Full Length Research Article

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Research Journal of Agricultural Sciences
Volume 17; Issue 02 (Mar–Apr 2026); pp 170–177

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

Kinetics of CXCLi2 Expression and Immune Response in Newly Hatched Kashmir Commercial Layer Chicks Challenged with *Salmonella enterica serovar* Typhimurium

Shabir Mir*[1]

[1] Division of Animal Genetics and Breeding, FVSc & AH Shuhama, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar - 190 006, Jammu and Kashmir, India

Received: 11 Feb 2026; Revised accepted: 19 Mar 2026

Abstract

Salmonella enterica serovar Typhimurium is a major avian pathogen that causes severe intestinal damage in young chicken leading to significant economic losses in the poultry industry. This study was conducted to examine the temporal expression of chemokine CXCLi2 gene in Kashmir Commercial Layer (KCL) breed of chicken following experimental infection with *Salmonella enterica serovar* Typhimurium. Sampling was conducted at 0, 1, 3, 5, 7, 9, 11, 13 and 15 days post infection (DPI). Quantitative real-time reverse transcription PCR (qRT-PCR) was employed to assess mRNA expression levels in the liver, spleen and caecum. This experimental analysis revealed a significant upregulation of CXCLi2 expression in infected birds relative to age matched uninfected controls. Tissue specific kinetics indicated a peak in gene expression until 5 DPI in liver and until 7 DPI in spleen and caecum followed by a marked decline through 15 DPI. Among the tissues examined caecum exhibited the highest expression followed by the spleen and liver. Correspondingly leukocyte counts demonstrated a progressive increase during the first week post infection which then gradually declined over the subsequent period. These findings highlight the tissue specific and time dependent nature of early immune response in KCL chickens to *Salmonella* Typhimurium infection and suggest that CXCLi2 plays a critical role in orchestrating initial host defence.

***Key words*:** *Salmonella*, KCL, CXCLi2 gene, mRNA expression, Leucocytes

Salmonellosis is one of the most prevalent food-borne bacterial diseases worldwide and is caused primarily by *Salmonella enterica* belonging to the enterobacteriaceae family. The disease affects millions of people annually and remains a major public health concern. Its significance stems from the organism's extensive global distribution, its ability to persist in diverse environmental conditions and its capacity to contaminate a wide range of food products throughout the farm-to-fork continuum. As a zoonotic pathogen, *Salmonella enterica* is frequently associated with farm animals such as poultry which act as important reservoirs. Contamination can occur at multiple stages including primary production, handling and preparation and hence making control challenging. Food borne diseases in humans caused by *Salmonella* remain a major public health concern worldwide and are commonly associated with the consumption of chicken meat contaminated with this bacterium [1]. *Salmonella* species are well recognized for their zoonotic potential and remain among the leading etiological agents of foodborne diseases worldwide [2]. Their persistent prevalence in human and animal populations continues to impose a substantial burden on global public health and economic systems [3]. The contamination of poultry products by *Salmonella enterica* serovar Typhimurium remains a significant driver of foodborne infections, frequently leading to widespread outbreaks and posing a persistent public health concern [4]. Only 10% of backyard layer feces publicized occurrence of *Salmonella* infection contrast to 76% of environmental surfaces, 33% of pooled cloacal swabs and 33% of feed samples. The major serovars detected were *Salmonella infantis* and *Salmonella* Typhimurium [5]. Therefore, the primary target of *Salmonella* control in poultry is to check these organisms from entering the food chain. *Salmonellae* are the leading cause of morbidity and mortality in poultry and lead to significant economic losses [6-7]. Careful Salmonella challenge research represents a vital milestone in understanding the genetics of infective disease resistance and offers a theoretical foundation for breeding *Salmonella pullorum* resistant chicken lines using marker-assisted selection (MAS), and postulates new understanding for salmonellosis research in humans and other animals [8].

