Changes in the Cytokine and Toll-Like Receptor Gene Expression Following Infection of Indigenous and Commercial Chickens With *Infectious bursal disease virus*

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Abstract A comparative study of cytokine and toll-like receptor (TLR) mRNA expression in 3 weeks old indigenous and commercial chickens infected with a very virulent strain of *Infectious bursal disease virus* (IBDV) was performed using a custom-made microarray chip. In uninfected indigenous chickens, the basal levels of interleukin (IL) 15 were lower and IL 16 was higher than their commercial counterparts. In the IBDV infected indigenous chickens, only IL16 gene expression was down regulated, while TLR3 expression was up regulated significantly. In the IBDV infected commercial chickens IL15, IL16 and TLR3 were down regulated. But, IL1- β , IL2, IL8, IL12, IL17, interferon (IFN)- α and β were significantly increased compared with the control. In IBDV infected indigenous chickens, IL15, IFN- γ , beta-defensin and TLR3 were up regulated compared to virus-infected commercial chickens. The results suggested that up regulation of TLR3, a ligand for double-stranded (ds) RNA probably could account for the possible clinical resistance in these birds. There was a 5.2 fold difference by quantitative real-time RT-PCR between indigenous and commercial chickens in TLR3 mRNA expression. Therefore, TLR3, a receptor for dsRNA could be a putative molecule that could play a role in

differential innate and adaptive immune responses to IBDV in commercial and indigenous chickens.

Keywords Microarray · Toll-like receptors · Cytokines · *Infectious bursal disease virus* · Real time PCR

Indigenous chickens are considered to be more disease resistant than their commercial counterparts in many parts of the world. Artificial breeding for production did not segregate the disease resistance parameters in commercial chicken production and hence they are immunologically under privileged. The resistance of indigenous chickens to many diseases indicates that they are immunologically superior. However, it is difficult to ascertain whether a single or a set of gene (s) define this property to indigenous chickens. Significant efforts have been directed toward identifying genetic variants in host genes conferring resistance to Marek's disease (MD) virus with the aim of understanding the molecular mechanism of resistance and integrating selection for such markers in a breeding program. Resistance to MD is affected by the innate immune system as well as the acquired immune responses [8]. A general attenuation of the immune response or a shift to a Th1 response, were shown to increase the susceptibility to MD.

Infectious bursal disease virus (IBDV), an avian virus of the family *Birnaviridae* causes highly contagious and immunosuppressive disease in young chickens between 3 to 6 weeks of age. In 3-weeks-old indigenous chickens, pathogenic strains of IBDV do not induce an overt clinical disease, whereas in age-matched commercial chickens, the same strain induces a very high mortality associated with clinical signs. Whether this is related to immunological factors conferring a higher degree of innate resistance to

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these chickens is not clearly understood. However, both types of birds respond vigorously with an antibody response. Therefore, the differences in innate immune response between commercial and indigenous chickens should provide an understanding of the disease resistance mechanisms.

The objective of this study was therefore to examine the differences in gene expression of innate immune response genes between these two groups of chicken after infection with IBDV. We analyzed the molecular transcription profile of selected innate immune response genes and validated one of the major up regulated genes by real-time polymerase chain reaction (PCR) assay. Our results showed that down regulation of type I interferon (IFN) response and up regulation of toll-like receptor (TLR) 3 may confer resistance against clinical disease in indigenous chickens.

A very virulent strain of IBDV isolated in the Department of Animal Biotechnology, Madras Veterinary College, Chennai (TN2001/3), India was used in these studies [20]. Stock virus in the form of 30% infective bursal suspensions was titrated for infectivity in 9–11 day-old embryonated chicken eggs.

Three-week old sero-negative commercial (white leg-horn) chickens were obtained from the Poultry Research Station, Tamil Nadu Veterinary and Animal Sciences University, Chennai. Indigenous (Aseel type) chickens were obtained from individual households from remote villages of Tamil Nadu whose date of hatch was known. The serum from both groups of birds were examined for antibodies to IBDV using the agar gel precipitation test (AGPT) with the infective bursal suspension as IBDV antigen and reference antiserum obtained from Central Veterinary laboratory, Weybridge, United Kingdom as positive control serum as described [19]. These chickens were housed in isolation rooms at the Department of Animal Biotechnology Animal Facility, Madras Veterinary College, Chennai, India.

