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Expression profile of Toll-like Receptors Pathway Genes in Chicken Erythrocytes Infected with *Mycoplasma synoviae*

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XYH and AK drafted the basic manuscript and analyzed the data. WXT contributed to conception and design of the research and reviewed the manuscript. YHB and RWF contributed to review the manuscript. RAM, MFQ, XYH and YW, participated in sample collection and laboratory testing. ARJ, DZ and MLQ participated in the data analysis and revised the manuscript. All authors read and approved the final manuscript.

Key words
Chicken, Erythrocytes, *Mycoplasma synoviae*, Toll-like receptors, Expression profiles.

ABSTRACT

Toll-like receptors (TLRs) are one of the important immunes signaling pathways that participate in the activation of host immune response after detecting microbial pattern molecules. Despite important expression profile TLRs genes in chicken erythrocytes, studies have been lacking. This study investigated the expression profile of TLRs pathway immune gene in chicken erythrocytes in response to *Mycoplasma synoviae*. The purpose of the current *in-vitro* study was to determine the chicken erythrocytes interaction with *M. synoviae* using a transmission electron microscope (TEM). The mRNA gene expression of *TLR1, 2, 3, 4, 5, 7, 15, MHC I, II,* and *MyD88* in *M. synoviae* infected chicken erythrocytes was determined using quantitative real-time PCR (qRT-PCR) at four different time intervals (0, 2, 6 and 10 h) post-infection and compared to uninfected controls. The mRNA expression of TLRs such as *TLR1, 2, 3, 15*, and *MHC II* were significantly upregulated at 6 and 10 h post-infection in infected chicken erythrocytes. However, significantly upregulated expression of *TLR5* and *MHC I* were noted at 2, 6, and 10 h while *TLR4* and *MyD88* mRNA expression was also significantly upregulated but at different time intervals. This study provides the first evidence of upregulated expression of TLR signaling pathway genes in *M. synoviae* infected chicken erythrocytes. These results provide new insights on *M. synoviae* infection resistance mechanisms and the role of TLR signaling immune genes in the control of the host immune response.

INTRODUCTION

The erythrocyte is the most abundant cell subset in blood circulation and functions mainly in the exchange and transportation of gases. Erythrocytes are the key in bactericidal cells, which perform pathogens clearance in the bloodstream. In certain species, such as avies, fishes and reptiles mature erythrocytes are nucleated and transcriptionally active (Morera *et al*., 2011). As a result, these erythrocytes contribute to other features of homeostasis such as immune system modulation (Morera and Mackenzie, 2011). Similarly, numerous studies showed that in immunology, an important role is played by chicken erythrocytes (Jahejo *et al*., 2020a; Niu *et al*., 2019). Furthermore, in erythrocytes, numerous toll-like receptors (TLRs) transcripts (*TLR2, 3, 4, 5, and 7*) were expressed constitutively (Paolucci *et al*., 2013).

Mycoplasma synoviae is a significant pathogen of domestic poultry that leads to huge economic losses in the poultry industry (Kleven, 2008; Umar *et al*., 2017). Infection mainly occurs as a subclinical causing respiratory and systemic disease, autoimmune disorders, and infectious synovitis in chickens (Kleven *et al*., 2003). Mycoplasmas have been reported in many studies as active players in host-pathogen interactions leading to alterations

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in cell death patterns (Obara and Harasawa, 2010). It has been reported that several immune genes are modulated in response to *M. synoviae* infection in chicken macrophages (Lavrič *et al*., 2007, 2008). More recently, nonphagocytic cell invasion capability has been found in *M. gallisepticum* and *M. synoviae* as another way of host defense evasion and persistence in the host (Much *et al*., 2002).

TLRs, the pattern recognition receptors (PRRs) key family, play an important role in host defense against invading pathogens by prompt innate recognition and inflammatory responses (Akira and Takeda, 2004; Kopp and Medzhitov, 1999). The PRRs play an essential role in the rapid initiation of the host's immune responses and the genetic identification of an invading microbe (Medzhitov *et al*., 1997; Rock *et al*., 1998) via recognition of pathogen-associated molecular patterns. TLRs have emerged as a main constituent of the vertebrate PRR repertoire. Upon activation, TLRs induce the expression of an extensive range of effector and immune regulatory molecules (Remer *et al*., 2003; Thoma-Uszynski *et al*., 2001) and immune cell types maturation (Akira *et al*., 2001; Banchereau *et al*., 1998; Brightbill *et al*., 2000; Hertz *et al*., 2001; Supajatura *et al*., 2001). Poultry TLRs are mainly located on the cells surface and cytoplasm (Brownlie, 2011), with different distributions, and thus perform different functions. Erythrocytes are one of the most numerous cells in the body, and there are TLRs expressed in the erythrocytes, TLRs are distributed inside and outside the cells. So TLRs play an irreplaceable role in the innate immune response of birds. TLRs have become the focus of biomedical research because these small molecule proteins can be used as one of the primary factors of the host's immune activation (Fitzgerald and Kagan, 2020).

Our earlier studies have also reported that in immunity against thiram induced TD chickens, or virus-infected erythrocytes, the main role had been played by chicken erythrocytes, which may also have a role in apoptosis, prostaglandin pathway especially (Jahejo *et al*., 2020a; Niu *et al*., 2019; Jia *et al*., 2018; Wang *et al*., 2018; Qadir *et al*., 2020a, b). Besides, chicken erythrocytes express TLRs and possess immune-related functions (Jahejo *et al*., 2020b; Jia *et al*., 2018). The current study examined the possibility that the expression of many immune-related genes in chickens erythrocytes respond to *M. synoviae* which may induce an inflammatory response. Little is known about TLR signaling immune-related genes that expressed in *M. synoviae* infected chicken's erythrocytes and how *M. synoviae* affects their genes expressions. Therefore, in the current study the interaction between *M. synoviae* and erythrocytes was determined. Furthermore, the results revealed the effect of *M. synoviae* strain on the mRNA expression of TLR signaling immune- related genes in chicken erythrocytes.