Salmonella enterica infection in chicken can strike at any age but the day-old chicks are very vulnerable to infections with *Salmonella enterica* subspecies. Older birds are more resistant to this infection as paralleled to the younger birds due to well-developed immune system and strong cell mediated and humoral immunity [9]. While the genus *Salmonella* is categorized by an extremely high degree of serological diversity, each of more than 2,500 known *Salmonella* serovars

***Correspondence to:** Shabir Mir, E-mail: shabir1982@gmail.com; Tel: +91 9419006793

Citation: Mir S. 2026. Kinetics of CXCLi2 expression and immune response in newly hatched Kashmir Commercial Layer chicks challenged with *Salmonella enterica serovar* Typhimurium. *Res. Jr. Agril. Sci.* 17(2): 170-177.

is potentially capable of producing gastroenteritis, with a few specific representatives being exceptionally threatening. The immune system is a host protection system encompassing many biological structures and processes within an organism that defends against the diseases. The pronounced mucosal immune response observed in chicks following *Salmonella* Typhimurium challenge indicates activation of both humoral and cellular immune pathways. These findings suggest the potential value of administering a natural immune-response stimulator at one day of age to enhance the chick's capacity to effectively combat infection and strengthen overall immunity [10]. Cytokines are dynamic proteins secreted by cells that play a fundamental role in the immune and inflammatory responses. They are the effector couriers of the innate and adaptive immune systems that initiate and manipulate the immune responses directed toward eliminating microbial pathogens. Chemokines are a category of cytokines that have chemo-attractant activities that govern the movement of immune cells [11]. Modern progress in cloning of avian cytokine and chemokine genes also stimulated additional analysis of pro-inflammatory cytokine and chemokine production during numerous viral and bacterial infections in chicken [12-15]. Certainly, changing patterns of cytokine mRNA expression have been validated during serovar Typhimurium or serovar enteritidis infection in avian organs like in spleen [16-17] and gut [18], and in avian cells, such as granulocytes [19] and T-cell subsets [20]. In *Salmonella pullorum* infection in chicken, significant down regulation of FBXW7 and LRBA genes proposed their probable function in controlling the infection [21].

Lot of advances such as vaccination, antibiotics and managemental interventions have been used to fight salmonellosis in birds, but have not showed to be highly successful and undergo major restraints. Widespread use of drugs leads to the development of multiple drug resistance and drug deposits in egg and meat, which is a reason of public health apprehension. Because of huge pathogenic exposure, vaccination or antibiotic treatments are not always efficient [22]. Vaccination is done for immunity induction but it also sometimes clues to morbidity and mortality. Use of improved vaccines is correlated with the evolution of virulence of many pathogens leading to increased disease losses until new generations of vaccines are introduced. Vaccination alone cannot succeed disease effectively but should be combined with disease resistance to exploit the defense against diseases. Genetic selection for improving disease resistance of birds appears to be an encouraging methodology [23-24]. Various selection strategies have been experienced boosting disease resistance of birds [25-28]. Therefore, under the given situation, possibly there is a greater necessity to focus our consideration to understand the genetics of diseases and immunity. Breeding for disease resistance is a feasible suggestion. Improvement made per generation is heritable and accumulative over generations. It safeguards natural resources and decreases repeated spending on health management. Hence, improvement in non-specific genetic disease resistance has been adjudged as one of the best long-term strategies.