Groups of 10 commercial or Aseel type indigenous chickens were infected with 100 µl of IBDV (105.0 log₁₀ EID₅₀) by the intra-ocular and intra-cloacal routes in divided volume [23]. Another group of 10 birds were kept as mock-infected controls and received a similar volume of phosphate buffered saline (PBS) by the same routes. Three days post-infection, all chickens were killed humanely by cervical dislocation. The bursa of Fabricius from these birds in each group was tested individually for the presence or absence of IBDV antigen by AGPT. The spleen from six infected (confirmed positive for IBDV antigen in their bursae) and six control chickens were pooled in batches of three samples each and used as two different samples for RNA extraction. The rest of the four birds of the infected group did not show presence of IBDV antigen by AGPT.

Not all birds infected might show antigen on day 3. It is possible that in these birds antigen could have been detected later than 3rd day post-infection.

RNA was isolated from control and infected spleen tissues using RNeasy mini kit (Qiagen). The quality and quantity of the RNA samples were examined by gel electrophoresis. For the microarray experiment, RNA was amplified using Express Art[®] mRNA amplification kit micro version (Artus GmbH, Germany) using 2.5 µg of total RNA. Amplified RNA was labeled with Cy3 Post-Labeling Reactive Dye Pack (GE Healthcare UK Limited, UK). Five µg of the labeled RNA was used for hybridization with the custom OciChip[™] (Ocimum Biosolutions, Hyderabad, India). Probe labeling and DNA microarray experiments were performed as described at <http://cmgm.stanford.edu/pbrown/protocols/index.html> [17]. Hybridized chips were scanned using Affymetrix 428[™] array scanner and the images were analyzed using Genowiz[™] software (Ocimum Biosolutions, Hyderabad, India). Fold change analysis was performed to detect differentially expressed genes among various conditions (Fold change cut-off of 1.50). Signal value for each gene/probe was taken by averaging intensity among triplicate values.

The RNA was labeled with Cy5 and hybridized with synthesized oligos specific for chicken interleukin (IL) 1 beta, IL2, IL6, IL15, IL16, IL17, IL18, IL12, IL8, interferon (IFN) alpha, IFN beta, IFN gamma, tumour necrosis factor (TNF) alpha, beta defensin, TLR 1, TLR3, TLR2 type 1, TLR type 2, TLR4, TLR5, TLR7, caspase 1 and caspase 2. GAPDH and beta actin were used as endogenous controls in a single channel microarray set up. The GenBank accession numbers of sequences used for preparation of oligos (Ocimum Biosolutions, Hyderabad, India) for imprinting on slides is given in Table 1. For each microarray, multiple scans were performed with a dual laser scanner. Then the Image[™] software (Biodiscovery, CA) was used to calculate the intensities of each spot. The Image[™] results were further processed using the MAVI Pro software (Ocimum Biosolutions, Hyderabad, India). This software combines the Image[™] data from multiple scans to one data set per microarray using linear regression analysis. For the ratio calculation, the normalized and background corrected intensities of the MAVI results were used. Ratios were calculated by dividing the experimental data (IBDV infected) by control data (untreated). Ratio values >1.5 represent up regulated gene expression while ratio values <0.5 represent down regulated gene expression. Ratio values from 0.5 to 1.5 represent genes that are not regulated at that time point. Four types of comparisons were performed using student's t test. Initially, the basal expression of all of the above genes between mock-infected commercial and indigenous control chickens was compared. Then the gene expression in each

Table 1 Expression levels of cytokines and toll-like receptor (TLR) genes in mock infected or *Infectious bursal disease virus* (IBDV) infected commercial and Indigenous chickens by OciChipTM microarray

