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Effect of CpG ODN on Infectious Bronchitis Virus Replication in Trachea and Kidneys of Chicken Embryos

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ABSTRACT

Toll like receptors (TLRs) are a major class of innate immune pattern recognition receptors that play a key role in the immune response and defense against infections**.** TLRs are essential for initiation of the innate immune responses and development of the adaptive immune responses. The immunomodulatory property of TLR agonists has led to their exploitation as both prophylactic agents to enhance host immunity to pathogens and adjuvants to induce robust immune response against vaccine antigens. The CpG DNA is the most widely used TLR agonist as an adjuvant in several infectious diseases of chicken such as fowl cholera, avian influenza and pathogenic *E. coli*. The present study was conducted on the hypothesis that the pre-treatment of specific pathogen free (SPF) embryonated chicken eggs (ECEs) with CpG ODN may reduce the viral load of infectious bronchitis virus (IBV) in the trachea and kidneys of ECEs. The antiviral activity of CpG ODN was tested in SPF ECEs. The CpG ODN was administered in 18 days old ECEs through allantoic route. The Dulbecco's phosphate buffered saline was given in the control group. The trachea and kidneys were collected after 24 and 72 h of IBV infection and quantification of IBV was done in the trachea and kidneys. The results revealed that the CpG ODN significantly reduced the IBV titer in trachea and kidneys of the treated group as compared to the control group. In conclusion, the CpG ODN may act as a good antiviral agent and an adjuvant for improvement and development of *in ovo* vaccines against IBV.

HIGHLIGHTS

- TLRs are innate immune pattern recognition receptors (PRRs) that play a key role in the immune response and defense against infections
- The CpG DNA is the most widely used TLR agonist as an adjuvant in several infectious diseases of chicken
- CpG ODN significantly reduced IBV load in trachea and kidneys of chicken embryos

Keywords: Toll Like Receptors, Infectious Bronchitis Virus, CpG ODN, antiviral and adjuvant

The toll like receptors (TLRs) are the pattern recognition receptors (PRRs) that detect the conserved and unique molecules present on the pathogens referred to as pathogen associated molecular patterns (PAMPs) (Medzhitov *et al.,* 1997) and are part of the innate immune system (Iqbal *et al.,* 2005). The TLRs are preferentially expressed on lymphocytes, dendritic cells (DCs) and macrophages. The engagement of TLRs on DCs links innate with adaptive immune system. Some TLR genes have been identified and characterized in chickens, such as TLR1A, 1B, 2A, 2B, 3, 4, 5, 7, 15 and 21 (Paul *et al.,* 2013). In chicken, various TLR agonists have been used as vaccine adjuvants (Huang *et al.,* 2011) and prophylactic agents (Stewart *et al.,* 2011; Gomis *et al.,* 2003). The endosomal TLRs like TLR3, 7 and 21 are useful targets against viral infections because

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they recognize nucleic acid part of microorganisms and induce type-I interferon (IFN). The CpG DNA is the most widely used TLR agonist as an adjuvant in several infectious diseases of chicken such as fowl cholera (Herath *et al.,* 2010), avian influenza (Wong *et al.,* 2009; Xiaowen *et al.,* 2009) and pathogenic *E. coli* (Gomis *et al.,* 2003). The antiviral activity of CpG ODN has been demonstrated by Barjesteh *et al.* (2014) in which the pre-treatment of chicken macrophages with CpG ODN reduced the ability of AIV to infect these cells. Pre treatment of embryonated chicken eggs with CpG ODN significantly reduced infectious bronchitis virus titer in allantoic fluid (Sharma *et al.,* 2021). Interaction of CpG ODN with TLR21 leads to the activation of B cells, macrophages, DCs, natural killer cell, and secretion of wide range of cytokines and chemokines (Gupta and Cooper, 2008; Klinman, 2004; Mena *et al.,* 2003; Nichani *et al.,* 2004).

