ISSN: 2356-7767

Detection of viral interference between Low Pathogenic Avian Influenza H9N2 subtype and Very Virulent NewCastle viruses on emboryonated chicken egg and tissue culture cells

M. Said; M. Samy; A. Halim Nour; EL sayed, M. and A. Halim Mohammed

Reference Laboratory for Veterinary Quality Control on poultry production (RLQP), Animal Health Research Institute (AHRI), Agriculture Research Centre (ARC)

Received in 6/1/2020
Accepted in 5/2/2020

Abstract
Viral respiratory diseases are the most divesting agent threatening the poultry industry in Egypt. As they entered the birds body via the same route so if mixed infection occurred there may be an evidence of possible interference. In this study we evaluated the degree of interference occurs by using quantitative Real Time Polymerase Chain Reaction (qrt-PCR) in case of introducing of low pathogenic Avian Influenza (LPAI) H9N2 subtype firstly followed by virulent NewCastle disease virus (VNDV) with 24 hours interval with challenge dose 10^4 EID_{50}/ ml for each inoculated viruses till 96 hours post inoculation (HPI) and vice versa, in embryonated chicken egg (ECE) and chicken embryo fibroblast (CEF) tissue culture cells. We concluded that in case of inoculation with VNDV following LPAI-H9N2, within 24 hours, there was an increased in the amount of VNDV yield, but when inoculation with VNDV before LPAI-H9N2, with 24 hours, interference phenomena occurred indicated by the negative effect of VNDV on LPAI-H9N2 growth.

Keywords: *QRT, PCR, LPAI, VNDV, ECE, CEF*

Introduction

Avian influenza (AI) and Newcastle disease virus (NDV) are two of the most diseases threatening poultry industry. In the Middle East, current epizootic of low pathogenicity avian influenza (LPAI) of the H9N2 subtype has been aid for the occurrence of (NDV - AI) mixed infections in backyard and all types of commercial poultry farms since the year 2000 **(Davidson *et al.,* 2014).**

Even flocks acquired high levels of immunity against NDV can develop vaccinal breaks of VNDV, drop in egg production and mortality proceedings often reported in combination with H9N2 infections **(Banet-Noach *et. al.,* 2007)**, symptomatic of a role of this pathogen in the constant circulation of NDV in the region.

Although H9N2 viruses alone can lead to moderate to severe respiratory infections in chickens, replicating beyond the respiratory and digestive tracts **(Gharaibeh, 2008; Mosleh *et. al.,* 2009)**, it has been thought that H9N2 viruses can either serve as immune-suppressive agents **(Xing *et al.,* 2009)** or cause epithelial damage at the level of the respiratory tract, encourage on secondary bacterial or viral infections **(Kishida *et al.,* 2004; Haghighat - Jahromi *et. al.,* 2008).**

The examination of the interaction between NDV and AI viruses under experimental settings give valuable vision about the impact of mixed infection on virulence, infectivity, virus shedding and sero-conversion, lead to modulate surveillance and diagnostic activities in the case of co-circulation of these diseases **(Bonfante *et. al.,* 2017).**

The presented literature conducted that H9N2 mixed infection studies only refers to experimental settings in which different combinations of H9N2 and either non-lethal pathogens or immune-suppressive agents are proven (**Kishida *et. al.,* 2004; Haghighat-Jahromi *et.***

***al.,* 2008; Kwon *et. al.,* 2008; Chu *et. al.,* 2016).**

Results available so far indicate that the clinical picture of mixed infected birds is increase, partially accounting for the interference between H9N2 disease outbreaks in the field and the single infection occur in experimental studies only **(Bonfante *et. al.,* 2017).**

The role of the H9N2 AI virus as co- factor in mixed infection with VNDV requires further studies. So in this study we simulate which could actually occur in the field in vitro by supposing that Newcastle virus entered to ECE & CEF cell line first then followed by H9N2 AI virus and vice versa with interval 24 hours.

Materials and Methods

Virus strain:

Four standard titrated viruses (of $10^4 EID_{50}$ titer/ml) were obtained from the repository of the Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP), Egypt. Purified Egyptian virus **A/Chicken/ Egypt /V1527/2018 (H9N2)** Avian Influenza sub type H9N2 live virus with Gen Bank Accession No **MK212394** and /or **NDV-B7-RLQP-CH-Eg-12** as NDV Genotype 7D live virus with Gen Bank Accession No **KM288609** . Virus strains were ten-fold serially diluted to get the inoculums concentrations (10^4 EID_{50}).

Egg inoculation :

Specific pathogen free eggs (SPF) 9-11 day old embryonated chicken eggs (ECE) were used for propagation of virus isolates and pathogenicity testing. They were obtained from (Koum-Oshiem SPF, Fayoum, Egypt). ECEs were inoculated via the allantoic sac according to the **OIE guidelines, 2015**.