MATERIALS AND METHODS

Erythrocyte collection

Blood was obtained from specific pathogen-free (SPF) chickens purchased from Longkol Company (Taigu, Shanxi). A total of 4 mL of fresh venous blood from the pterygoid vein of adult SPF chicken was drawn and mixed. To the 4 mL Histopaque-1119 solution (Sigma–Aldrich, Oakville, ON), the diluted blood was carefully added following centrifugation at 2000 r/min for 20 min. Consequently, the leukocytes and platelets were removed from the supernatant. Later procedures were done as described previously by Kabanova *et al*. (2009). Moreover, pure erythrocytes were obtained by a procedure as described by Niu *et al*. (2018).

Treatment of mycoplasma

A total of 8 mL of FM-4 mycoplasma culture medium was taken during the log phase (the medium has just turned yellow). The concentration of mycoplasma was about 1×10^{6}~1×10^{7}/ mL, followed by centrifugation at 12000 r/min for 15 min, and the supernatant was discarded. After washing twice with PBS, the sample was recentrifuged at 12000 r/min for 10 min. Lastly, cells were then cultured in 98% Dulbecco's Modified Eagle Medium (DMEM) (Solarbio, Beijing, China), added with 2% fetal bovine serum (FBS) and 2% chicken serum (Longkol, Shanxi, China).

Experimental infection of chicken erythrocytes (CER)

Total 50 µL of erythrocytes were obtained from SPF chicken and drawn in sixteen 2 mL centrifuge tube containing a cell maintenance solution and distributed into four groups *i.e.,* 0, 2, 6, and 10 h. The experiment was performed in an Animal Biosafety Level 2 Laboratory. To an experimental group, 100 µL of *M. synoviae* was added into all the four experimental groups with the addition of 900 µL of DMEM to make the final volume 1050 µL. To another four groups *M. synoviae* was not added and was designated as control group. The cells were then cultured at 37°C in 5% CO_2 incubator, and each of the respective group was taken out at 0, 2, 6, and 10 h, respectively after centrifugation for 10 min at 2000 r/min. Cell supernatant was discarded and cells were washed three times with PBS and stored in a refrigerator at −80°C upcoming experiments.

Transmission electron microscope (TEM) for interaction between M. synoviae *and erythrocytes*

Erythrocytes were isolated at 2000 r/min for 10 min and washed 3 times with phosphate buffer, fixed in osmium tetroxide for 2 h at room temperature, and prestained in acetabarbitone for 10 min. After dehydration through the graded ethanol series, the samples were embedded

in Spurr's resin. Sections were prepared and then stained with uranyl acetate and lead citrate. Finally, the samples were sent to Shanxi Medical University for testing where, the ultrastructure was observed in JEM-1011 (JEOL Ltd., Tokyo, Japan) TEM.

Extraction of RNA, cDNA synthesis and qRT-PCR

From both experimental and control groups at 0, 2, 6, and 10 h fresh blood samples were obtained. From obtained erythrocytes of each group total RNA was isolated by RNAiso Plus (9109; Takara Bio Inc., Dalian, China) according to the instructions of the manufacturer. The PrimeScript RT reagent Kit (RR047A; Takara Bio Inc., Dalian, China) was used to reverse transcribe RNA into cDNA, by following the instructions of the manufacturer's recommended protocol. The cDNA samples were then stored at −20°C after dilution at 1:10 in RNase-free water. For the expression analysis of TLR family genes, qRT-PCR was performed using kit of TaKaRa SYBR Premix Ex TaqTM II (RR820A; Takara Bio Inc., Dalian, China) by the QuantStudio™ 6 (Applied Biosystems, America). Primer designing of TLR family genes was done by using Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA) according to NCBI gene coding sequences and manufactured by Shanghai Generay Biotech Co., Ltd. (Shanghai, China). Primer sequences, annealing temperature for this experiment along with accession numbers are shown in Table I. Thermal cycling parameters used for qRT-PCR were previously described by Niu *et al*. (2018). The expression profile of TLR family genes relative to the housekeeping gene 18S rRNA were calculated by the QuantStudio™ 6 Flex Real-Time PCR System Software (Applied Biosystems, USA).

Statistical analysis

The real time PCR data was calculated using $2^{-\Delta\Delta Ct}$ method. The data obtained between control and experiment groups for each time point post-infection was employed

Table I.- Primer sequences and accession numbers used in quantitative RT-PCR.

Gene targeted	Primers sequences (5′→3′)	Sizes (bp)	Annealing temp.	Accession No.
TLR 1	F: GATGATACGAAGGTCAGACT	100	55˚C	NM_001007488
	R:CAGACTTAGAGGCTCATACA			
TLR 2	F:ACCTGGCCCATAACAGGATA	100	55˚C	AB046119
	R:ATGGAGCTGATTTGGTTGGA			
TLR 3	F:GCCTAAATATCACGGTACTC	100	55˚C	NM_001011691
	R:CACAACAGTGGTAGTGATCA			
TLR 4	F:AGTCTGAAATTGCTGAGCTCAAAT	190	55˚C	NM_001030693
	R:GCGACGTTAAGCCATGGAAG			
TLR 5	F:CTGCCAAATCTTCGTGTCTT	100	55˚C	FJ915552
	R:ACAGACGGAGTATGGTCAAA			
TLR 7	F: GGTGTTAGCCACGTGCTTAG	100	55˚C	NM_001011688
	R: CCATCCCTGTGCTGATAGAG			
TLR 15	F:CTCACAGCACAATGCCTACATCC	100	55˚C	NM_001037835
	R:TCCCAAGCAAAGAGATAGAGCCC			
MHC I	F:TGCCGTGGTTCGTGATTGTG	138	55˚C	KT337504
	R:TCTGCGTCTGTCCATTCCAG			
MHCII	F:TGCCCGAAACCGACCGTCTG	160	55˚C	NM001318995
	R:TCCAGCACCACCAGCACCTG			
MyD88	F:ATGGAAGCCAAGCCAGAGTT	144	55˚C	XM015287208
	R:ACAGCGCACCAGAAGGGTAT			
18SrRNA	F:TTCCGATAACGAACGACAC	139	55˚C	FM165414
	R:GACATCTAAGGGCATCACAG			

to Two-way ANOVA followed by Tukey's Multiple Comparison test to perform statistical analysis. All graphs were accomplished using GraphPad Prism 5. Significant differences were measured once the *P* value was $P < 0.05$, $P < 0.01$ or $P \leq 0.001$.