Infectious bronchitis (IB) is a highly contagious disease of chicken that causes huge economic losses to poultry industry worldwide. It is caused by infectious bronchitis virus belonging to the genus *Coronavirus* of the Family *Coronaviridae*.A number of live and inactivated vaccines are available for the control of IB virus (IBV) infections. However, the vaccine strain used in the vaccine may not always be protective. Therefore, the outbreaks of IBV are very common in vaccinated flocks in India. The emergence of new variant serotypes and genotypes of IBV in the field is frequent because of mutations and/or recombination of the hyper variable regions of the S1 gene which do not cross-protect (Chhabra *et al.,* 2015). Small changes in the amino acid sequences of the spike protein can result in the generation of new antigenic types, quite different from existing vaccine types (Adzharia *et al.,* 1997) and may require a homologous vaccine. This may be the probable reason of vaccine failure against IBV in the vaccinated flock. The *in ovo* vaccination has been recognized as an alternative choice for the vaccination in poultry. However, there are some problems with the *in ovo* vaccination like low immunogenicity in case of killed vaccines and embryo lethality in case of live vaccines. These challenges may be resolved by the use of TLR agonist like CpG ODN. The TLR agonists may act as an immune enhancer with killed vaccine or reduce embryo mortality by enhancement of innate immune response in live vaccines (Rautenschlein *et al.,* 2002). The CpG ODN may also be used as an adjuvant with existing IBV vaccine. The present study was therefore, conducted to evaluate the effect of CpG ODN on the IBV replication in trachea and kidneys of chicken embryos in SPF ECEs.

MATERIALS AND METHODS

TLR ligand

The CpG ODN (Class-B TLR) agonist was commercially procured from the InvivoGen, California (USA).

Embryonated Chicken Eggs

The zero (0) day old specific pathogen free (SPF) embryonated chicken eggs (ECEs) were procured from Immunetic Lifesciences Private Limited, Una, HP, India and kept in egg incubator upto 18 days in Department of Veterinary Microbiology LUVAS, Hisar at 37°C with relative humidity about 60%. The ECEs were turned eight times a day.

Infectious bronchitis virus

The IBV isolate, IBV3 Hisar 2018, isolated in the Department of Veterinary Microbiology, LUVAS (Sharma *et al.,* 2020) was used in the present study. The bulk production of IBV3 Hisar 2018 was done in 9-11 days old SPF ECEs through intra-allantoic route and virus titration done subsequently. The end point titers were expressed as 50 % embryo infective doses (EID_{50}) per ml (Reed and Muench, 1938). The allantoic fluid was checked for contamination of Newcastle disease virus, infectious laryngeotracheitis virus, mycoplasma, bacterial or fungal contamination. The presence of IBV in allantoic fluid was re-confirmed by S1 (partial) gene based PCR (Cavanagh *et al.,* 2001) and used for further experiments.

Experimental design

Six SPF ECEs (18 day old) were inoculated with CpG ODN @ 4 µg/egg through allantoic route. The control group of six SPF ECEs was treated with Dulbecco's phosphate buffered saline (DPBS). After 24 h of incubation, the eggs were inoculated with 100 µl of IBV3 Hisar 2018 ($10^{4.8}$ EID_{50}) and incubated again. The eggs were candled at 24 h intervals for checking the viability of their embryos. The tissue samples (trachea and kidneys) were collected in

TRIzol at 24 and 72 h post IBV inoculation. The tissues from two infected or control embryos were pooled to generate one sample. The viral load in tissue samples was quantified by real time PCR (RT-qPCR). The IBV nucleocapsid (*N*) gene progression in cellular RNA extracted from tissue was used as an indicator of IBV infection progression.

Isolation of total RNA from tissue samples

The RNA isolations from tissue samples (trachea and kidneys) were done by the TRIzol method, according to the manufacturer's protocol. Briefly, the collected tissue (100 mg) was homogenized with 750 µl TRIzol by pipetting several times, incubated at room temperature for 3 min and 200 µl chloroform added. The tube was shaken vigorously for 15 s and centrifuged at 8,000 g for 15 min at 4 °C for phase separation. The aqueous phase was transferred carefully into new tube. To this 500 µl isopropanol was added and centrifuged at 8,000 g for 15 min at 4 °C for precipitation of RNA. The supernatant was removed; RNA pellet was washed carefully with 75 % ethanol and centrifuged at 8000 g for 5 min at 4 °C. The ethanol was removed and RNA pellet dissolved in nuclease free water (NFW), warmed at 55-60 °C for complete dissolution and then stored at -20 °C, until further used. The purity and concentration of the isolated RNA was checked by measuring $OD_{260}/_{280}$ in a UV spectrophotometer using the following formulae:

