet also added in the preservation of the antigen. The present findings agreed with the findings of Scheffer, 1931.

Table 1. Comparative study of different antigens with slide agglutination test

Types sera	Types of antigens (Agglutination shown within 0-60 seconds)				
	Indian	Hungarian	Japanese	Local	Netherlands
1. Hungarian salmonella pullorum	6	6	3	7	6
2. Murex polyvalent O	17	35	15	15	17
3. Known Sera (Positive)	20	75	22	22	20
4. Unknown (Negative control)	120	120	120	120	120

Preservation of antigen :

The keeping quality of the stained antigen at different temperature and transport system showed that within six months its keeping quality was good. After one year without refrigeration there was little change in sensitiveness. These results agreed with the findings of the Schaffer, 1931. It was also observed that antigen showed better sensitivity after one weeks of preparation and retained up to six months.

Table 2. Incidence of salmonella infection in different farms

No. of govt. farms	No. of non-govt. farms	No. of commercial breed farms	No. of blood samples tested	Positive cases (%)
7	–	–	611	132 (2.86%)
–	2	–	570	–
–	–	2	300	30 (10%)

Results of whole blood agglutination test :

It is reveled from the findings that a total number of 4611 blood were tested from the government and 570 samplers from non-government farms and found 132 (2.87) and 0% positive reaction whereas in commercial farm twelve hundred samples tested and found 120 (10%) sera agglutinations.

From table 3, in government farms the highest incidence was 7.5% in Central breeding farm, Dhaka, followed by Jessore Regional farm (4.8%), Tangail Regional farm(4.28%) and Kishorgong Regional farm (2.15%). In case of Noakhali and Chittagong poultry farms 320 and 600 birds were examined and none were found positive.

The blood samples were also collected from non-government organization of BRAC unit from Rajshahi of which samples were tested and reactors were not found. The incidence of salmonella organism was high (10%) in imported broiler and layer breeds. From India. Ghosh and Panda. 1988 reported that the incidence rate was 4.05% in Orissa which has the more or less similar findings with us. The incidence in Central Poultry farm is quite alarming because most of the farms get their chick from this farm and there is chance to spread the disease whole country. On the other hand, the commercial farms are spreading disease in the whole country. So, we strongly recommend to test the whole flock, government farms and commercial hatcheries with pullorum antigens developed by us.

Table 3. Incidence of salmonella on the basis of farms

Name of the farm	No. of sample tested	Positive cases (%)
Rajshahi Regional poultry farm	1160	0
Tangail Regional poultry farm	350	15 (4.28)
Kishorgong Regional poultry farm	556	12 (2.15)
Jessore Regional poultry farm	625	30 (4.8)
Noakhali Regional poultry farm	320	0
Dhaka Central poultry farm	1000	75 (7.5)
Chittagong Regional poultry farm	600	0
BRAC unit Rajshahi	330	0
BRAC unit Charghat	240	0
Commercial farms	1200	120 (10%)

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