HARINGHATA BLACK CHICKEN SHOWS RESISTANCE TO PATHOGENIC E. coli BY HIGH IMMUNE-EFFECTOR ACTIVITIES

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ABSTRACT

To substantiate the popular notion regarding disease-resistance potentiality of native breed of poultry against pathogens, immune responses in one native breed of India (Haringhata Black, HB), one of best adapted exotic breed of backyard system (Rhode Island Red, RIR) and commercial broiler birds against virulent E. coli were assessed upon experimental inoculation. Immunological parameters measured were superoxide production by heterophils, macrophage phagocytic activity, in vitro lymphoproliferation and antibody production against the antigens of E. coli. HB and RIR birds showed more cellular immune responses than that of broiler. Moreover, anti-E. coli antibody production was observed highest in HB than RIR and broiler as assessed by ELISA. Clinical manifestations in form of diarrhoea, roughened feather, rise of temperature as observed in broiler and RIR were not detected in HB birds. It is concluded that high immune responses shown by HB birds against virulent E. coli might be the driving factor of disease resistance as opposed to broiler and RIR birds where clinical symptoms were discernible.

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1 Introduction

The poultry and farm animals are considered as major reservoir of *E. coli* and *Salmonella* throughout the world without any clinical syndrome (Girlich et al., 2007; Carattoli, 2008; Watson et al., 2012; Samanta et al., 2014a). However, mode of disease resistance generated in native breeds of poultry against *E. coli* and its underlying immunological mechanism is not yet known (Samanta et al., 2014b). Therefore, it is quite expedient to study immune response patterns in native breeds of poultry to explore their scale of disease resistance against pathogenic *E. coli* (Samanta et al., 2015). Further, reports on antigenic characteristics of the target pathogen are scarce that might be useful while considering immunodiagnostic and/or immunoprophylactic preparations against such pathogen (Roy et al., 2015). With this background the present study was conducted to assess cellular and humoral immune responses upon experimental inoculation of pathogenic *E. coli* in one native breed (Haringhata Black, HB), one of best adapted exotic breed of backyard system (Rhode Island Red, RIR), and commercial broiler birds in West Bengal, a major backyard and native breed rearing state in India.

2 Materials and Methods

2.1 Experimental bird

Rhode Island Red, Haringhata black and Broiler birds (3 months old) were used as experimental birds procured from the University Farm at Mohanpur, Nadia. Each variety of birds was divided into two groups viz. control group & sensitized group, and each group contained 6 birds.

2.1 Experimental design

All the experimental birds were given normal feed (Amrit feed\(^TM\), India) containing maize, soyabean, ground nut cake, mineral mixture, vitamin up to the end of the experiment with *ad libitum* feed and water. The period of observation was started from the day of inoculation (0-day) with subsequent post inoculation periods, viz. 0, 2\(^{nd}\), 7\(^{th}\), 14\(^{th}\) and 21\(^{st}\) days after inoculation. The Institute Ethics Committee of West Bengal University of Animal and Fishery Sciences, India approved this study.

2.3 Sensitization of experimental bird

After 7 days of acclimatization in experimental cages of the Department, all the birds of one group (sensitized) of each variety were inoculated (IP) with virulent field isolate of ESBL producing *E. coli* [strain- SK-3, serogroup - O62, genotype- bla _TEM_ (+ve), bla _CTX-M_ (-ve), bla _SHV_ (-ve)] bacteria (10\(^5\) CFU/ml dose). The field isolate (SK-3) was obtained from a local broiler (29 days old) which was suffering from diarrhoea, fever, roughened feather and was un-responding to higher group of cephalosporin antibiotics. Another group of birds were kept as control. The birds were maintained for observation up to 21 days. Neither the sensitized group nor the control group of birds in the present study for each category (HB, RIR, Broiler) received any kind of antibiotic during the study period.

2.4 Antigen

The somatic soluble antigen of the *E. coli* isolate (SK-3) was extracted by ultrasonication on ice at 150W with repeating duty cycles and 0.5 sec pulse pressure for two min with 30 sec interval (five times) using an ultrasonicator (Hielscher Ultrasonics GmbBH, Germany). The soluble sonicated extract was centrifuged at 10,000 g for 30 min at 4°C and the supernatant was collected as antigen as described earlier (Choi et al., 1989). The somatic soluble antigen was kept at -20°C for further use.

2.5 Protein Estimation

Protein concentration of somatic soluble antigen was estimated using commercially available protein estimation kit (Merck Biosciences, India). The absorbance was measured at 660 nm by UV-VIS Spectrophotometer (TechComp, Taiwan).

