



Evaluation of immune response of broiler chicks against *Salmonella* Gallinarum infection

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The present study was performed to evaluate the pattern of immune response of susceptible broiler chicks after *Salmonella* Gallinarum infection. Experimentally infected birds with positive strains of *Salmonella* Gallinarum which remained alive were used. Immune responses in them were studied on day 14th, 21st and 28th days post-infection. Six (6) control chicks were also examined.

Total lymphocyte count: The method described by Chauhan and Verma (1983) was used. Peripheral blood was collected from the wing vein of 8 recovered and 6 control chicks. Two ml of diluted blood was layered slowly over 1 ml of Ficoll-paque and kept in a glass tube of 8 mm diameter. The tubes were centrifuged at 400 *g* for 30 min in a refrigerated centrifuge. The lymphocytes and monocytes formed a white layer, which was separated completely with a Pasteur pipette and the volume of suspended fluid was measured. The number of cells per unit volume of the suspension was counted with haemocytometer and was expressed as lymphocyte count per microlitre (μ l) of blood. The counts from recovered and control chicks were made on 14th, 21st and 28th days post-infection.

Evaluation of cell mediated immune response: Direct fluorescent antibody technique was used for T-lymphocyte count as per Chauhan and Verma (1983).

Raising of anti-thymocyte serum: Anti-thymocyte serum was raised in two chinchilla rabbits as per the methods described by Shivanandan and Maheshwaran (1980) with slight modification. The viable cell count was made by trypan blue dye exclusion technique as per the method described by Hudson and Hay (1978) and the number of cells in suspension was adjusted to 1×10^9 cell/ml. Over 90% cells were found to be viable. For immunization, 3 injections of thymocyte cell suspension were given to the rabbits. Each

injection contained 1×10^9 viable cell/ml. Primary inoculation to the rabbits was done intramuscularly into the thighs and all the foot pads after emulsification of cell suspension with equal amount of Freund's complete adjuvant (1 ml cell suspension + 1 ml adjuvant). The second and third injections @1/rabbit without adjuvant were given intravenously on 21st and 28th days. Rabbits were bled one week of last injection. Sera were collected, pooled and heated in water bath at 56°C for 30 min to inactivate the complement. Inactivated anti-thymocyte serum was stored at 0°C. The anti-thymocyte serum was absorbed as per Albini and Wick (1974). After adsorption the chicken RBCs were removed by centrifugation. Anti-thymocyte serum was extensively cross adsorbed with 3 volumes of bursal cells at 4°C overnight to remove the antibodies against antigenic components of B-cells, if any. The bursa cells were removed by centrifugation after adsorption. Thereafter, 1 ml of anti-thymocyte serum was finally adsorbed with 2 ml of chicken γ (gamma)-globulin to remove antibodies against immunoglobulin surface determinants. Isolation and purification of immunoglobulin was carried out as per Nowotny (1979) with minor modifications. Adsorbed and purified immunoglobulins against thymocytes (anti-thymocyte serum) were conjugated to FITC as per Johnstone and Thorpe (1982) and Chauhan and Verma (1983) with slight modification. The protein content of immunoglobulin was determined according to Lowry *et al.* (1951). FITC (0.05 mg/mg of protein) was added to the immunoglobulin solution. The mixture was stirred with magnetic stirrer overnight at 4°C for complete conjugation. The mixture was passed through Sephadex G-25 column already equilibrated with 0.15 M PBS, pH 7.2. Fractions of elute of 5 ml were collected in succession. Two colored fractions were visible during elution. The first colored fraction containing conjugated globulins were retained. The conjugated globulin was stored at –20°C in small vials for future use. The optimum dilution of conjugate was determined by serial 2-fold dilutions of conjugate on thymocyte smear. The working dilution of anti-thymocyte serum was 1: 10 of the original conjugate.

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As described earlier, lymphocytes separated from 8 recovered and 6 control chicks on 14th, 21st and 28th days post-infection were considered for absolute count. Smears of lymphocytes were fixed in chilled acetone for 10 min and air-dried. After drying, they were washed twice or thrice with 0.15 M PBS, pH 7.2 and stained with 2–3 drops of 1: 10 diluted anti-thymocyte globulin conjugated with FITC and incubated in a moist chamber at 37°C to prevent the slides from drying. Slides were removed from incubation after 2 h, washed twice or thrice with 0.15 M PBS, pH 7.4. Then 1–2 drops of 50% glycerin buffer saline, pH 7.4 were poured over the smear and then it was covered with cover slip. This smear was observed under fluorescent microscope. At first, total number of lymphocytes was counted in a field under tungsten light and then the numbers of fluorescing cells were determined in the same field under UV-light. Percentage of T-lymphocytes was calculated. The number of T-lymphocytes/μl blood was calculated on the basis of percentage of T-lymphocytes counted.