RESULTS

Interaction between M. synoviae *and erythrocytes*

The interaction of *M. synoviae* and erythrocytes is shown in Figure 1B by having endosome. While, non-infected (control) erythrocytes was distributed uniform cytoplasm as shown in control section of Figure 1A.

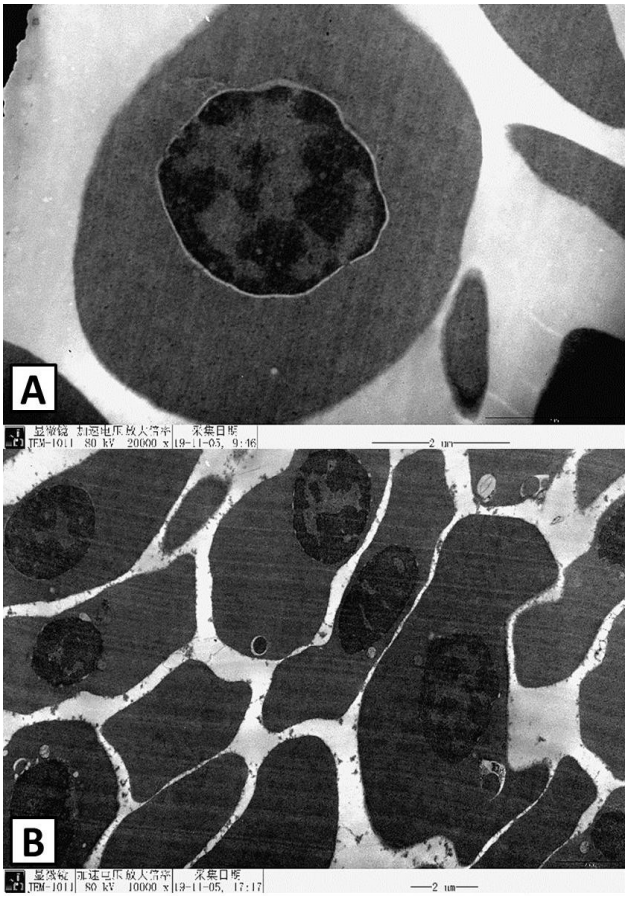

Fig. 1. The transmission electron micrograph (TEM) revealed intearction between erythrocytes with *M. synoviae.*

Expression of TLR pathway genes in chicken erythrocytes infected with M. synoviae

The relative mRNA expression levels of TLRs signaling pathway genes in chicken erythrocytes following infection with *M. synoviae* were analyzed and presented in Figure 2 to investigate the degree of effect on gene expression in chicken erythrocytes after interaction with *M. synoviae*. This study determined that the expression levels of TLRs signaling pathway genes were significantly varied at different time intervals such as 0, 2, 6, and 10 h. The qRT-PCR results indicated that relative mRNA expression of *TLR4, 5, 7,* and *MHC I* were significantly upregulated at 0 h ($P < 0.05$), compared to the control group erythrocytes. Moreover, at 2 h, the expression of *TLR4, MyD88, MHC I* and *MHC II* were significantly upregulated ($P < 0.05$). Relative mRNA expression of *TLR1, 2, 3, 5, 7, 15, MHC I,* and *MHC II* were significantly up-regulated ($P < 0.05$) compared to a control group at 6 h. Furthermore, the expression of *TLR1, 2, 3, 7* and *MHC* II in infected erythrocytes at 10 h were also significantly upregulated ($P < 0.05$) while *TLR4* expression was significantly downregulated ($P < 0.05$) at 10 h compared with the control group.

DISCUSSION

M. synoviae infection emerges to cause considerable financial loses to worldwide poultry producers. *M. synoviae* infection controls numerous immune genes in chicken macrophages (Lavrič *et al*., 2007, 2008) and apoptotic genes in chicken chondrocytes (Dušanić *et al*., 2012). Recent studies indicate that certain *M. gallisepticum* strains may invade cells, such as chicken erythrocytes and embryonic fibroblasts (Winner *et al*., 2000; Vogl *et al*., 2008). Therefore in the current study, we revealed that chicken erythrocytes have an important role in immunity.

The current study results proposed that transcripts for the *TLR1, 2, 3, 4, 5, 15, MHC I, MHC II* and *MyD88,* were constitutively expressed in *M. synoviae* infected chicken erythrocytes. Therefore, this result offers a novel perspective for poultry health because; targeting such TLRs for therapeutic purposes can be one of the ways to defend chickens from infection of mycoplasmas, particularly, *M. synoviae*. Based on expressed TLRs repertoire, erythrocytes possibly can respond to both bacterial and viral pathogens. Interestingly, the repertoire of TLRs expressed in erythrocytes is identical to that of various kinds of leukocytes (Iqbal *et al*., 2005).