MATERIALS AND METHODS

Chicks, design and sampling

For this experimental study, 120 day-old chicks from Kashmir Commercial Layer (KCL) birds were undertaken. They were reared strictly under sanitized conditions at the Experimental House of Faculty of Veterinary Science and Animal Husbandry, SKUAST-Kashmir, Shuhama Srinagar. The environment of both experimental and control groups was managed such that it remained same for all chicks in the experiment. Commercial feed in the powder form during the first week exclusive of antibiotics or other feed additives and drinking water was offered adlibitum. Cleaning and feeding regimens were organized, which prevented cross-contamination excellently throughout the trial. To confirm that the chicks were free from *Salmonella* infection, faecal swabs were taken and examined bacteriologically for uncovering carrier state. The swabs were inoculated in freshly prepared selenite broth followed by plating on BGA and MacConkey's agar and the growth were observed after 24-36 hours of incubation at 37˚C. Based on culture and biochemical studies, chicks found *Salmonella* negative were used for further analyses. Chicks were separated into two groups infected and control group. Infected group were orally challenged using the 1.0 ml of bacterial culture containing $2x10^8$ CFU/ml of *Salmonella* Typhimurium. The control age matched group were mock infected with 1.0 ml of an un-inoculated nutrient broth. For validation of infection faecal swabs were taken 12 hours after infection and cultured in TTB and incubated for 18 hours at 42˚C. At each point of time chicks were sacrificed and tissues samples were taken. Tissues of liver, spleen and caecum were stored in RNA Later at -80^0 C until use for RNA extraction. The rest material was disposed by burial. After the trials were concluded, the premises used for the study purpose was sanitized by fumigation with formaldehyde and potassium permanganate solution.

Bacterial strains

Salmonella enterica serovar Typhimurium culture (KwikStik, LOT 180-171-1, REF 0180P, ATCC^{R}51812^{TM}) procured from HIMEDIA Laboratories Pvt. Ltd. was used for experimenting the dose response relationship and regulating the inoculum necessary for inducing infection in chicks. Established *Salmonella enterica serovar* Typhimurium colonies were inoculated in nutrient broth and kept in shaking incubator (140 rpm) at 37ºC overnight. Serial dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} were made using PBS as diluent. 100 µl of 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} were spread on BGA plates and kept overnight in incubator at 37º C.

RNA extraction and cDNA synthesis

Total RNA was extracted from the samples of each individual bird by TrizolTM method (Invitrogen, USA) following the manufacturer's instructions. Prior to cDNA synthesis, RNA samples were run on 1% agarose gel to check quality of RNA and presence of any DNA impurity. Contaminating DNAs were digested using DNase1 kit (Sigma, USA). CDNA synthesis in all samples was completed using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit using oligodT primers by using manufacturers protocol. For authentication of cDNA and primers, conventional PCR was run using cDNA as template. The primers used for amplification of CXCLi2 gene were F 5′-GCCCTCCTCCTGGTTTCAG-3′ and R 5′-TGGCACCGCAGCTCATT-3′ and for β actin gene F 5′-TGGCATTGCTGACAGGAT-3′ and R 5′-CTGCTTGCTGATCCACAT-3′ previously testified [29-30].