Graft versus host reaction (GVHR): The procedure described by Chauhan *et al.* (1984) was followed. The chicks were weighed and sacrificed after 96 h of injection. The spleens of chicks were removed and weighed. Spleen indices were collected with the help of formula given below:

$$\text{S.I.} = \frac{\text{Spleen weight (in g)}}{\text{Body wt. of inoculated chicks (in g)}} \times 100$$

Nitroblue tetrazolium (NBT) salt test with spleen smear: The test was performed as per Reddy and Rajan (1985) and Singh *et al.* (1990). This test was performed on the day of termination of experiment after sacrificing the experimental chicks i.e. on 28th day post-infection. The cells in smears incubated with NBT salt solution were examined for NBT

positive macrophages with formazan deposits in their cytoplasm and calculated from 400 macrophages in each smear based on their morphological character.

Evaluation of humoral immune response: β-lymphocyte count: The method described for β- lymphocyte count was followed with slight modification. Anti-bursal cell serum (ABS) was cross adsorbed with thymocytes to remove the antigens against antigenic components of T-cells, if any. The other procedural details for purification, conjugation and staining were similar as described for T-lymphocyte count.

Total lymphocyte count: The mean values of total lymphocyte count in broiler chicks in group 1 (infected) and group 2 (control) are presented in Table 1. The values obtained on all 3 days of examination from infected chicks were highly significant ($P < 0.01$) when subjected to 't' test.

In the birds and mammals, the increase in the number of circulating lymphocytes may be an indication of stimulation of immune competence of the animals. Yanluzov (1977) recorded that after inoculation of killed *S. Gallinarum* vaccine in 5 months old Canadian Leghorn pullets, the white cell counts were similar after infection or vaccination and consisted of a brief rise in leukocytes, pseudoeosinophils and fall in these counts in comparison with unrelated controls on first and second days. The lymphocyte count increased again between 4th and 25th days post-infection.

Detection of cellular immune response: The mean T-lymphocyte counts of infected and control groups are presented in Table 2. T-cells showed characteristic greenish yellow fluorescence when stained with anti-thymocyte serum tagged with FITC. Values obtained on all three days of examination and the average values of pooled data of all three days of examination from infected groups were found to be highly significant ($P < 0.01$).

Table 1. Effect of *Salmonella* Gallinarum infection on mean total lymphocyte count* in broiler chicks

Group	Days post-infection			
	14	21	28	Overall
1 (infected)	13875.00 ^a ±143.34 (8)	14475.00 ^a ±91.43 (8)	14875.00 ^a ±96.42 (8)	14408.33 ^a ±08.50 (24)
2 (control)	13300.00 ^b ±62.36 (6)	13366.67 ^b ±50.92 (6)	13516.67 ^b ±49.55 (6)	13394.44 ^b ±39.17 (18)
t-value	3.68**	10.59**	12.53**	7.79**

* per ml of blood; (** $P < 0.01$); values bearing different superscripts in a column for an effect differed significantly; figures in parentheses indicate the number of observations.

Table 2. Effect of *Salmonella* Gallinarum infection on mean T-lymphocyte count* in broiler chicks

Group	Days post-infection			
	14	21	28	Overall
1 (infected)	7509.95 ^a ±65.45 (8)	8818.09 ^a ±21.48 (8)	9637.10 ^a ±100.74 (8)	8650.05 ^a ±187.33 (24)
2 (control)	6946.72 ^b ±68.35 (6)	7060.95 ^b ±34.97 (6)	7215.97 ^b ±37.07 (6)	7074.55 ^b ±39.64 (18)
t-value	5.95**	42.87**	22.56**	7.19**

*per ml of blood; (** $P < 0.01$); values bearing different superscripts in a column for an effect differed significantly; figures in parentheses indicate the number of observations.

Table 3. GVHR showing mean splenic indices (SI) in recipient chicks

Source of donor lymphocytes	Weight of spleen (g)	Body weight (g)	S.I.
Group 1 (infected)	0.0304±0.0020(48)	35.2583±0.4444(48)	0.0847±0.0064(48)
Group 2 (control)	0.0155±0.0004(36)	33.8972±0.5666(36)	0.0458±0.0009(36)
Group 3 (negative control)	0.0063±0.0007(6)	30.0533±0.5258(6)	0.0191±0.0019(6)

Figures in parentheses indicate the number of observations.

With cyclophosphamide immunosuppression, Cameron *et al.* (1974) reported no reduction in tuberculin sensitivity and immune response against *S. Gallinarum* infection suggesting the possible role of cell-mediated immunity against Fowl typhoid, but the response was found unaffected when birds were thymectomized or treated with Anti-thymocyte serum. Jaiswal *et al.* (1981) while working with live *S. Gallinarum* Freund's complete adjuvant vaccine concluded that cell-mediated immunity which is attributed to be thymus dependent in chicks might play a major role in the resistance. Yanluzov (1977) had reported a brief rise in the count of leukocytes, pseudoeosinophils and lymphocytes at 8th hour and a fall in these counts (in comparison with unrelated controls) on first and second days and again an increase in lymphocyte count between 14th and 25th days after inoculation of a killed *S. Gallinarum* vaccine in 5 months old Canadian Leghorn pullets.