It has reported from current study that chicken erythrocytes infected with *M. synoviae* strain respond to the TLRs pathway genes when compared with an uninfected group. This study first reported that mRNA expression of TLRs and other immune-related genes show upregulation at a certain time interval when chicken erythrocytes were infected with *M. synoviae* strain. In the current study, it was observed the upregulation of *TLR1* and 2 at 6 and 10 h indicating that infection severity was enhanced with the

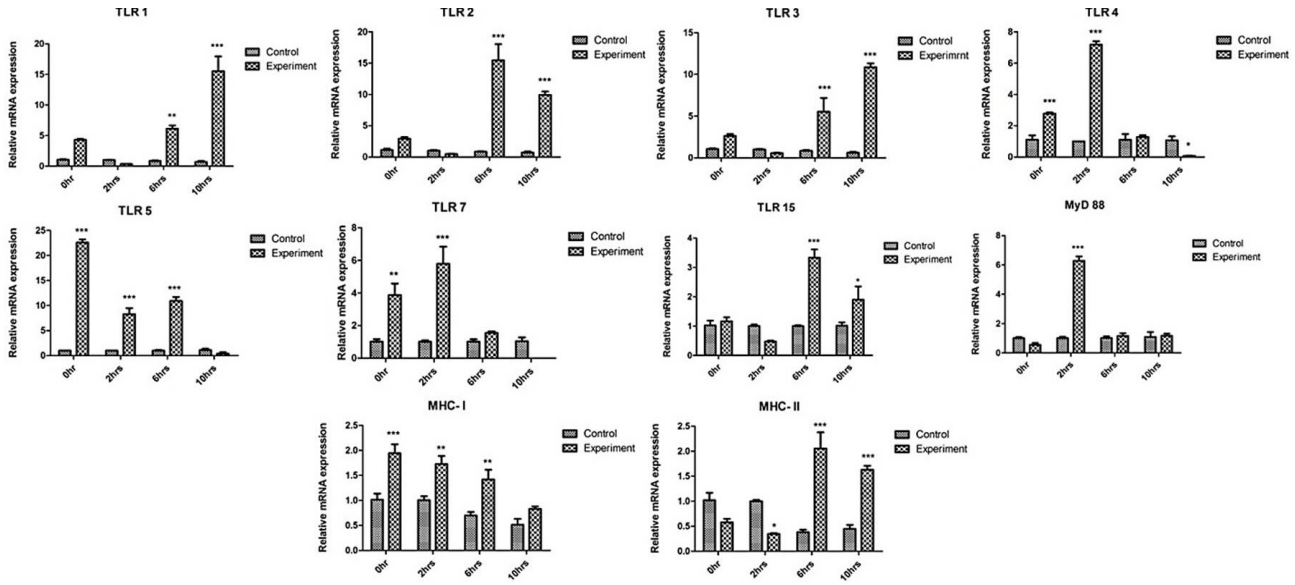

Fig. 2. The expression pattern toll-like receptors pathway genes in chicken erythrocytes on 0, 2, 6, and 10 h. This experimental study group was as follows: the control group (Con, erythrocytes treated with PBS, n = 3) and the experiment group (Exp, erythrocytes infected with *M. synoviae*, n = 3). Expression levels of TLR pathway genes were relatively calculated to that of *18S rRNA* the housekeeping genes via qRT-PCR. SEM (standard error of mean) represented, by error bars. Bars with asterisks indicate a significant (*$P < 0.05$, **$P < 0.01$, ***$P \leq 0.001$) Up or downregulated relative mRNA expression when compared to uninfected control group.

passage of time which may be due to decreased immunity level of birds so, infection was more severe in later hour. Therefore, such results suggested that *M. synoviae* have an influential effect on gene expression of TLRs, as in many cases at certain time interval genes expression was significantly upregulated. Previously related to current study similar kind of studies was carried on for the observation of TLR genes expression in chicken in response to *Clostridium perfringin* infection (Lu *et al*., 2009), *Salmonella* and *Campylobacter* infection (Meade *et al*., 2009). Therefore previous studies are also in agreement with our study results in regard to upregulation of *TLR1* and *TLR2* in response to bacterial infections in chickens (Lu *et al*., 2009). Thus, the observed upregulation of *TLR1* and *TLR2* may be a direct consequence of initial *TLR4* activation followed by self-downregulation (Higgs *et al*., 2006).

TLR3 plays a key role in infected host immune response. The TLR3 role in identifying dsRNA, NF-κB pathway activation and induction of type I IFN production in chicken has been described (Schwarz *et al*., 2007; Karpala *et al*., 2008). In this study, the significant upregulation of *TLR3* at 6 and 10 h in infected chicken erythrocyte was detected. Interestingly, in previous studies there were no evidences regarding mRNA expression of *TLR3* in chicken erythrocytes in response to bacterial infections. Therefore, the altered expression of *TLR3* in the current study has been linked to increased responsiveness to bacterial infection. Furthermore, such results suggest that chicken *TLR3* is constitutively expressed in infected chicken erythrocytes and may contribute to the innate immune response induction against bacterial infections in *in-vitro*.

Upon gene expression profile of *TLR4* and *TLR7* in chicken erythrocytes, results revealed that their mRNA expression was significantly upregulated at 0 and 2 h, post-infection showing that severity of infection is higher at early time interval and decreased with passage of time. These results were in agreement with data reported about upregulation of *TLR7* in chicken (Yilmaz *et al*., 2005). Whereas, significant differential expression of *TLR4* helps to detect the capability of different microorganisms' growth and entry in different chicken tissues, therefore an expression of *TLR4* activates intracellular signaling via the adaptor MyD88 (O'Neill, 2006). Moreover, in the chicken spleen, the mRNA expression of *TLR4* and *TLR7* was also reported upregulated in response to bacterial infection (*Clostridium perfringen*) (Lu *et al*., 2009), and suggested that *TLR4* and *TLR7* plays an important role in innate immune response to *M. synoviae* infection. Thus upregulation indicates that chicken erythrocytes meets criteria necessary to be considered an immunological organ as has been suggested for chicken erythrocytes in general.