Gene	Accession number	Indigenous chicken mock infected vs commercial mock infected		Indigenous chicken IBDV infected vs village chicken mock infected		Commercial IBDV infected vs commercial mock infected		Indigenous chicken IBDV infected vs commercial chicken IBDV infected	
		Ratio	P-value	Ratio	P-value	Ratio	P-value	Ratio	P-value
Beta actin	L08165	-1.12	0.72	1.09	0.70	-2.17	0.19	2.10	0.19
Beta defensin	AF033336	-1.73*	0.02	1.17	0.30	-1.68	0.01	1.13	0.37
Caspase 1	AF031351	1.09	0.82	-1.35	0.20	-1.84	0.36	1.48	0.09
Caspase 2	U64963	-1.12	0.74	-1.26	0.11	-2.32	0.25	1.63	0.17
GAPDH	NM204305	-1.83	0.01	-1.26	0.13	-3.52	0.00	1.53	0.06
Interferon alpha	GGU07868	1.17	0.28	-1.13	0.31	1.44	0.08	0.71	0.04
Interferon beta	X92479	1.27	0.07	1.02	0.87	2.05	0.11	0.63	0.09
Interferon gamma	GGU27465	-1.33	0.29	1.63	0.11	7.31	0.19	-8.91	0.15
Interleukin 1beta	Y15006	1.32	0.34	-1.38	0.29	1.32	0.09	0.72	0.034
Interleukin 12	AY262751	-1.12	0.37	-1.11	0.47	1.13	0.68	0.71	0.38
Interleukin 15	AF152927	-2.24	0.04	-1.15	0.13	-5.52	0.03	2.13	0.04
Interleukin 16	AB104417	1.54	0.01	-2.92	0.01	-3.76	0.00	1.97	0.06
Interleukin 17	AY920750	1.18	0.81	-1.12	0.86	-1.92	0.75	2.02	0.72
Interleukin 18	AJ277865	-1.14	0.32	1.11	0.43	-1.12	0.36	1.09	0.47
Interleukin 2	AF000631	-9.84	0.44	7.17	0.42	-2.23	0.60	1.62	0.70
Interleukin 6	AJ309540	-1.80	0.11	-1.03	0.89	-3.22	0.11	1.73	0.10
Interleukin 8	NM205018	1.13	0.52	-1.18	0.42	1.53	0.04	0.62	0.02
TLR 2 type 1	AB050005	-1.42	0.03	-1.06	0.01	-1.41	0.03	0.93	0.37
TLR2 type 2	AB046533	-1.58	0.27	-1.12	0.02	-1.63	0.26	0.91	0.31
TLR1	AB109401	-1.16	0.26	-1.18	0.26	-1.09	0.12	0.79	0.03
TLR 3	NM001011691	-1.81	0.11	1.87	0.00	-3.99	0.04	4.11	0.01
TLR 4	AY064697	1.16	0.14	-1.21	0.11	1.48	0.02	0.65	0.04
TLR 5	AJ626848	-1.12	0.32	-1.24	0.07	-1.23	0.16	0.88	0.26
TLR 7	AJ632302	-1.00	0.96	-1.07	0.33	1.14	0.14	0.81	0.05
TNF alpha	M80573	1.25	0.00	1.03	0.40	1.92	0.13	0.66	0.17

* Values in bold face indicate significant change in expression

type of IBDV infected chickens with its respective mock-infected controls was compared. Then the ratios between the gene expression of IBDV infected commercial and indigenous chickens were compared to determine the differential regulation of genes in these two types of birds.

To confirm the microarray results of TLR3 up regulation, quantitative real time RT-PCR (qRT-PCR) using Sybr Green I chemistry was performed. The primer sequences were derived from TLR 3 chicken sequence (NM 001011691) or from chicken beta actin sequences (NM 205518). The TLR 3 forward and reverse primer sequences are 5'-GCCGCAGCATAGCATCATAC-3' and 5'-TGAG TGAGGTGACAGCAAGG-3' respectively. The chicken beta actin forward and reverse primer sequences are 5'-GAGAAATTGGTGACATCA-3' and 5'-CCTGAAC CTCTCATTGCCA-3' respectively. Total RNA was extracted from the infected bursae of commercial and

indigenous chickens using TRIzol (Invitrogen, USA) and cDNA was synthesized using the High capacity cDNA archive kit (Applied Biosystems Inc., USA). β -actin was used as an endogenous control for RT-PCR. The PCR mix for Sybr Green I assay consisted of 10 μ l of the Sybr Green I PCR master mix (Applied Biosystems), 1 μ l (1 pmol) each of forward and reverse primers and 100 ng cDNA adjusted in water in 8 μ l to give a total reaction volume of 20 μ l. Each reaction was prepared in triplicate. A no-template control (NTC) was prepared using only DEPC water (instead of cDNA) as above. The plate was centrifuged in a refrigerated (4°C) centrifuge at 560 rpm for 3–5 min to remove air bubbles. qRT-PCR was (Applied Biosystems Inc., USA, Model 7500) performed using universal cycling condition consisting of one cycle at 50°C for 2 min, one cycle at 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 1 min. The Ct values were

recorded for both the target and endogenous controls. The data was accepted only when the NTC had no amplification. The relative amount of target sequence in the indigenous Vs commercial chicken samples was calculated using the comparative Ct (Δ Ct) value. The difference in the expression levels of TLR3 between the indigenous and commercial birds was calculated as $2^{-\Delta\Delta C_t}$.