2.6 Leucocyte functional assay

To measure non-specific immune response of poultry birds against pathogenic *E. coli*, oxidative radical production by heterophils was determined by the nitroblue tetrazolium (NBT) (SRL, India) reduction assay at different days post inoculation (DPI) period (Siwicki et al., 1998).

2.7 Isolation of PBMC

Heparinised blood was diluted with PBS at 1:1 ratio. For isolation of peripheral blood mononuclear cell (PBMC), diluted blood was layered onto HiSep\(^TM\) (Himedia, India) at the ratio of 2:3 (2 part of HiSep and 3 parts of cell suspension) and centrifuged for 30 min at 400g (Chung & Secombs, 1988). Using density gradient centrifugation, mononuclear cells from blood of poultry of the three varieties were isolated at different DPI as earlier. Enumeration of viable cells was done using Trypan blue dye exclusion method. In most of the cases > 90 % of the cells were viable.

2.8 Phagocytosis assay

*In vitro* phagocytosis made by blood monocytes of the inoculated poultry birds was examined in different DPI as per Yoshida et al. (1993). The Phagocytic activity (PA) was determined as per Findlay & Munday (2000) using the following formula-

\[
PA = \frac{\text{Number of phagocytic cell}}{\text{Total number of phagocytes}} \times 100 \text{ (expressed as %)}
\]
2.9 Stock solutions for ConA and bacterial antigen

Stock solution of concanavalin A (Con A) was prepared at the concentration of 1 mg/ml of the growth medium (RPMI-1640), filtered through a sterile membrane filter (0.22µ) and stored at -20°C for future use. Stock solution of bacterial somatic soluble antigen was prepared at the concentration of 40µg/ml with the growth medium (RPMI).

2.10 Lymphoproliferation Assay (LPA)

PBMC (2 x 10^6 cells/ml) were suspended in RPMI-1640 and 100 µl of cell suspension was dispensed into wells of 96-well tissue culture plates. The final volume of each well was made up to 200 µl with somatic antigen (20µg/ml), Con A (10 µg/ml) (Sigma-Aldrich, USA) in positive control well and growth medium in negative control well. Finally, the plate was incubated at 37°C for 72 hr under 5% CO₂ tension. The colorimetric 4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, USA) was used to determine the proliferation of blood lymphocytes in response of somatic antigen.

2.11 Indirect Enzyme Linked Immunosorbent Assay (i-ELISA)

To detect anti- E. coli antibody in serum samples of RIR, HB and broiler birds (collected at 21 DPI), plate ELISA was performed based on the principle of indirect-ELISA as per Mockett et al. (1987) with some modifications.

2.12 Statistical analysis

The results of leukocyte functional assay, phagocytosis assay and lymphoproliferation assay were expressed as the mean±standard error of mean (SEM) and analysed using SPSS 21 (SPSS Inc. Chicago, USA).

3 Results and Discussion

Present study was aimed to detect changes in immune effector activities in three breeds of poultry viz. Haringhata Black, a native breed of India, Rhode Island Red, a backyard breed and broiler bird upon experimental inoculation of pathogenic E. coli isolate. The study revealed the comparative role of immune effector activities in three different poultry breeds by which they can resist pathogenic E. coli. The E. coli strain (SK-3) was selected for experimental inoculation because it was isolated from a local diseased broiler. Further, the isolate possessed bla TEM gene which is one of the major ESBL genes produced by E. coli (bla TEM, bla SHV, bla CTX-M) and the bacteria of reservoir poultry chiefly harbours bla TEM (Olsen et al., 2014).

The broiler birds inoculated with pathogenic E. coli showed the clinical syndrome such as high fever, roughened feather and diarrhea within 7 days. In RIR birds intensity of diarrhea was less than the broiler birds. HB chickens didn’t show any observable clinical sign and symptom. After 30 days, sensitized broiler birds died of the infection, although both of the sensitized HB and RIR birds survived. Probably the higher disease resistance potential in HB birds was responsible for the development of less severe clinical manifestation which was investigated in present study.

Super oxide anion produced by heterophils in E. coli inoculated Haringhata Black, Rhode Island Red and Broiler birds was significantly (p<0.05) higher during first 15th DPI, however, lower in 21st DPI in all the variety in comparison to the control birds (Table-1). Higher super oxide anion production by leucocytes of all the sensitized birds was corroborated with earlier works where significantly enhanced super oxide anion production was reported in treated chicken and turkey than control groups (Lowry et al., 2005; He et al., 2008; Paul et al., 2012).

Table 1 Assessment of super oxide production by blood leukocytes of E. coli inoculated (sensitized) and control birds at different days post inoculation by NBT reduction test.