TLR5 is activated via bacterial flagellins and is highly

conserved in vertebrate species that play an important role in the first-line defense against bacterial pathogens and in immune homeostasis (Faber *et al*., 2018). The Appropriate function of TLR5 is, thus, essential for the timely immune response activation during many pathogenic bacterial infections (Iqbal *et al*., 2005). It was determined in current study that in response to *M. synoviae* strain infection the mRNA expression of *TLR5* was significantly upregulated at 0, 2, and 6 h while not significant at 10 h post-infection revealing that severity of infection decreased at later stage of infection. Previously it was reported that *TLR5*is highly expressed in the spleen, tonsils, lung, kidney, intestine, heart, testis, liver, and immune cells (Iqbal *et al*., 2005a; Leveque *et al*., 2003). Therefore, the upregulated expression in chicken erythrocytes may reveal that *TLR5* was involved in response to *M. synoviae* infection.

TLR15 is unique in avian species, and its exact function is currently unidentified. In the current study, we examined that *TLR15* expression was significantly upregulated in chicken erythrocytes infected with *M. synoviae* strain at 6 and 10 h post-infection, which was consistent with the previous studies results of *TLR15*expression in the chickens shown upregulation in response to *M. synoviae* induced infection at an early stage of infection (Oven *et al*., 2013). Previously, upregulated expression of *TLR15* was reported after stimulation of cells with live and heat-killed Gram-positive and Gram-negative bacteria, usually isolated from chickens, but not with equine specific pathogen *Rhodococcus equi*, therefore indicating that *TLR15* might respond specifically to avian pathogens (Nerren *et al*., 2010), furthermore, it was also reported previously that *TLR15* was highly expressed in the bursa of Fabricius and bone marrow (Higgs *et al*., 2006). Therefore it is revealed that the erythrocytes have a vital role in mediating responses to *M. synoviae* infection. Similar to the report of Ciraci *et al*. (2011), the upregulation of both *TLR15* and *MyD88* expression in the chicken erythrocytes suggested that the response of *TLR15* to *M. synoviae* may operates in a MyD88-dependent manner.

This study result also revealed the significant upregulation of *MyD88* mRNA gene expression in chicken erythrocytes in response to *M. synoviae* strain. Previously it was reported that *MyD88* gene was constitutively expressed on almost all tissues (Hardiman *et al*., 1997). Likewise, it was shown previously that in the spleen and thymus *MyD88* gene was expressed higher, which is consistent with results of mouse and humans (Hardiman *et al*., 1996, 1997). Previous studies also suggested an important role of *MyD88* gene expression in NF-κB activation *in-vitro* in the chicken innate immune response to bacterial infections (Qiu *et al*., 2008). Recently it was also stated that all TLR3 employ the MyD88 dependent pathway (O'Neill, 2006). The significant upregulation of *MyD88* suggested that this adaptor molecule plays an important role in the TLR signaling pathway and induces an innate immune response to *M. synoviae* infection.

In current study the MHC mRNA expression in chicken erythrocytes as observed was significantly increased when *M. synoviae* infected erythrocytes were compared with the uninfected control group which suggests that *MHC I* and *II* gene expression has been influenced by bacterial infections. Similar studies were reported formerly that associations of different MHC haplotypes in the chicken are responsible for the actions of vaccination and disease challenges (Briles *et al*., 1983; Bacon and Witter, 1995). Furthermore, to several microorganisms including bacterial (Joiner *et al*., 2005) and viral (Bacon *et al*., 2004; Boonyanuwat *et al*., 2006) the chicken MHC genes play an important role in disease resistance and susceptibility to bacterial infectious agents.

CONCLUSION

This *in-vitro* study provided the first evidence that *M. synoviae* interacts with chicken erythrocytes which can also constitutively express several different TLRs and other immune-related genes. The expression of such TLRs mediated pathway genes can be upregulated in response to *M. synoviae* infection in chicken erythrocytes at the early phase of infection at different time intervals. Future studies may be planned at exploring the further role of erythrocyte in TLR mediated response in chicken.

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Statement of conflict of interest

The authors declared that there is no conflict of interest regarding the publication of this article.

REFERENCES

Akira, S., Takeda, K. and Kaisho, T., 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.,* **2**: 675–680.

https://doi.org/10.1038/90609

Akira, S. and Takeda, K., 2004. Toll-like receptor signalling. *Nat. Rev. Immunol.,* **4**: 499–511. https://doi.org/10.1038/nri1391

Bacon, L.D. and Witter, R.L., 1995. Efficacy of Marek's disease vaccines in MHC heterozygous chickens: MHC congenic x inbred line F1 matings. *J. Hered.,* **86**: 269–273. https://doi.org/10.1093/oxfordjournals.jhered.a111580

Bacon, L.D., Hunter, D.B., Zhang. H.M., Brand, K. and Etches, R., 2004. Retrospective evidence that the MHC (B haplotype) of chickens influences genetic resistance to attenuated infectious bronchitis vaccine strains in chickens. *Avian Pathol*., **33**: 605–609. https://doi.org/10.1080/03079450400013147

Banchereau, J. and Steinman, R.M., 1998. Dendritic cells and the control of immunity. *Nature,* **392**: 245–252. https://doi.org/10.1038/32588

Boonyanuwat, K., Thummabutra, S., Sookmanee, N., Vatchavalkhu, V., Siripholvat, V. and Mitsuhashi, T., 2006. Influences of MHC Class II haplotypes on avian influenza traits in Thai indigenous chicken. *J. Poult. Sci.,* **43**: 120–125. https://doi.org/10.2141/jpsa.43.120

Brightbill, H.D. and Modlin, R.L., 2000. Toll-like receptors: molecular mechanisms of the mammalian immune response. *Immunology,* **101**: 1–10. https://doi.org/10.1046/j.1365-2567.2000.00093.x