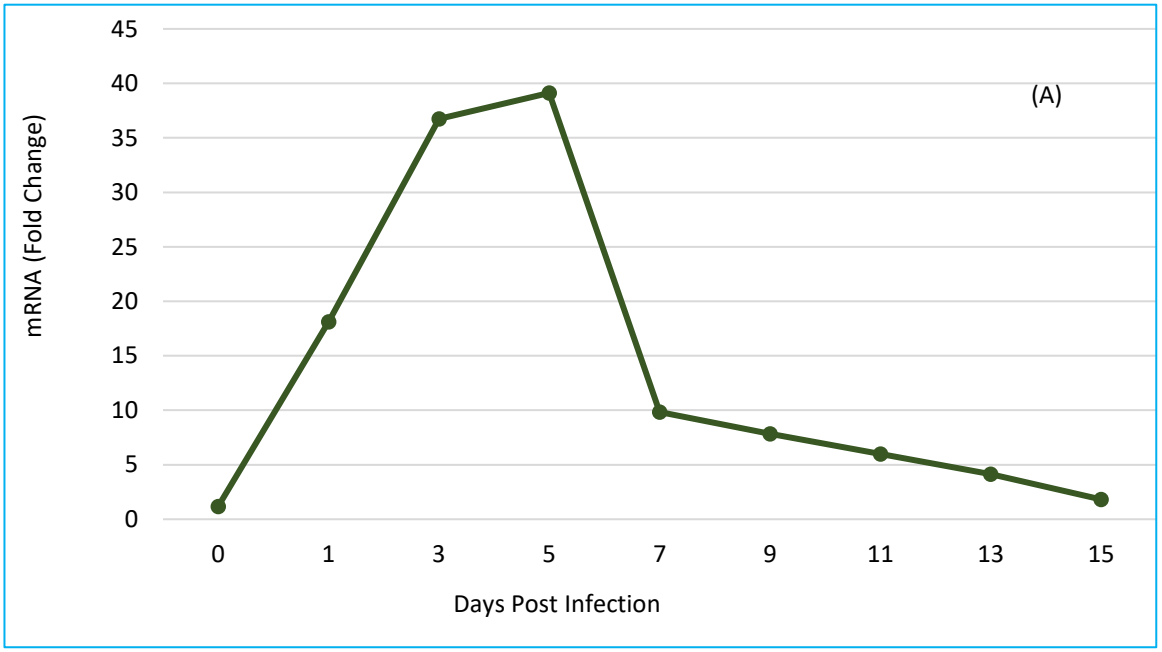
Quantitative real time PCR analysis

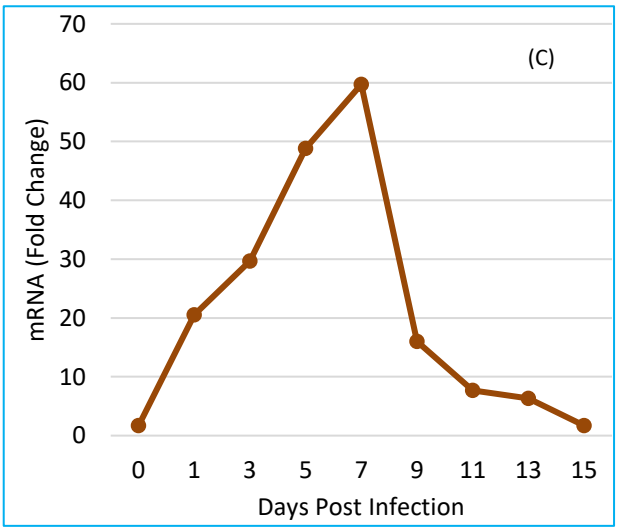
The mRNA expression levels of CXCLi2 gene in different tissues of infected and age matched mock-infected birds were determined by Real-Time (RT)-PCR (RocheTM, Germany) at nine different points (0,1,3,5,7,9,11,13 and 15 days post infection) for all the three breeds. β actin gene was

used as internal control with the following temperature-time profile given in (Table 1). The comparative C_T method also known as $\Delta\Delta C_T$ method [31] was used to get the results for comparative quantitation.

Table 1 qPCR conditions for RT-qPCR of *β* actin and CXCLi2 genes

Programme	Temperature °C	No. of cycles	Time
Pre-incubation	95	1	5 min
Amplification	95	40	20 sec
	63 (β actin)		15 sec
	60 (CXCLi2)		
	72		15 sec (single)
Melting	95	1	5 sec
	70		1 min
	95		Continuous
Cooling	40	1	30 sec





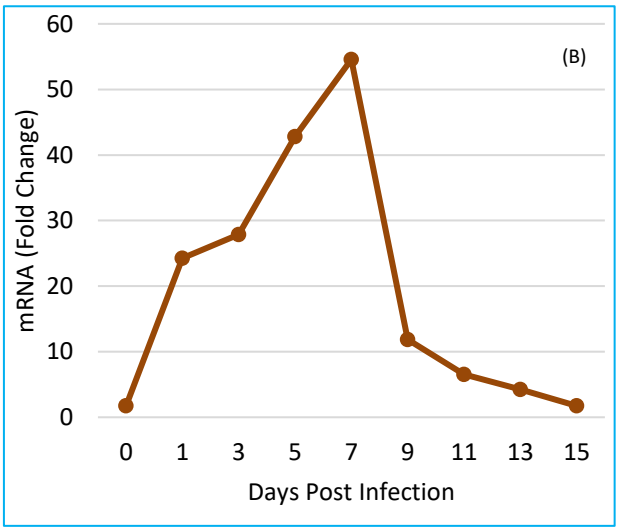


Fig 1 Changes in CXCLi2 mRNA expression in KCL chicks following *Salmonella* Typhimurium infection in (A) Liver, (B) Spleen, and (C) Caecum Across Nine Time Points (0, 1, 3, 5, 7, 9, 11, 13, and 15 DPI)