The results of the microarray experiment are given in Table 1. On comparison of the basal expression levels of the analyzed gene set between mock-infected commercial and indigenous chickens, differential expression was seen only with respect to IL15, IL16 and beta defensin. In indigenous chickens, while the basal levels of IL15 and beta defensin were lower and IL16 was higher than the commercial chickens. In the IBDV infected indigenous chickens, only IL16 gene expression was down regulated, while TLR3 expression was up regulated significantly ($P < 0.01$) compared to mock-infected birds. In the IBDV infected commercial chickens IL15, IL16 and TLR3 and beta-defensin were down regulated significantly ($P < 0.05$). But, IL8 was significantly increased compared to their mock-infected counterparts ($P < 0.05$). When the gene expression of IBDV infected indigenous chickens were compared with the virus infected commercial chickens, IL15 (2-fold) and TLR3 (4-fold) were up regulated in infected indigenous chickens ($P < 0.05$).

To validate the micro array results of TLR3, qRT-PCR for TLR3 mRNA was performed, and results are shown in Table 2. There was a 5.2 fold difference between the indigenous and commercial chickens in TLR3 expression. The histopathological lesions observed in virus-infected commercial and indigenous chickens were comparable in severity (data not shown) showing evidence of loss of bursal architecture, lymphoid depletion and cellular infiltration.

This study was undertaken to identify the possible immune resistance mechanism seen in indigenous chickens to clinical IBDV in terms of differentially expressed cytokine(s) or TLR molecules. In this context, indigenous chickens are those that are grown under natural environments given no premixed feed and commercial chickens as those reared under captivity with definite feed formulations.

By using a custom chicken microarray, several genes with differential expression associated with host resistance to IBDV were identified. One of the genes (TLR3) was also subjected to RT-PCR that confirmed the results of microarray. Microarray analysis and qRT-PCR were performed on day 3 post-infection to correspond with the known peak of IBDV acute phase [10, 11]. The basal expression levels of IL15 and IL16 were different between mock-infected indigenous Vs commercial chickens with inherent increased IL-16 expression in indigenous chickens indicating inherent genetic differences between these two groups of chickens. Chicken IL15 has been shown to be a T cell growth factor [13] and can potentially enhance cell mediated immunity. But, chicken IL-16 has been shown to be preferentially expressed in lymphoid tissues such as bursa of Fabricius and chemotactic to splenic T lymphocytes [16]. Therefore, a higher IL-16 level in indigenous chickens can offer a selective advantage by enhancing B cell development and T cell chemotaxis in the bursa of Fabricius.

Depending on the virus strain, IBDV-infection may induce transient up-regulation of IFN- γ , IL-2, and IL-6, while cytokines such as IL-1 β and type I IFNs may be down-regulated temporarily [3, 21]. In IBDV-infected indigenous chickens, IL16 was significantly down regulated compared to mock-infected chickens, suggesting a major role for this cytokine in IBDV infection.

In IBDV-infected indigenous chickens, IL15 and TLR3 were up regulated compared to their age-matched commercial counterparts. In general, IL-15 mRNA expression is increased in the presence of classical microbial activators of macrophage function [2]. IBDV infects and replicates in macrophages leading to the production of pro-inflammatory cytokines [9, 18]. Therefore, selective up regulation of IL-15 can enhance Th-1 type immunity in indigenous chickens. While the levels of TLR3 were increased in indigenous chickens, its level was reduced in commercial chickens contributing to the most potent differential effect seen between these two groups of chickens. This differential response was also validated by quantitative qRT-PCR. Thus, TLR3 could be a putative molecule that could play a major role in differential innate and adaptive immune responses to IBDV in commercial and indigenous

Table 2 Expression of toll-like receptor 3 (TLR3) mRNA in indigenous and commercial chickens infected with *Infectious bursal disease virus* (IBDV) in comparison with the endogenous control gene, beta actin