Tissue culture cells:

Chicken Embryo Fibroblast **(CEF)** cell cultures were prepared from 18-day-old ECE. Thus, the muscles of the embryos were collected, washed with PBS, cell were maintained in DMEM containing 10% fetal bovine serum (FBS) and antibiotic concentration according to standard procedure in 6 well micro plates. The confluent cells were washed twice with PBS and infected with 100 ul of 10^4 EID50 from each virus and incubated 1 h at 37°C with 5% CO2 and after that discard the supernatant fluid and adding 1 ml MD containing 2% serum and incubated at 37°C with 5% CO2 environment. Supernatant fluid collected at different time point 24 h, 48 h, 72 h and 96 h post infection and stored at -70°C until titration by HA test. One uninfected well in each plate was considered as negative control.

Haemagglutination assay

The haemagglutination assay was performed each 24 HPI on ECE &CEF culture cell line according to **OIE 2015** guide line.

Quantitative Real Time PCR (qRT-PCR):

Viral RNA was extracted by using QIAamp viral RNA mini kit **(Qiagen, Germany)** in accordance with manufacturer's instructions. AgPathID One-Step RT-PCR **(Thermo scientific, USA)** and specific oligonucleotide primers and taqman probes assays were used for detection and quantification of AIV-H9N2 **(Ben Shabat *et al.,* 2010).** And F gene of NDV **(Wise *et al.,* 2004).** To determine AIV-H9N2 and NDVviruses' titers, a standard curve for each virus was generated using titrated viruses in SPF-ECE. The qRT-PCR reaction volume was 25 µl containing 4.5 µl of extracted RNA, 12.5 µl 2 × One-step RT-PCR ready mix, 1 µl RT enzyme, 0.5 µl of 50 pmol of both forward and reverse primers, 0.125 µl of specific probe of 30 pmol conc. And 6 nuclease free water. The thermal profile included a reverse transcription step at 50 °C for 30 min followed denaturation step at 95 °C for 15 min. The PCR cycling was performed in 40 cycles of denaturation at 95 °C for 15 s, annealing (53 °C for NDV) and (54°C for AIV-H9) for 30 s and extension at 72 °C for 10 s. QRT-PCR was performed using Step One Plus Real-Time PCR machine **(Applied Biosystem Thermo scientific, USA)**

Results

Table (1). Results of HA activity of ECE for VNDV:

Hours post inoculation (HPI)	H9N2 Control	NDV Control	H9N2+NDV	NDV + H9N2
24	0	4	5	5
48	5	5	7	7
72	7	5	7	8
96	8	6	8	8

HA was expressed by $\log^2$

Table (2). Results of HA activity of CEF for VNDV**:**

Hours post inoculation (HPI)	H9N2 Control	NDV Control	H9N2+NDV	NDV + H9N2
24	0	3	4	4
48	4	4	6	6
72	6	6	6	7
96	7	5	7	7

HA was expressed by $\log^2$

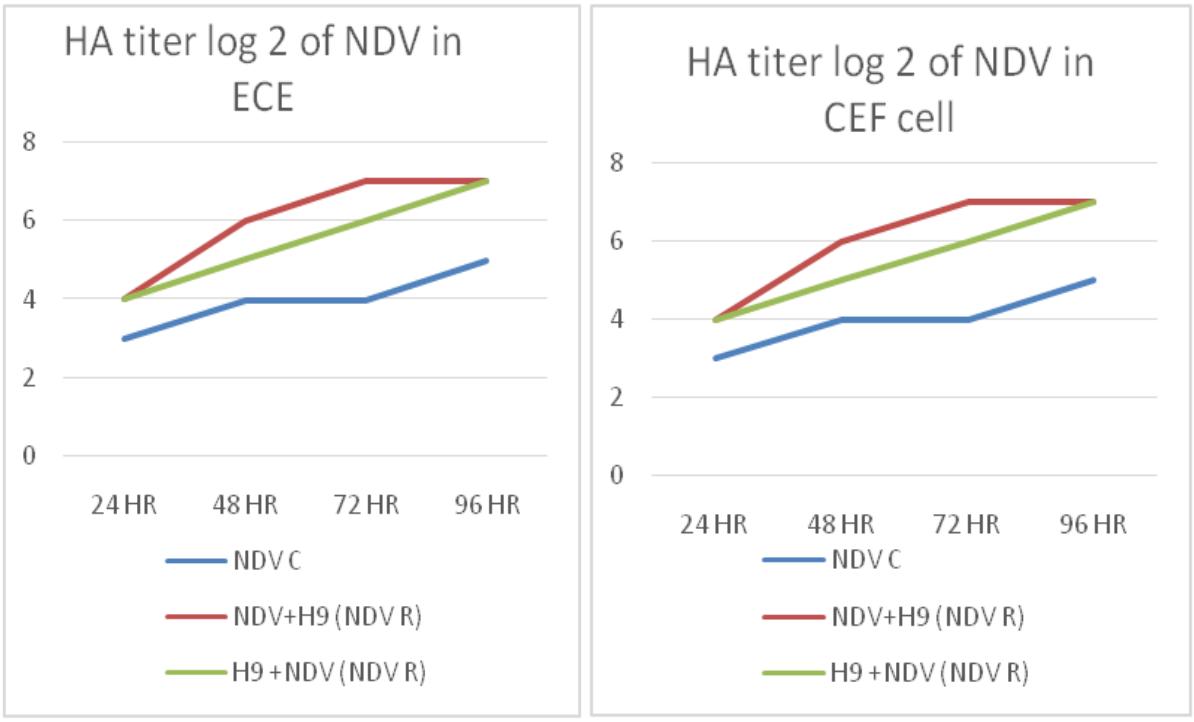

Fig. (1): Results of HA activity of NDV

The results from tables (1&2) and fig (1) showed that in case of VNDV firstly inoculated then followed by H9N2with 24h interval (VNDV +H9N2) the HA log titer was increased by 3log at 72 HPI than control while VNDV was secondary inoculated (H9N2 + NDV) the HA log titer was increased by 2log at 72 HPI.