Briles, W.E., Briles, R.W., Taffs. R.E. and Stone H.A., 1983. Resistance to a malignant lymphoma in chickens is mapped to subregion of major histocompatibility (B) complex. *Science,* **219**: 977–979. https://doi.org/10.1126/science.6823560

Brownlie, R. and Allan, B., 2011. Avian toll-like receptors. *J. Cell Tissue Res.,* **343**: 121–130. https://doi.org/10.1007/s00441-010-1026-0

Ciraci, C. and Lamont, S.J., 2011. Avian-specific TLRs and downstream effector responses to CpG-induction in chicken macrophages. *Dev. Comp. Immunol.*, **35**: 392-398. https://doi.org/10.1016/j.dci.2010.11.012

Dušanić, D., Berčič, R.L., Cizelj, I., Salmič, S., Narat, M. and Benčina, D., 2009. Mycoplasma synoviae invades non-phagocytic chicken cells *in vitro*. *Vet. Microbiol.,* **138**: 114–119. https://doi.org/10.1016/j.vetmic.2009.02.014

Dušanić, D., Benčina, D., Oven, I., Cizelj, M., Benčina. A. and Narat, M., 2012. *Mycoplasma synoviae* induces upregulation of apoptotic genes, secretion of nitric oxide and appearance of an apoptotic phenotype in infected chicken chondrocytes. *Vet. Res.,* **43**: 7. https://doi.org/10.1186/1297-9716-43-7

Faber, E., Tedin, K., Speide, Y., Brinkmann, M.M. and Josenhans, C., 2018. Functional expression of TLR5 of different vertebrate species and diversification in intestinal pathogen recognition. *Scient. Rep*., **8**: 11287. https://doi.org/10.1038/s41598-018-29371-0

Fitzgerald, K.A. and Kagan, J.C., 2020. Toll-like receptors and the control of immunity. *J. Cell.,* **6**: 1044–1066. https://doi.org/10.1016/j.cell.2020.02.041

Hardiman, G., Jenkins, N.A., Copeland, N.G., Gilbert, D.J., Garcia, D.K., Naylor, S.L., Kastelein, R.A. and Bazan, J.F., 1997. Genetic structure and chromosomal mapping of MyD88. *Genomics,* **45**: 332–339. https://doi.org/10.1006/geno.1997.4940

Hardiman, G., Rock, F.L., Balasubramanian, S., Kastelein, R.A. and Bazan, J.F., 1996. Molecular characterization and modular analysis of human MyD88. *Oncogene,* **13**: 2467–2475.

Hertz, C.J., Kiertscher, S.M., Godowski, P.J., Bouis, D.A., Norgard, M.V., Roth M.D. and Modlinm, R.L., 2001. Microbial lipopeptides stimulate dendritic cell maturation via Toll-like receptor 2. *J. Immunol.,* **166**: 2444–2450. https://doi.org/10.4049/jimmunol.166.4.2444

Higgs, R., Cormican, P., Cahalane, S., Allan, B., Lloyd, A.T. and Meade, K., 2006. Induction of a novel chicken Toll-like receptor following *Salmonella enterica* serovar Typhimurium infection. *Infect. Immun.,* **74**: 1692–1698. https://doi.org/10.1128/IAI.74.3.1692-1698.2006

Iqbal, M., Philbin, V.J. and Smith, A.L., 2005a. Expression patterns of chicken Toll-like receptor mRNA in tissues, immune cell subsets and cell lines. *Vet. Immunol. Immunopathol.,* **104**: 117–127. https://doi.org/10.1016/j.vetimm.2004.11.003

Iqbal, M., Philbin, V.J., Withanage, G.S., Wigley, P., Beal, R.K., Goodchild, M.J., Barrow, P., McConnel, I., Maskell, D.J., Young, J., Bumstead, N., Boyd, Y. and Smith, A.L., 2005. Identification and functional characterization of chicken toll-like receptor 5 reveals a fundamental role in the biology of infection with *Salmonella enterica* serovar Typhimurium. *Infect. Immun.,* **73**: 2344–2350. https://doi.org/10.1128/IAI.73.4.2344-2350.2005

Jahejo, A.R., Zhang, D., Niu, S., Mangi, R.A., Khan, A., Qadir, M.F., Khan, A., Chen, H.C. and Tian, W.X., 2020a. Transcriptome-based screening of intracellular pathways and angiogenesis related genes at different stages of thiram induced tibial lesions in broiler chickens. *BMC Genom.,* **21**: 1–15. https://doi.org/10.1186/s12864-020-6456-9

Jahejo, A.R., Bukhari, S.A.R., Jia, F.J., Raza, S.H.A., Shah, M.A., Rajput, N., Ahsan, A., Niu, S., Ning,

G.B., Zhang, D., Bi, Y.H., Wang, Q.H., Tian, W.X. and Han, L.X., 2020b. Integration of gene expression profile data to screen and verify immune-related genes of chicken erythrocytes involved in Marek's disease virus. *Microb. Pathog.,* **148**: 104454. https://doi.org/10.1016/j.micpath.2020.104454

Jia, F.J., Zhang, N., Li, X., Ning, G.B., Zhang, D., Li, H.Q., Ma, H.L., Hao, W.F., Gao, W.W., Zhao, Y.J., Gao, S.M., Li, G.L., Li, J.H., Yan, F., Gao, R.K. and Tian, W.X., 2018. The effection of recombinant protein GSTA3 on the transcription of erythrocytes immune related genes in Tibial dyschondroplasia broiler induced by thiram. *Acta Vet. Zootec. Sin.,* **49**: 811–817.