<table>
<thead>
<tr>
<th>Types of bird</th>
<th>Status of bird</th>
<th>0-day</th>
<th>2nd day</th>
<th>7th day</th>
<th>14th day</th>
<th>21st day</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB</td>
<td>Sensitized</td>
<td>0.426±0.009⁷</td>
<td>1.892±0.005⁷</td>
<td>1.745±0.007⁷</td>
<td>1.085±0.183⁷</td>
<td>0.490±0.003⁷</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.413±0.003⁷</td>
<td>0.399±0.002⁷</td>
<td>0.406±0.001⁷</td>
<td>0.418±0.009⁷</td>
<td>0.421±0.005⁷</td>
</tr>
<tr>
<td>RIR</td>
<td>Sensitized</td>
<td>0.321±0.004⁷</td>
<td>1.576±0.003⁷</td>
<td>1.559±0.005⁷</td>
<td>1.168±0.040⁷</td>
<td>0.315±0.008⁷</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.318±0.004⁷</td>
<td>0.347±0.006⁷</td>
<td>0.325±0.007⁷</td>
<td>0.321±0.008⁷</td>
<td>0.319±0.011⁷</td>
</tr>
<tr>
<td>Broiler</td>
<td>Sensitized</td>
<td>0.495±0.022⁷</td>
<td>1.128±0.001⁷</td>
<td>1.221±0.004⁷</td>
<td>1.022±0.029⁷</td>
<td>0.530±0.005⁷</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.487±0.010⁷</td>
<td>0.469±0.004⁷</td>
<td>0.413±0.003⁷</td>
<td>0.492±0.010⁷</td>
<td>0.471±0.010⁷</td>
</tr>
</tbody>
</table>

HB- Haringhata Black, RIR- Rhode Island Red
Results shown are mean of six observations ± SEM
The mean bearing different superscript in the same row and column differs significantly (p<0.05)
Table 2 Phagocytic activity of PBMC of *E. coli* inoculated (sensitized) and control birds at different days post inoculation

<table>
<thead>
<tr>
<th>Types of bird</th>
<th>Status of bird</th>
<th>0-day</th>
<th>2nd day</th>
<th>7th day</th>
<th>14th day</th>
<th>21st day</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HB</strong></td>
<td>Sensitized</td>
<td>15±0.032&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22±0.023&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21±0.023&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19±0.024&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27±0.033&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>15±0.023&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16±0.021&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14±0.026&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14±0.031&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16±0.028&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>RIR</strong></td>
<td>Sensitized</td>
<td>18±0.024&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24±0.022&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25±0.021&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24±0.025&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30±0.031&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>17±0.026&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15±0.030&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16±0.032&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15±0.032&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16±0.027&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Broiler</strong></td>
<td>Sensitized</td>
<td>13±0.023&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19±0.022&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18±0.028&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17±0.028&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13±0.023&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>12±0.031&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11±0.033&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12±0.032&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11±0.034&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14±0.024&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

HB-Haringata Black, RIR-Rhode Island Red

Results shown are mean of six observations ± SEM

The mean bearing different superscript in the same row and column differ significantly (p<0.05)

The phagocytic activity (PA) of PBMC of *E. coli* inoculated and control HB, RIR and Broiler birds are presented in Table - 2. The sensitized broiler birds showed little higher PA values (19±0.022) at 2nd DPI which was decreased (13±0.023) in 21<sup>st</sup> DPI. Whereas, sensitized HB and RIR birds showed higher PI values at 2<sup>nd</sup> (22±0.023 and 24±0.022), 7<sup>th</sup> (21±0.023 and 25±0.021), 14<sup>th</sup> (19±0.024 and 24±0.025) and 21<sup>st</sup> (27±0.033 and 30±0.031) DPI than the control birds. In short, higher phagocytic activity of HB and RIR PBMC was observed than the broiler birds and it was maintained up to 21<sup>st</sup> DPI. Similar significant higher phagocytic potential of macrophages in treated chicken was reported by previous workers against *E. coli* (Qureshi, 1998).