$OD_{260}/_{280}$ = 2.00 RNA preparation is pure (No protein or phenol contamination)

OD_{260} = 1.00 (Approx. 40 µg of RNA/ml)

Preparation of complementary DNA

The total RNA isolated from tissue samples were used for the preparation of cDNA employing Revertaid™ First Strand cDNA Synthesis Kit (Thermo Scientific, USA) following manufacturer's instructions. Briefly, total RNA (5 µg) and 1 µl of random hexamer primer were made up to 12 µl with NFW, incubated at 25 °C for 5 min and8 µl of reaction mixture (containing 5 X Reaction buffer, 4 µl; Ribolock™ RNase inhibitor, 1 µl; 10 mM dNTPs mix, 2 µl; and MoMuLV Reverse Transcriptase,1 µl)added. The tube was mixed gently by spinning, incubated at 42 °C for 60 min for cDNA synthesis and the reaction terminated by heating at 70 °C for 5 min. The cDNA product was stored at -20 °C, until further used.

Real-time quantitative polymerase chain reaction

The differential mRNA expression in trachea and kidneys was assessed by real-time quantitative PCR (RT-qPCR) using a PikoReal Real-Time PCR (Thermo Scientific) and QuantiTect SYBR Green PCR Kit (Qiagen, Germany). The RT-qPCR was performed in a reaction volume of 20 µl (containing cDNA diluted to 1:10 in NFW, 2µl; SYBR® Green master mix,10 µl; Forward primer *N* gene, 0.5 µl; Reverse primer *N* gene, 0.5 µl; and RNase-free water, 7 µl). All the non-treatment controls and treated samples were put in triplicate on the same plate. The RT-qPCR cycling conditions were set as initial denaturation cycle at 95 °C for 15 min; followed by 45 cycles each of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 76 °C for 30 s. The final step was to obtain a melt curve for the PCR products to determine the specificity of amplification. The relative expression of viral *N* gene was calculated relative to the expression of the *β-actin* gene and expressed as n-fold increase or decrease relative to the control samples (Dar *et al.,* 2009). The cycle at which the sample amplicon reporter dye concentration crossed the pre-set threshold was recorded as the cycle threshold (Ct) value. The data of RT-qPCR were analyzed by $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001) to derive the relative fold change in mRNA expression of IBV *N* gene. The primers used in RT-qPCR for the expression of the IBV viral *N* gene and *β-actin* gene have been depicted in Table 1.

Table 1: Primers used for Real time quantitative PCR analysis

Genes	Primer Sequence (5′-3′)	References
IBV *N* gene	F:GAAGAAAACCAGTCCCAGA TGCTTGG	Dar *et al*. (2009)
	R: GTTGGAATAGTGCGCTTGC AATACCG	
β-actin gene	F: CAA CAC AGT GCT GTC TGG TGG TA	Paul *et al*. (2011)
	R: ATC GTA CTC CTG CTT GCT GAT CC	

STATISTICAL ANALYSIS

The mean relative expression for each group was statistically analyzed by one way ANOVA [least significance difference (LSD) and Duncan's Test] for 'T' distribution (P value) using SPSS (16.0) Software. For statistical analysis of virus replication data in two different groups, independent t-test was performed to check the difference between two groups at different hours significant at $P<0.05$ (Snedecor and Williams, 1989).

RESULTS AND DISCUSSION

IBV propagation

The characteristic embryo changes were seen three days after inoculation of the virus in SPF ECEs. Only slight movement of a dwarfed embryo was observed during candling. When the inoculated egg was opened from the broad end, the chicken embryo was seen curled into a spherical form with feet deformed and compressed over the head with the thickened amnion adhered to it.

Effect of CpG ODN on viral load in trachea and kidneys

The embryos treated with CpG ODN had lower viral load in trachea and kidneys as compared with DPBS treated embryos at 24 and 72 h post infection (Table 2).

Table 2: Relative fold change in IBV N gene mRNA transcript expression in trachea and kidneys after treatment of embryonated chicken eggs with CpG ODN

Relative fold change in *N* gene mRNA expression ($2^{-\Delta\Delta Ct}$) post infection (Mean ±SE)		
Organs	**24 h post infection**	**72 h post infection**
Trachea	0.788±0.028*	0.777±0.022*
Kidneys	0.612±0.050*	0.725±0.072*

The statistical analysis of each treatment and control group was done using independent sample t-test to compare the mean of two different groups using SPSS (16.0) and significant difference ($P<0.05$) between a test group and the DPBS group are indicated by *. There were six replicates in each group.