WBC's estimation

WBC's were estimated by using haematological analyzer (Melet Schloesing Laboratories MS4S).

Statistical analysis

The amount of target gene, normalized to an endogenous reference gene comparative to calibrator is given as $2^{-\Delta\Delta CT}$ method where ΔΔCT corresponded to the difference between the CT measured for the mRNA level of each tissue and the CT measured for the mRNA level of the reference gene, $\Delta CT = CT_{(CXCLi2)} - CT_{(\beta\ actin)}$, C_T represents thresh hold cycle i.e., the cycle number at which fluorescence is detected. The data were analyzed by three factorial design using R-Software. Alterations were considered statistically significant if $p \leq 0.05$.

RESULTS AND DISCUSSION

Clinical signs

The administration of *Salmonella enterica serovars* Typhimurium inoculated orally at a dose of $2x10^8$ CFU/ml per bird produced the all-out clinical symptoms but did not result in mortality. Noticeable symptoms were dullness, clear depression, progressive weakness, sealed eyes, hesitancy to move, in-appetence, amplified thirst, ruffled feathers, drooping of wings, lowering of head and diarrhoea.

Postmortem pathological changes associated with Salmonella infection

On postmortem examination, there were strong haemorrhages of the intestines. Congestion of liver in infected birds was seen during the whole experimental length. Also, liver was enlarged with bronze discoloration, prominent necrotic foci were also seen on liver and elevated white nodular lesions on cardiac ventricles in almost all infected birds. Spleen and lungs showed congestion and haemorrhages. Inflamed caeca with cheesy necrotic material were also seen.

Dynamic expression profile of CXCLi2 in various tissues during post infection period

CXCLi2 gene mRNA fold expression presented dynamic processes in the course of Salmonella infection. The mRNA fold expression in liver of Kashmir Commercial Layer (KCL) chicks increased up to 39.12 fold till 5th day post infection (DPI) and onwards decreased to 1.83 folds. In spleen, the mRNA CXCLi2 gene expression was characterized by a significant peaking fold expression during first week post infection from 1.75 to 54.57 folds but decreased during second week from 11.88 to 1.74 folds. On interpreting the values of $\Delta\Delta C_T$ in caecum on day 0, 1, 3, 5, 7, 9, 11, 13 and 15 day post infection, mRNA fold expression increased to 59.71 upfolds on 7th day post infection and decreased during second week (from 16.00 to 1.72 folds). The overall mean fold expression levels in liver, spleen and caecum were 13.870 ± 1.839, 19.514 ± 2.459 and 21.347 ± 2.717, respectively and were found statistically significant to each other ($p<0.05$) indicating higher expression in caecum followed by spleen and liver. The mRNA fold expression levels on day 15th were comparable to first day post infection in spleen and caecum but were on higher side in liver tissues (Fig 1, Table 2).