Graft versus host reaction (GVHR)

Mean splenic indices of inoculated chicks were presented in Table 3. Analysis of variance revealed that mean values of group 1 differed significantly ($P<0.01$) with group 2 and Group 3 (Table 3). The critical difference test or 'd.t'. (analysis of variance) revealed that splenic indices of all 3 groups differed significantly ($P<0.01$) from each other. Analysis of data also indicated that body weight did not affect the SI as there were nonsignificant differences between the body weights of chicks of groups 1, 2 and 3.

Lamont and Smith (1984) also used to study GVH spleenomegaly for evaluation of CMI response in chicken. In the present study, GVHR was used to study the T-cell dependent immune response in *S. Gallinarum* infection. The SI (Table 3) was highest in infected group followed by control group. SI of infected group as compared to control group and negative control group were significant whereas the values of control and negative control groups did not differ significantly. As per the critical difference test of the same data SI of all the 3 groups differed significantly ($P<0.01$) from each other. It is concluded that the reaction in the spleen could be attributed to lymphocytes and not to the Tyrode's solution used for suspending the lymphocytes. Therefore, the nonsignificant difference between control and negative control was only due to the variation in the number of observations recorded. The highest SI in the infected group in GVHR spleenomegaly could be a measurement of

increased CMI response in the chicks recovered after *S. Gallinarum* infection.

Nitroblue tetrazolium (NBT) salt test: The mean number and percentage of NBT-positive cells in the two experimental groups are presented in Table 4. The higher counts were recorded in group 1 (infected). The 't' test revealed that the two groups differed significantly from each other ($P<0.01$).

The functional activity of splenic macrophage was observed by performing the NBT salt reduction test on splenic smears of experimental chicks. The observation was significant ($P<0.05$) indicating increased activity of splenic macrophages in *S. Gallinarum* infection. Allan and Duffus (1971) observed increase in the total WBCs, heterophils and monocytes or circulating macrophages counts 2–3 times than the normal value while chicks suffering from acute infection of *S. Gallinarum*. With the increase in phagocytic activity in *S. Gallinarum* infection, the authors are of the opinion that macrophages play an active role in protection against Fowl typhoid. This is a general agreement in both medical and veterinary field that bacterial infection enhances the NBT positivity and therapeutic amelioration of the condition reduces the number of NBT positive cells to almost normal level (Khalifa *et al.* 1984).

Detection of humoral immune response: The mean values of B-lymphocyte in broiler chicks in group 1 (infected) and group 2 (control) are represented in Table 5. When subjected to 't' test the difference in B-cell count was not significant on all 3 days of examination.

Bursa dependent lymphocytes (B-cells) are the precursors of humoral immunity. Though rise in counts of B-cells in infected chicks was statistically non-significant, the present findings regarding B-cell count and its correlation in pathogenesis of *S. Gallinarum* infection appear to be the only of its kind to illustrate the role of circulating B-cells in the pathogenesis and protection.

Table 4. Effect of *Salmonella* Gallinarum infection on mean number of NBT positive cells in broiler chicks

Group	NBT positive cells	Percentage of NBT positive cells
1 (infected)	133.57±5.02 (7)	35.27 (33.40%)±0.76 (7)
2 (control)	113.40±4.49 (5)	32.14 (28.35%)±0.73 (5)
t-value	2.99*	2.98*

(* $P<0.05$); Figures in parentheses indicate the number of observations.

Table 5. Effect of *Salmonella* Gallinarum infection on mean B-lymphocyte count* in broiler chicks

Group	Days post-infection			
	14	21	28	Overall
1 (infected)	3528.99±26.92 (8)	3511.03±25.11 (8)	3501.21±26.88 (8)	3513.75±15.70 (24)
2 (control)	3445.26±48.16 (6)	3489.42±64.48 (6)	3548.03±32.26 (6)	3495.24±31.44 (18)
t-value	1.52NS	0.31NS	1.12NS	0.57NS

*per ml of blood; NS, not significant; figures in parentheses indicate the number of observations.

SUMMARY

The present study was performed to evaluate the pattern of immune response of susceptible broiler chicks after *Salmonella* Gallinarum infection. The study indicated that both antibody and cell mediated immune responses are operative in protection against *Salmonella* Gallinarum infection. From the present study, it was concluded that vaccine formulation having ability to stimulate both the cellular and humoral immune responses can be developed against fowl typhoid for effective immunization of poultry.

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