Lymphocyte proliferation up conA and antigen stimulation of blood leukocytes of experimental birds are expressed as stimulation index (Table -3). Sensitized HB birds showed similar SI values on the first day (0.413 & 0.359) with that of control birds (0.401 & 0.343) but it significantly (p<0.05) differed on 14<sup>th</sup> and 21<sup>st</sup> DPI. An increase in stimulation index was noticed on 14<sup>th</sup> DPI (0.478 & 0.462) and 21<sup>st</sup> DPI (0.522 & 0.517) post inoculation. Sensitized RIR birds showed similar SI values on the first day (0.413 & 0.359) with that of control birds (0.439 & 0.285) but it significantly (p<0.05) differed on 14<sup>th</sup> and 21<sup>st</sup> DPI. An increase in stimulation index was noticed on 14<sup>th</sup> DPI (0.478 & 0.327) and 21<sup>st</sup> DPI (0.534 & 0.473). Sensitized broilers showed similar SI values (0.237 & 0.192) with that of control (0.194 & 0.183) on 0-day but it significantly (p<0.05) differed on 14<sup>th</sup> DPI. An increase in stimulation index was noticed on 14<sup>th</sup> DPI (0.363 & 0.301) but that decreased at 21<sup>st</sup> DPI (0.218 & 0.183). Similar significant enhanced proliferation of blood leukocytes was detected in treated broiler chicken (Lee et al., 2007). Moreover, in vitro lymphocyte proliferation of peripheral blood leukocytes (PBL) was reported in treated broiler chicken that gave 100% protection in IBD virus challenge (Tayade et al., 2006). In the present study, high specific cellular stimulation (in form of lymphocyte proliferation) was noted up to 21<sup>st</sup> DPI in HB birds than RIR and broiler that might be related to its enhanced immune potential.

The seroreactivity expressed as O.D. values were 0.82±0.021 in sensitized HB birds, 0.74±0.032 in sensitized RIR and 0.47±0.017 in sensitized broiler birds (Figure 1) with experimental sera at 1:400 dilution, respectively. Whereas, the O.D. values were detected from 0.58±0.032; 0.53±0.022 and 0.43±0.025, respectively for the same range of dilution with the sera of control birds. The humoral immune -effector activity measured by indirect ELISA to detect anti- *E. coli* antibody in serum samples of RIR, HB, and broiler birds showed enhanced seroreactivity in all the sensitized than the control birds. Similar enhanced seroreactivity in *E. coli* sensitized birds were observed by Mukherjee (2006) and Mitra (2007) using i-ELISA. In the present study, highest seroreactivity was noted in HB birds compared to RIR and broiler birds indicating more immune-potential of the breed.

Table 3 Lymphoproliferation assay of PBMC of *E. coli* inoculated (sensitized) and control birds at different days post inoculation

<table>
<thead>
<tr>
<th>Types of bird</th>
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<th>0-day</th>
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<th>14th day</th>
<th>21st day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ConA</td>
<td>Antigen</td>
<td>ConA</td>
<td>Antigen</td>
<td>ConA</td>
</tr>
<tr>
<td><strong>HB</strong></td>
<td>Sensitized</td>
<td>0.41±0.009&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.359±0.016&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.471±0.017&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.402±0.021&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.40±0.006&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.343±0.008&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.400±0.009&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.384±0.006&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>RIR</strong></td>
<td>Sensitized</td>
<td>0.43±0.006&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.285±0.009&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.421±0.014&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.327±0.013&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.42±0.010&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.273±0.003&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.422±0.010&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.264±0.003&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Broiler</strong></td>
<td>Sensitized</td>
<td>0.23±0.010&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.192±0.004&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.269±0.002&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.301±0.006&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.19±0.007&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.183±0.005&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.231±0.005&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.175±0.006&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

HB-Haringata Black, RIR-Rhode Island Red

Results shown are mean of six observations ± SEM

The mean bearing different superscript in the same row and column differ significantly (p<0.05)
Thus, the present study showed enhanced cellular and humoral immune effector activities in native and backyard poultry breed than the test broiler birds against pathogenic *E. coli*. Similarly, Munir et al. (2010) detected a virulent Newcastle disease virus in backyard birds without any clinical syndrome probably due to enhanced immune effector activities which was unexplored in the conducted study. Further, in a comparative study between a native chicken (Erlang mountainous chicken) and commercial broiler in China, native chicken breed showed higher resistance against Marek’s disease virus than the broiler due to difference in expression pattern of transcription factor (*IRF-3*) and interferon (*IFN-β*) genes associated with genetic background (Feng et al., 2013). Emam et al. (2014) also detected the possible role of genetic background in different immune response (*in vitro* blood mononuclear cell proliferation) between native chicken and broiler birds against sheep red blood cell and *Brucella* antigen.

**Conclusions**

The present study detected dynamic changes in cellular and humoral immune responses in all the poultry breeds/strain used upon exposure to pathogenic *E. coli* during study period. However, HB birds showed higher cellular and humoral immune responses against pathogenic *E. coli* than the other test birds that corroborates their resistance from experimental infection. Continuation of the study may affirm the disease resistance potential of this native breed at genetic level.

**Acknowledgements**

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**Conflict of Interests**

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

**References**