Joiner, K.S., Hoerr, F.J., Santen, V.E. and Ewald, S.J., 2005. The avian major histocompatibility complex influences bacterial skeletal disease in broiler breeder chickens. *Vet. Pathol.,* **42**: 275–281. https://doi.org/10.1354/vp.42-3-275

Kabanova, S., Kleinbongard, P., Volkmer, J., Andrée, B., Kelm, M. and Jax, T.W., 2009. Gene expression analysis of human red blood cells. *Int. J. med. Sci.,* **6**: 156. https://doi.org/10.7150/ijms.6.156

Karpala, A.J., Lowenthal, J.W. and Bean, A.G., 2008. Activation of the TLR3 pathway regulates IFNbeta production in chickens. *Dev. Comp. Immunol*., **32**: 435–444. https://doi.org/10.1016/j.dci.2007.08.004

Kleven, S.H., 2003. *Mycoplasma synoviae* infection. In: *Diseases of poultry*, 11 editions (eds. Y.M. Saif, H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. Mc Dougald and DE. Swayne). Iowa State University Press, Ames, pp. 756–766.

Kleven, S.H., 2008. Control of avian mycoplasma infections in commercial poultry. *Avian Dis.,* **52**: 367–374. https://doi.org/10.1637/8323-041808-Review.1

Kopp, E.B. and Medzhitov, R., 1999. The toll-receptor family and control of innate immunity. *Curr. Opin. Immunol.,* **11**: 13–18. https://doi.org/10.1016/S0952-7915(99)80003-X

Lavrič, M., Benčina, D., Kothlow, S., Kaspers, B. and Narat, M., 2007. *Mycoplasma synoviae* lipoprotein MSPB, the N-terminal part of VlhA haemagglutinin, induces secretion of nitric oxide, IL-6 and IL-1β in chicken macrophages. *Vet. Microbiol.,* **121**: 278–287. https://doi.org/10.1016/j.vetmic.2006.12.005

Lavrič, M., Maughan, M.N., Bliss, T.W., Dohms, J.E., Benčina, D., Keeler, Jr. C.L. and Narat M., 2008. Gene expression modulation in chicken macrophages exposed to *Mycoplasma synoviae* or *Escherichia coli. Vet. Microbiol.,* **126**: 111–121. https://doi.org/10.1016/j.vetmic.2007.06.011

Leveque, G., Forgetta, V., Morroll, S., Smith, A.L., Bumstead, N. and Barrow, P., 2003. Allelic variation in TLR4 is linked to susceptibility to *Salmonella enteric* serovar Typhimurium infection in chickens. *Infect. Immun.,* **71**: 1116–11124. https://doi.org/10.1128/IAI.71.3.1116-1124.2003

Lu, Y., Sarson, A.J., Gong, J., Zhou, H., Zhu, W., Kang, Z., Yu, H., Sharif, S. and Han, Y., 2009. Expression profiles of genes in toll-like receptor-mediated signaling of broilers infected with *Clostridium perfringens. Clin. Vaccine Immunol.,* **16**: 1639–1647. https://doi.org/10.1128/CVI.00254-09

Meade, K.G., Narciandi, F., Cahalane, S., Reiman, C., Allan, B. and O'Farrelly, C., 2009. Comparative in vivo infection models yield insights on early host immune response to *Campylobacter* in chickens. *Immunogenetics,* **61**: 101–110. https://doi.org/10.1007/s00251-008-0346-7

Medzhitov, R., Preston-Hurlburt, P. and Janeway, Jr. C.A., 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature,* **388**: 394–397. https://doi.org/10.1038/41131

Morera, D., Roher, N., Ribas, L., Balasch, J.C., Doñate, C., Callol, A., Boltaña, S., Roberts, S., Goetz, G. and Goetz, F.W., 2011. RNA-Seq reveals an integrated immune response in nucleated erythrocytes. *PLoS One,* **6**: e26998. https://doi.org/10.1371/journal.pone.0026998

Morera, D. and MacKenzie, S.A., 2011. Is there a direct role for erythrocytes in the immune response. *Vet. Res.*, **42**: 89. https://doi.org/10.1186/1297-9716-42-89

Much, P., Winner, F., Stipkovits, L., Rosengarten, R. and Citti C., 2002. *Mycoplasma gallisepticum*: Influence of cell invasiveness on the outcome of experimental infection in chickens. *FEMS Immunol. Med. Microbiol*., **34**: 181–186. https://doi.org/10.1111/j.1574-695X.2002.tb00622.x

Nerren, J.R., He, H., Genovese, K. and Kogut, M.H., 2010. Expression of the avian-specific toll-like receptor 15 in chicken heterophils is mediated by gram-negative and gram-positive bacteria, but not TLR agonists. *Vet. Immunol. Immunopathol.,* **136**: 151–156. https://doi.org/10.1016/j.vetimm.2010.02.017

Niu, S., Jahejo, A.R., Jia, F., Li, X., Ning, G.B., Zhang, D., Ma, H., Hao, W., Gao, W., Zhao, Y., Gao, S., Li G., Li, J., Yan, F., Gao, R., Bi, Y., Han, L., Gao, G.F. and Tian W.X., 2018. Transcripts of antibacterial peptides in chicken erythrocytes infected with Marek's disease virus. *BMC Vet. Res.,* **14**: 363. https://doi.org/10.1186/s12917-018-1678-7

Niu, S., Jahejo, A.R., Jia, F., Li, X., Ning, G.B., Zhang,

D., Ma, H., Hao, W., Gao, W., Zhao, Y., Gao, S., Li G., Li, J., Yan, F., Gao, R., Chen, H.C. and Tian, W.X., 2019. The mRNA expression of host defense peptides in chicken erythrocytes are highly related to tibial dyschondroplasia and induced by recombinant Glutathione-S-Transferase A3 protein. *Pakistan J. Zool*., **51**: 1475–1482. https://doi.org/10.17582/journal.pjz/2019.51.4.1475.1482