Type of chickens	Mean ($\pm$ SD) Ct values for β -actin	Mean ($\pm$ SD) Ct values for TLR3	Mean ΔC_t	$\Delta\Delta C_t$	$2^{-\Delta\Delta C_t}$
Commercial control	22.44 $\pm$ 0.040	31.54 $\pm$ 0.022	9.1	1.84	0.279
Commercial IBDV infected	21.25 $\pm$ 0.03	32.19 $\pm$ 0.043	10.94		
Indigenous control	22.36 $\pm$ 0.043	31.96 $\pm$ 0.061	9.6	-1.01	2.013
Indigenous IBDV infected	22.25 $\pm$ 0.011	30.84 $\pm$ 0.013	8.59		

chickens. Toll-like receptors are a major group of trans-membrane receptors that are involved in the detection of several pathogens. Viral double-stranded RNA (dsRNA) is a pathogen-associated molecular pattern that is recognized by TLR3 and several cytosolic sensors, such as protein kinase R, 2'-5'-oligoadenylate synthetases, and the recently identified RNA helicases RIG-I (retinoic acid-inducible gene I) and MDA5 (melanoma differentiation-associated gene 5) [14, 15, 25]. TLR3 is considered a "danger" receptor with a pleiotropic potential in innate and adaptive immunity.

TLR3 plays key roles in antiviral responses through cross-priming of multiple physiological pathways, including the activation of IFN responses, NF-kappa B and mitogen-activated protein kinase (MAPK) pathways, as well as the caspase cascade [22]. In our study, the expression of TLR3 was up regulated significantly in indigenous chickens implying possible roles of TLR3 in dsRNA-initiated antiviral responses in IBDV-infected spleen cells. The altered expression of TLR3 has been linked to increased responsiveness to viral infection, which was reported in a number of viruses, such as respiratory syncytial virus [4], vaccinia virus [6] and hepatitis C virus (HCV) [12]. Up regulation of TLR3 has also been described for respiratory viruses such as Rhino virus, Influenza virus, and Sendai virus [1, 5, 7].

Another molecule that was differentially expressed was the beta-defensin. This molecule probably acts by blocking of viral cellular entry possibly because of destabilization/disintegration of the viral envelope. Beta-defensins may promote adaptive immune responses by recruiting dendritic and T cells to the site of microbial invasion through interaction with CCR6, a chemokine receptor [24]. In the commercial chickens infected with IBDV, beta defensin was down regulated. The histopathological changes in the infected bursae of these birds did not show any differences in lesion scores between these two groups of chickens. This is probably due to the very virulent nature of the virus isolate, circumventing the innate and adaptive immune responses in establishing virus replication and immunopathology.

In summary, we have shown for the first time that some local innate effectors including TLR3 and IL15 are differentially up regulated in the spleen of indigenous chickens 3 days post-infection. The innate antiviral effect induced through TLR3 up regulation and engagement and Th1 type immunity by IL15 in indigenous chickens probably confer innate resistance to IBDV-induced clinical disease in these native birds.