The TLRs are one of the types of PRRs, have a central role in innate immunity as they recognize conserved molecular pattern in the microbes (Jin and Lee, 2008) and also help in activation of adaptive immune response. TLR ligands have been tried as adjuvants along with many bacterial and viral vaccines in mammals as well as birds. In the present study, an *in vivo* model was established to study the effect of TLR ligands on IBV replication in trachea and kidneys using SPF ECEs. The IBV was selected as a model because of its economic importance and highly infectious nature. The IBV infection is distributed worldwide and its control is very difficult because of presence of multiple serotypes and variants of virus emerging continuously, which are not cross protecting each other (Cavanagh and Naqi, 2003). For protection against IBV through a successful vaccination program, it is essential to identify the prevalent genotypes in the region and to determine the role of TLR ligands in enhancing the protective potential of IBV vaccine. Since the development of a new vaccine is a very difficult task, so it becomes equally important to improve the efficacy and delivery of existing IBV vaccine.

Several studies have suggested that CpG ODN stimulated different immune cells of humans, animals &poultry and led to the production of different cytokines and other effector molecules, including nitric oxide, IFN-α, IFN-γ, IL-1, IL-6 and TNF-α, which play an important roles in CpG ODN induced immunity (Harandi *et al.,* 2003; Jeurissen *et al.,* 2000; Schlaepfer *et al.,* 2004; Xie *et al.,* 2003). Chicken infected with *Salmonella Enteritis* induced activation of IFN-γ and IL-1β, stimulated chicken heterophils and mediated phagocytosis (Kogut *et al.,* 2003).In the present study, it was observed that the pre-treatment of ECEs with CpG ODN had significantly reduced the IBV load in trachea and kidneys of chicken embryos. It has been demonstrated earlier that the treatment of ECEs with CpG ODN had significantly up regulated the expression of IFN-γ, IL-1β, IL-6, IL-8 and OAS in the spleen of chicken embryos (Dar *et al.,* 2009). These genes may inhibit IBV replication in trachea and kidneys of chicken embryos. These results have also been supported by Barjesteh *et al*. (2014), wherein the intranasal administration of CpG ODN in chickens induced antiviral genes like IFN-α, IFN-β,interferon stimulating gene, melanoma differentiation-associated protein 5and interferon inducible transmembrane in trachea. These antiviral genes may further inhibit the virus replication in trachea and kidneys of chicken embryos. The treatment of tracheal organ culture with CpG ODN up-regulated the

expression of different immune response gene like IL-1β, iNOS, IRF-7, IFN-α, IFN-β and OAS at different time points (Barjesteh *et al.,* 2016). The CpG ODN activated monocytes, macrophages, and dendritic cells to secrete various Th1 cytokines, which in turn activated the T_H and NK cells to secrete a broad range of cytokines (Ballas *et al.,*1996) yielding strong antigen specific immune responses (Sparwasser and Lipford, 2000).Thus, it is speculated that the *in ovo* administration of CpG ODN in chickens will help in enhancement of innate immune response and possible protection against viral infection in neonatal chickens before development of protective vaccine response against viral infections.

CONCLUSION

In conclusion, the CpG ODN demonstrated significant reduction in IBV load in trachea and kidneys of chicken embryos. The CpG ODN induced the expression of different antiviral and pro-inflammatory cytokines in chicken embryos responsible for inhibition of IBV replication in trachea and kidneys. The CpG ODN may enhance neonatal immunity without inducing disease pathology may be helpful in the replacement of antibiotics and reduction of early chick mortality (Haygreen *et al.,* 2005).On the basis of above findings in the present study, it has been demonstrated that the CpG ODN may act as a good antiviral agent, immune modulation agent and adjuvant in *in ovo* vaccines against IBV.

REFERENCES

Adzhar, A., Gough, R.E., Haydon, D., Shaw, K., Britton, P. and Cavanagh, D. 1997. Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain. *Avian Pathol.,* **26**(3): 625-40.

Ballas, Z.K., Rasmussen, W.L. and Krieg, A.M. 1996. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. The *J. Immunol.,* **157**(5): 1840-5.