Table 2 CXCLi2 gene expression levels in different tissues (liver, spleen and caecum) at different time points (0,1,3,5,7,9,11,13, and 15 DPI) post *Salmonella* Typhimurium infection

Tissue	Liver	Spleen	Caecum
DPI (day post infection)			
0	1.18	1.75	1.68
1	18.13	24.25	20.53
3	36.76	27.86	29.65
5	39.12	42.81	48.84
7	9.85	54.57	59.71
9	7.84	11.88	16.00
11	5.98	6.54	7.67
13	4.14	4.23	6.32
15	1.83	1.74	1.72
Overall mean	$13.870^m \pm 1.839$	$19.514^n \pm 2.459$	$21.347^o \pm 2.717$

Means with different superscripts differ significantly ($p<0.05$)

Alterations in leukocyte profile following Salmonella Typhimurium infection

The overall WBC count displayed most diverse change in the infected groups up to 7th day post infection (DPI). After 7th day post infection (DPI), the mean WBC's count started to decline. The mean count continued higher in case of the infected group as compared to un-infected respective controls throughout our experimental study. Within the control group, the overall mean was 24.463±0.224 and in infected group overall mean value was 31.802±0.747. Mean WBC's values differed significantly ($p<0.05$) between infected and control groups. The mean count was lower in uninfected chicks compared to infected group (Fig 2-3, Table 3). These findings indicate that infection induces a significant but transient elevation in WBC count, reflecting an active immune response followed by gradual normalization over time.

Table 3 Comparison of *Salmonella* Typhimurium uninfected to infected KCL chicks on WBC counts (10^3/µl) at different days (0,1,3,5,7,9,11,13, and 15)

DPI (day post infection)	Control group	Infected group
0	23.465	23.682
1	24.132	28.461
3	24.263	34.421
5	24.681	38.432
7	24.832	39.042
9	24.154	35.420
11	24.681	31.684
13	24.941	28.642
15	25.021	26.431
Overall mean	$24.463^j \pm 0.224$	$31.802^k \pm 0.747$

Means with different superscripts differ significantly ($p<0.05$)

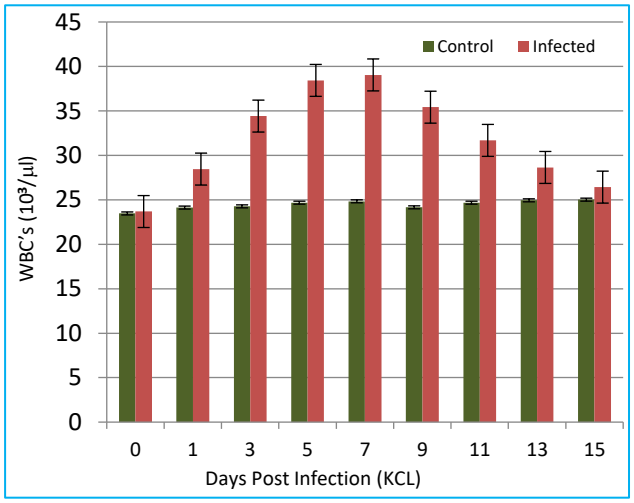


Fig 2 Impact of experimental *Salmonella* Typhimurium infection on white blood cell counts (10^3/µl) in control versus infected groups

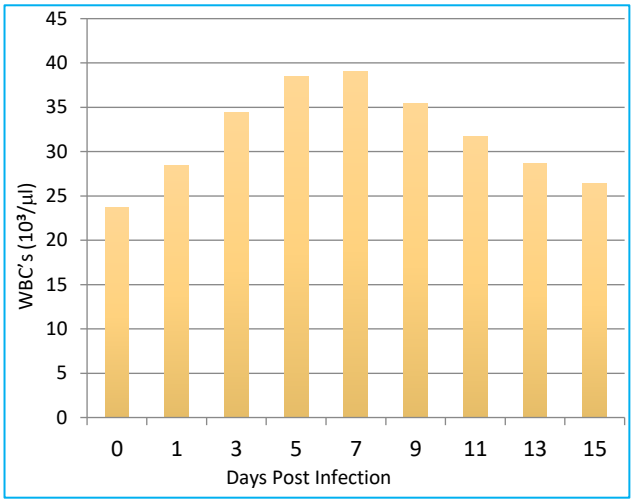


Fig 3 Temporal comparison of WBC Counts (10^3/µl) at nine intervals after induction of *Salmonella* Typhimurium infection