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References

- Boehme KW, Compton T. Innate sensing of viruses by toll-like receptors. *J. Virol.* 2004;78:7867–73.
- Doherty TM, Seder RA, Sher A. Induction and regulation of IL-15 expression in murine macrophages. *J. Immunol.* 1996; 156:735–41.
- Eldaghayes I, Rothwell L, Williams A, Withers D, Balu S, Davison F, Kaiser P. Infectious bursal disease virus: strains that differ in virulence differentially modulate the innate immune response to infection in the chicken bursa. *Viral Immunol.* 2006;19:83–91.
- Groskreutz DJ, Monick MM, Powers LS, Yarovinsky TO, Look DC, Hunninghake GW. Respiratory syncytial virus induces TLR3 protein and protein kinase R, leading to increased double-stranded RNA responsiveness in airway epithelial cells. *J. Immunol.* 2006;176:1733–40.
- Guillot L, Le Goffic R, Bloch S, Escrivo N, Akira S, Chignard M, Si-Tahar M. Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J. Biol. Chem.* 2005;280:5571–80.
- Harte MT, Haga IR, Maloney G, Gray P, Reading PC, Bartlett NW, Smith GL, Bowie A, O'Neill LA. The poxvirus protein A52R targets Toll-like receptor signaling complexes to suppress host defense. *J. Exp. Med.* 2003;197:343–51.
- Hewson CA, Jardine A, Edwards MR, Laza-Stanca V, Johnston SL. Toll like receptor 3 is induced by and mediates antiviral activity against rhinovirus infection of human bronchial epithelial cells. *J. Virol.* 2005;79:12273–9.
- Kaiser P, Underwood G, Davison TF. Differential cytokine responses following Marek's disease virus infection of chickens differing in resistance to Marek's disease. *J. Virol.* 2003;77: 762–8.
- Khatir M, Palmquist JM, Cha RM, Sharma JM. Infection and activation of bursal macrophages by virulent infectious bursal disease virus. *Virus Res.* 2005;113:44–50.
- Kim IJ, Karaca K, Pertile TL, Erickson SA, Sharma JM. Enhanced expression of cytokine genes in spleen macrophages during acute infection with infectious bursal disease virus in chickens. *Vet. Immunol. Immunopathol.* 1998;61:331–41.
- Kim IJ, You SK, Kim H, Yeh HY, Sharma JM. Characteristics of bursal T lymphocytes induced by infectious bursal disease virus. *J. Virol.* 2000;74:8884–92.
- Li K, Foy E, Ferreón JC, Nakamura M, Ferreón AC, Ikeda M, Ray SC, Gale M Jr, Lemon SM. Immune evasion by hepatitis C virus NS3/4A protease mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc. Natl. Acad. Sci. USA.* 2005;102:2992–7.
- Lillehoj HS, Min W, Choi KD, Babu US, Burnside J, Miyamoto T, Rosenthal BM, Lillehoj EP. Molecular, cellular, and functional characterization of chicken cytokines homologous to mammalian IL-15 and IL-2. *Vet. Immunol. Immunopathol.* 2001;82:229–44.
- Medzhitov R. Recognition of microorganisms and activation of the immune response. *Nature.* 2007;449:819–26.
- Meylan E, Tschopp J. Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. *Mol. Cell.* 2006; 22:561–9.
- Min W, Lillehoj HS. Identification and characterization of chicken interleukin-16 cDNA. *Dev. Comp. Immunol.* 2004;28: 153–62.

17. Mueller A, O'Rourke J, Grimm J, Guillemin K, Dixon MF, Lee A, Falkow S. Distinct gene expression profiles characterize the histopathological stages of disease in *Helicobacter*-induced mucosa-associated lymphoid tissue lymphoma. *Proc. Natl. Acad. Sci. USA*. 2003;100:1292–7.
18. Palmquist JM, Khatri M, Cha RM, Goddeeris BM, Walcheck B, Sharma JM. *In vivo* activation of chicken macrophages by infectious bursal disease virus. *Viral Immunol*. 2006;19:305–15.
19. Raj GD, Thangavelu A, Elankumaran S, Koteeswaran A, Venugopalan AT. Quantitative counter-immunoelectrophoresis for estimation of antibodies to infectious bursal disease virus. *Vet Res. Commun*. 1994;18:289–93.
20. Ramadass P, Thiagarajan V, Parthiban M, Senthil Kumar TM, Latha D, Anbalagan S, Krishnakumar M, Nachimuthu K. Sequence analysis of infectious bursal disease virus isolates from India: phylogenetic relationships. *Acta Virol*. 2003;47:131–5.
21. Rautenschlein S, Yeh HY, Njenga MK, Sharma JM. Role of intrabursal T cells in infectious bursal disease virus (IBDV) infection: T cells promote viral clearance but delay follicular recovery. *Arch. Virol*. 2002;147:285–304.
22. Schroder M, Bowie AG. TLR3 in antiviral immunity: key player or bystander? *Trends Immunol*. 2005;26:462–8.
23. Thangavelu A, Raj GD, Elankumaran S, Manohar BM, Koteeswaran A, Venugopalan AT. Pathogenicity and immunosuppressive properties of infectious bursal disease virus field isolates and commercial vaccines in India. *Trop. Anim. Health Prod*. 1998;30:167–76.
24. Yang W, Molenaar A, Kurts-Ebert B, Seyfert HM. NF-kappa B factors are essential, but not the switch, for pathogen-related induction of the bovine beta-defensin 5-encoding gene in mammary epithelial cells. *Mol. Immunol*. 2006;43:210–25.
25. Yoneyama M, Fujita T. Function of RIG-I-like receptors in antiviral innate immunity. *J. Biol. Chem*. 2007;282:15315–8.