Barjesteh, N., Alkie, T. N., Hodgins, D. C., Nagy, É. and Sharif, S. 2016. Local innate responses to TLR ligands in the chicken trachea. *Viruses*, **8**(7): 207.

Barjesteh, N., Behboudi, S., Brisbin, J. T., Villanueva, A. I., Nagy, É. and Sharif, S. 2014. TLR ligands induce antiviral responses in chicken macrophages. *PloS one*, **9**(8): e105713.

Cavanagh, D. and Naqi, S.A. 2003. Infectious bronchitis. *In:* Saif, Y.M. (Ed.), Diseases of Poultry, 11th ed. University Press, Ames, Iowa, pp. 101–119.

Cavanagh, D., Mawditt, K., Sharma, M., Drury, S.E., Ainsworth, H.L., Britton, P. and Gough, R.E. 2001. Detection of a coronavirus from turkey poults in Europe genetically related to infectious bronchitis virus of chickens. *Avian Pathol.,* **30**(4): 355-368.

Chhabra, R., Forrester, A., Lemiere, S., Awad, F., Chantrey, J. and Ganapathy, K. 2015. Mucosal, cellular, and humoral immune responses induced by different live infectious bronchitis virus vaccination regimes and protection conferred against infectious bronchitis virus Q1 strain. *Clin. Vaccine Immunol.,* **22**(9): 1050-1059.

Dar, A., Potter, A., Tikoo, S., Gerdts, V., Lai, K., Babiuk, L. A. and Mutwiri, G. 2009. CpG oligodeoxynucleotides activate innate immune response that suppresses infectious bronchitis virus replication in chicken embryos. *Avian Dis.,* **53(**2): 261-267.

Gomis, S., Babiuk, L., Godson, D.L., Allan, B., Thrush, T., Townsend, H. and Potter, A. 2003. Protection of chickens against Escherichia coli infections by DNA containing CpG motifs. *Infect. Immun.,* **71**(2): 857-863.

Gupta, K. and Cooper, C. 2008. A review of the role of CpGoligodeoxynucleotides as toll-like receptor 9 agonists in prophylactic and therapeutic vaccine development in infectious diseases. *Drugs in R & D.*, **9**(3): 137-145.

Harandi, A. M., Eriksson, K. and Holmgren, J. 2003. A protective role of locally administered immunostimulatory CpG oligodeoxynucleotide in a mouse model of genital herpes infection. *J. Virol.,* **77**(2): 953-962.

Haygreen, L., Davison, F. and Kaiser, P. 2005. DNA vaccines for poultry: the jump from theory to practice. *Expert Rev. Vaccines,* **4**(1): 51-62.

Herath, C., Kumar, P., Singh, M., Kumar, D., Ramakrishnan, S., Goswami, T.K., Singh, A. and Ram, G.C. 2010. Experimental iron-inactivated Pasteurella multocida A: 1 vaccine adjuvanted with bacterial DNA is safe and protects chickens from fowl cholera. *Vaccine*, **28**(11): 2284-2289.

Huang, Y., Temperley, N.D., Ren, L., Smith, J., Li, N. and Burt, D.W. 2011. Molecular evolution of the vertebrate TLR1 gene family-a complex history of gene duplication, gene conversion, positive selection and co-evolution. *BMC Evol. Biol.,* **11**(1): 149.

Iqbal, M., Philbin, V. J. and Smith, A. L. 2005. Expression patterns of chicken Toll-like receptor mRNA in tissues, immune cell subsets and cell lines. *Vet. Immunol. Immunopathol*., **104**(1-2): 117-127.

Jeurissen, S.H., Boonstra-Blom, A.G., Al-Garib, S.O., Hartog, L. and Koch, G. 2000. Defence mechanisms against viral infection in poultry: a review. *Vet. Q.*, **22**(4): 204-208.

Jin, M.S. and Lee, J.O. 2008. Structures of the toll-like receptor family and its ligand complexes. *Immunity*, **29**(2): 182-191.

Klinman, D.M. 2004. Use of CpGoligodeoxynucleotides as immunoprotective agents. *Expert Opin. Biol. Ther.,* **4**(6): 937-46.