In the present analysis, *Salmonella enterica serovar* Typhimurium culture was used for studying the dose response association and standardizing the dose prerequisite for inducing the infection in chicks. Confirmed *Salmonella enterica serovar* Typhimurium colonies were inoculated in nutrient broth and different infection doses were prepared using serial dilution mode. For induction of Salmonellosis in chicks, infectious dose of 2×10^8 CFU/ml induced signs and symptoms characteristics of Salmonellosis. Oral infection route has been found to be more effective compared to other inoculation means [32]. Following oral infection of one week old chicks with 2×10^8 CFU/ml of *Salmonella* Typhimurium, clinical signs appeared within 2 days post infection which progressively increased in duration-dependent routine and is in agreement to findings of Pas *et al.* [33] who observed parallel clinical signs at similar dosage. The clinical symptomology comprised were dullness, obvious depression, closed eyes and reluctance to move. On seventh day post infection, the chicks exhibited advanced weakness, inappetence, improved thirst, ruffled feathers, drooping of wings, lowering of head and diarrhoea. Similar clinical signs have also been coexisted in *Salmonella gallinarum* infection in chicken [34-37]. Postmortem examination of the *Salmonella* Typhimurium infected chicks showed bronze discoloration of liver, hemorrhagic and prominent necrotic foci on liver, elevated white nodular lesions on ventricles, fibrinous exudate in pericardial sac, congestion of intestines and lungs, swollen caeca with cheesy necrotic material and also enlargement of liver possibly from the hepatic damage. These findings are well supported by other conclusions in which necrotic and hemorrhagic foci have been discovered on liver in Salmonella infected chicken [38]. Adult chicken infected with *Salmonella gallinarum*, lesions reported included were bronze discoloration of the liver [39] and congestion in lungs [34], [40]. *Salmonella* colonization and invasion beyond gastrointestinal tract leads to proliferation in the macrophage phagocyte system (MPS) of the liver and spleen and eventual distribution to a variety of internal tissue sites and subsequent widespread bacteremia [41]. Invasion of intestinal epithelial cells by *Salmonella* Typhimurium provokes a series of pathological modifications affecting intestinal fluid and electrolyte regulation [42].

The mRNA expression levels of CXCLi2 gene in liver, spleen and caecum displayed a weighty increase till day 5-7th post infection followed by a continual drop till 15th day post infection. Caecal expression levels were comparatively more than that of spleen and liver. Amplified expression of immune response genes up to 7-9 DPI could be endorsed to elicitation of cellular immune response/acquired immunity period of infection for early clearance of the *Salmonella* infection [43]. Many fold surge in immune response gene expression in caecum could be narrated to more Salmonella colonization and proliferation in caecum matched to spleen and liver. It has been also observed that CXCLi1 mRNA expression increased markedly through days 5-7th post-infection followed by a gradual decline up to day 15. Notably expression levels were substantially higher in the caecum than in the spleen or liver. Concurrently, progressive histopathological lesions were evident in the small and large intestines, including the colon of chicks inoculated with *Salmonella* Typhimurium, with severity increasing over the course of the experiment [44]. Immune response gene expression in spleen accountable for early clearance of the pathogen could be due to huge volume of macrophages present in the spleen [45] and mutations in NRAMP1 gene giving rise to uncontrolled bacterial growth in reticulo endothelial system during the early infection period [46]. Immune response gene expression in liver accountable for bacterial clearance could be attributed to late stage (relocation of bacteria through blood to liver) proliferation of *Salmonella* in liver [41]. Infection of chickens with *Salmonella enterica* has been shown to disrupt metabolic homeostasis, resulting in oxidative stress and exacerbated inflammatory responses. Moreover, elevated levels of biomarkers associated with hepatocellular carcinoma were observed, suggesting a potential progression toward liver pathology and increased risk of malignancy [47]. Cheeseman *et al.* [48] observed up-regulation of CXCLi1 and CXCLi2 genes mRNA expression and macrophage cell populations in caeca of *Salmonella enteritidis* infected young chicken. Setta *et al.* [29] observed increased expression of CXCLi1 and CXCLi2 genes in caecal tonsils of freshly hatched chickens infected with *Salmonella enteritidis* compared to upregulated expression of LITAF in *Salmonella gallinarum* infected birds. Continuous exposure to environmental stressors and diverse microbial challenges likely contributes to the development of a more resilient and responsive immune system in these breeds, enabling them to mount a more effective defence against *Salmonella* Typhimurium infection. These observations highlight the pivotal influence of genetic factors in modulating immune gene expression profiles during *Salmonella* infection with important implications for breeding strategies aimed at enhancing disease