Obara, H. and Harasawa, R., 2010. Nitric oxide causes anoikis through attenuation of E-cadherin and activation of caspase-3 in human gastric carcinoma AZ-521 cells infected with *Mycoplasma hyorhinis*. *J. Vet. Med. Sci.,* **72**: 869–874. https://doi.org/10.1292/jvms.09-0573

O'Neill, L.A., 2006. How toll-like receptors signal: What we know and what we don't know. *Curr. Opin. Immunol.,* **18**: 3–9. https://doi.org/10.1016/j.coi.2005.11.012

Oven, I., Rus, K.R., Dušanić, D., Benčina, D., Keeler, Jr. C.L. and Narat, M., 2013. Diacylated lipopeptide from *Mycoplasma synoviae* mediates TLR15 induced innate immune responses. *Vet. Res.,* **44**: 99. https://doi.org/10.1186/1297-9716-44-99

Paolucci, S., Barjesteh, N., Wood, R.D. and Sharif, S., 2013. Chicken erythrocytes respond to toll-like receptor ligands by upregulating cytokine transcripts. *Res. Vet. Sci.,* **95**: 87–91. https://doi.org/10.1016/j.rvsc.2013.01.024

Qadir, M. F., Han X.Y., Qiao, M.L., Y. Wang, Y., Zhang, D., Bi, Y.H., Jahejo, A.R., Cheng, Q.Q. and Tian, W.X., 2020a. Expression of prostaglandins-related genes in erythrocytes of chickens infected with H9N2 subtype of avian influenza virus. *Pakistan J. Zool.,* **53**: 1417-1424. https://doi.org/10.17582/journal.pjz/20200707170711

Qadir, M. F., Han, X.Y., Qiao, M.L., Cheng, Q.Q., Mangi, R.A., Jahejo, A.R., Khan, A., Bi, Y.H. and Tian, W.X., 2020b. Profiling of Apoptosis-Related Genes in Erythrocytes of Chickens Infected with Avian Influenza Virus (H9N2 Subtype). *Pakistan J. Zool*., **2020**: 1–8. https://doi.org/10.17582/journal.pjz/20200803180858

Remer, K.A., Brcic, M. and Jungi T.W., 2003. Toll-like receptor-4 is involved in eliciting an LPS-induced oxidative burst in neutrophils. *Immunol. Lett.,* **85**: 75–80. https://doi.org/10.1016/S0165-2478(02)00210-9

Rock, F.L., Hardiman, G., Timans J.C., Kastelein R.A. and Bazan J.F., 1998. A family of human receptors structurally related to *Drosophila* Toll. *Proc. natl. Acad. Sci.,* **95**: 588–593. https://doi.org/10.1073/pnas.95.2.588

Schwarz, H., Schneider, K., Ohnemus, A., Schwarz, H., Schneider, K., Ohnemus, A., Lavric, M., Kothlow, S., Bauer, S., Kaspers, B. and Staeheil, P., 2007. Chicken toll-like receptor 3 recognizes its cognate ligand when ectopically expressed in human cells. *J. Interferon Cytokine Res*., **27**: 97–101. https://doi.org/10.1089/jir.2006.0098

Supajatura, V., Ushio, H., Nakao, A., Okumura, K., Ra, C. and Ogawa, H., 2001. Protective roles of mast cells against enterobacterial infection are mediated by Toll-like receptor 4. *J. Immunol.,* **167**: 2250–2256. https://doi.org/10.4049/jimmunol.167.4.2250

Thoma-Uszynski, S., Stenger, S., Takeuchi, O., Ochoa, M.T., Engele, M., Sieling, P.A., Barnes, P.F, Rollinghoff, M., Bolcskei, P.L., Wagner, M., Akira, S., Norgard, M.V., Belisle, J.T., Godowski, P.J., Bloom, B.R. and Modlin, R.L., 2001. Induction of direct antimicrobial activity through mammalian toll-like receptors. *Science,* **291**: 1544–1547. https://doi.org/10.1126/science.291.5508.1544

Umar, S., Munir, M.T., Ur-Rehman, Z., Subhan, S., Azam, T. and Shah, M.A.A., 2017. Mycoplasmosis in poultry: Update on diagnosis and preventive measures. *World Poult. Sci. J.,* **72**: 17–28. https://doi.org/10.1017/S0043933916000830

Vogl, G., Plaickner, A., Szathmary, S., Stipkovits, L., Rosengarten, R. and Szostak, M.P., 2008. *Mycoplasma gallisepticum* invades chicken erythrocytes during infection. *Infect. Immun.,* **76**: 71–77. https://doi.org/10.1128/IAI.00871-07

Wang, C., Niu, S., Jahejo, A., Jia, F., Li, Z., Zhang, N., Ning, G., Zhang, D., Li, H. and Ma, H., 2018. Identification of apoptosis-related genes in erythrocytes of broiler chickens and their response to thiram-induced tibial dyschondroplasia and recombinant glutathione-S-transferase A3 protein. *Res. Vet. Sci.,* **120**: 11–16. https://doi.org/10.1016/j.rvsc.2018.08.001

Winner, F., Rosengarten, R. and Citti, C., 2000. *In vitro* cell invasion of *Mycoplasma gallisepticum*. *Infect. Immun.,* **68**: 4238–4244. https://doi.org/10.1128/IAI.68.7.4238-4244.2000

Qiu, Y., Shen, Y., Li, X., Ding, C. and Ma, Z., 2008. Molecular cloning and functional characterization of a novel isoform of chicken myeloid differentiation factor 88 (MyD88). *Dev. Comp. Immunol.,* **32**: 1522–1530. https://doi.org/10.1016/j.dci.2008.05.016

Yilmaz, A., Shen, S., Adelson, D.L., Xavier, S. and Zhu, J.J., 2005. Identification and sequence analysis of chicken Toll-like receptors. *Immunogenetics,* **56**: 743–753. https://doi.org/10.1007/s00251-004-0740-8