Kogut, M. H., Rothwell, L. and Kaiser, P. 2003. Differential regulation of cytokine gene -expression by avian heterophils during receptor-mediated phagocytosis of opsonized and nonopsonized Salmonella enteritidis. *J. Interferon Cytokine Res.,* **23**(6): 319-327.

Medzhitov, R., Preston-Hurlburt, P. and Janeway, C.A. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature*, **388**(6640): 394-397.

Mena, A., Nichani, A.K., Popowych, Y., Godson, D.L., Dent, D., Townsend, H.G., Mutwiri, G.K., Hecker, R., Babiuk, L.A. and Griebel, P. 2003. Innate immune responses induced by CpG oligodeoxyribonucleotide stimulation of ovine blood mononuclear cells. *Immunology*, **110**(2): 250-257.7.

Nichani, A.K., Kaushik, R.S., Mena, A., Popowych, Y., Dent, D., Townsend, H.G., Mutwiri, G., Hecker, R., Babiuk, L.A. and Griebel, P.J. 2004. CpG oligodeoxynucleotide induction of antiviral effector molecules in sheep. *Cell. Immunol.,***227**(1): 24-37.

Paul, M.S., Brisbin, J.T., Abdul-Careem, M.F. and Sharif, S. 2013. Immunostimulatory properties of Toll-like receptor ligands in chickens. *Vet. Immunol. Immunopathol*., **152**(3-4): 191-199.

Paul, M.S., Mallick, A.I., Haq, K., Orouji, S., Abdul-Careem, M.F. and Sharif, S. 2011. *In vivo* administration of ligands for chicken toll-like receptors 4 and 21 induces the expression of immune system genes in the spleen. *Vet. Immunol. Immunopathol*., **144**(3-4): 228-237.

Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res.,* **29**(9): e45-e45.

Rautenschlein, S., Sheikh, A.M., Patnayak, D.P., Miller, R.L., Sharma, J.M. and Goyal, S.M. 2002. Effect of an immunomodulator on the efficacy of an attenuated vaccine against avian pneumovirus in turkeys. *Avian Dis.,* **46**(3): 555-561.

Reed, L.J. and Muench, H. 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Epidemiol*., **27**(3): 493-497.

Schlaepfer, E., Audigé, A., von Beust, B., Manolova, V., Weber, M., Joller, H., Bachmann, M.F., Kundig, T.M. and Speck, R.F. 2004. CpGoligodeoxynucleotides block human immunodeficiency virus type 1 replication in human lymphoid tissue infected ex vivo. *J. Virol.,* **78**(22): 12344-12354.

Sharma, B.K., Kakker, N.K., Bhadouriya, S. and Chhabra, R., 2020. Effect of TLR agonist on infections bronchitis virus replication and cytokine expression in embryonated chicken eggs. *Mol. Immunol.,* **120**: 52-60.

Sparwasser, T. and Lipford, G.B. 2000. Consequences of bacterial CpG DNA-driven activation of antigen-presenting cells. In: *Immunobiology of Bacterial CpG-DNA* (pp. 59-75). *Springer,* Berlin, Heidelberg.

Snedecor, G.W.C., William, G., 1989. Statistical methods. George W. Snedecor and William G. Cochran (No. QA276. 12. S6313 1989.).

Stewart, C.R., Karpala, A.J., Lowther, S., Lowenthal, J.W. and Bean, A.G. 2011. Immunostimulatory motifs enhance antiviral siRNAs targeting highly pathogenic avian influenza H5N1. *PloS one*, **6**(7): e21552.

Wong, J.P., Christopher, M.E., Viswanathan, S., Karpoff, N., Dai, X., Das, D., Sun, L.Q., Wang, M. and Salazar, A.M., 2009. Activation of toll-like receptor signaling pathway for protection against influenza virus infection. *Vaccine*, **27**(25-26): 3481-3483.

Xiaowen, Z., Qinghua, Y., Xiaofei, Z. and Qian, Y. 2009. Co-administration of inactivated avian influenza virus with CpG or rIL-2 strongly enhances the local immune response after intranasal immunization in chicken. *Vaccine*, **27**(41): 5628-5632.

Xie, H., Raybourne, R. B., Babu, U. S., Lillehoj, H. S. and Heckert, R.A. 2003. CpG-induced immunomodulation and intracellular bacterial killing in a chicken macrophage cell line. *Dev. Comp. Immunol*., **27**(9): 823-834. 51