resistance [49]. Significant increases in IL-12 and IL-17 levels were observed at 6 hours post-infection ($p<0.05$) in the caecal tonsils of chickens challenged with *Eimeria tenella*. This rapid cytokine response indicates an early activation of local immune mechanisms within the lymphoid tissue, highlighting the prompt engagement of the host's mucosal defence following infection [50].

In infected groups, overall mean value of WBC count increased significantly ($P<0.05$) from day one post infection to day seven compared to respective uninfected controls. Significant increase ($P<0.05$) in total white blood cell witnessed post-infection was corresponding the one reported by Berchieri [51], who attributed the surge in leukocyte count to fast exponentiation of *Salmonella gallinarum* inside the phagocytes, with subsequent cell lysis and release of the bacterium into extracellular section inducing robust immune response. The elevated serum concentrations of the pro-inflammatory cytokines IL-6, IL-16, and IL-21 in challenged chicks reflect a vigorous mucosal immune response to *Salmonella* Typhimurium infection. These cytokines serve as critical mediators of inflammation and play integral roles in coordinating immune defence mechanisms at mucosal interfaces [52]. The leukocytosis documented in this study corresponded with the period of manifestation of the clinical signs (depression, somnolence, anorexia, ruffled feathers and greenish to yellowish diarrhoea) of fowl typhoid in the infected birds. This finding corresponded to reports of Berchieri [50] and Freitas Neto *et al.* [53]. In addition, the probable bacterial attack of the target organs, such as the liver, spleen, kidneys, and ovarian follicle might cause rise in peripheral blood leukocytes as an inflammatory answer. Leucocytosis due to comparative heterophilia initial in response to the *Salmonella* challenge might be attributed to them being part of natural immunity and cellular defense anti-microbial infections and in reaction to acute inflammatory and degenerative/necrotic changes in internal organs, and due to bone marrow hyperplasia [54]. Their relocation to inflammatory sites could also be a reason for their drop in blood level at advanced times [55]. During invasive Salmonella infection, PAMPs and DAMPs elicited the innate immune system hints to stimulation and recruitment of neutrophils and macrophages and the production of pro-inflammatory cytokines [56]. The lymphopenia observed post-infection in the infected chicks might be due to stress of infection with *Salmonella,* inducing adrenal gland release of cortical hormones that destroy the lymphocytes [57]. In related conclusions by Shah *et al.* [58] in poultry establish that WBC, heterophil and lymphocyte count in infected group increased significantly ($p<0.05$) as related to uninfected ones. These outcomes are in agreement with the observations made by Madhuri and Sudana [59], Kokosharov [60] and Shah *et al.* [58] who noticed significant ($P<0.05$) increase in WBC counts in infected groups compared to their respective controls.

CONCLUSION

In conclusion**,** our results indicate that during *Salmonella* infection mRNA expression levels exhibited marked upregulation followed by a subsequent decline irrespective of tissue type. Among the tissues examined caecum showed the greatest expression followed by the spleen and liver. Additionally, leukocyte counts increased consistently during the first week post infection and then gradually declined.

Acknowledgment

We are extremely grateful to the Department of Microbiology and Immunology, College of Veterinary Sciences and Animal Husbandry, SKUAST-Kashmir, Shuhama, Srinagar Jammu & Kashmir, India, for providing the experimental animal house and the necessary facilities for this study.

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