Table (3). ECE daily deaths were recorded as following:

Hours post inoculation (HPI)	H9N2 Control	NDV Control	H9N2+ NDV	NDV + H9N2
24	0/5	0/5	1/5	1/5
48	1/5	1/5	2/5	1/5
72	2/5	2/5	1/5	2/5
96	2/5	2/5	1/5	1/5

Table (4). qRT-PCR results of embryonated chicken egg (ECE) for detection of No. of viruses copies:

Hours post inoculation (HPI)	H9N2			NDV		
	Control	1st	2nd	Control	1st	2nd
24	0	0	0	$2*10^6$	$6*10^6$	$4*10^6$
48	$8*10^6$	$1.9*10^6$	$0.9*10^6$	$2*10^6$	$9*10^6$	$6*10^6$
72	$14*10^6$	$2*10^6$	$1*10^6$	$2.4*10^6$	$10*10^6$	$10*10^6$
96	$18*10^6$	$2*10^6$	$1*10^6$	$3.8*10^6$	$12*10^6$	$12*10^6$

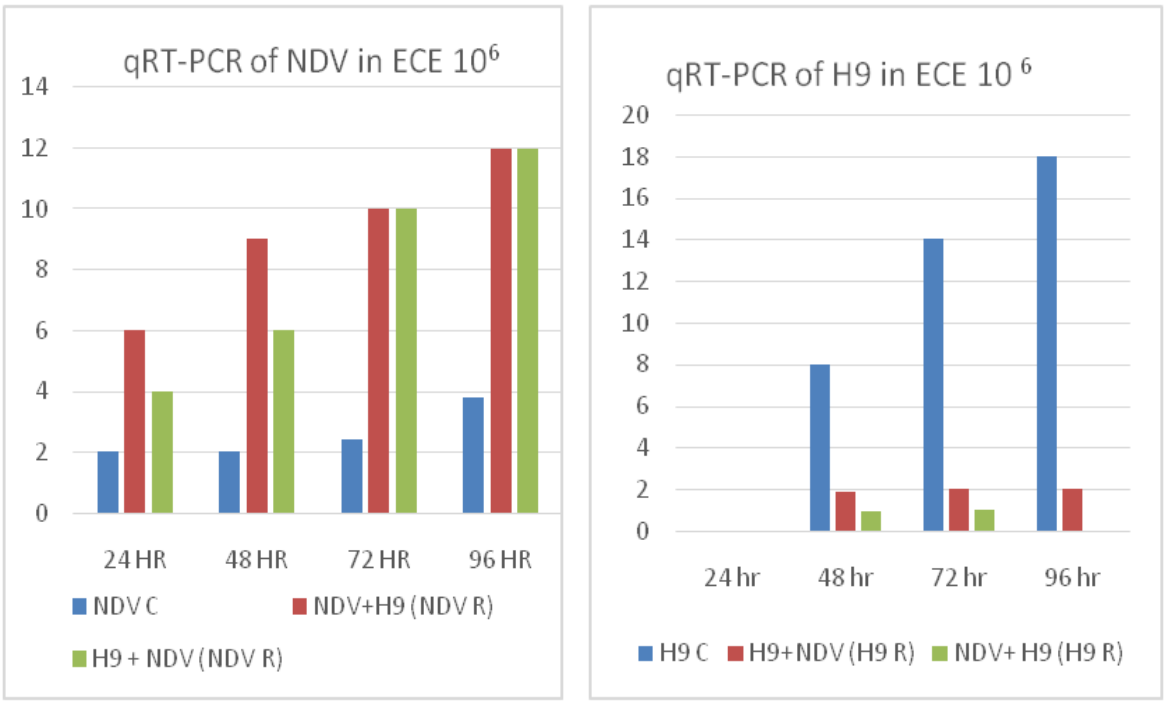

Fig. (2): qRT-PCR results of embryonated chicken egg (ECE) for detection of No. of viruses copies

Table (5). qRT-PCR results of chicken embryo fibroblast cell line (CEF) for detection of No. of viruses copies:

Hours post inoculation (HPI)	H9N2			NDV		
	Control	1st	2nd	Control	1st	2nd
24	0	0	0	$2*10^5$	$6*10^5$	$4*10^5$
48	$8*10^5$	$1.9*10^5$	$0.9*10^5$	$2*10^5$	$9*10^5$	$6*10^5$
72	$14*10^5$	$2*10^5$	$2*10^5$	$2.4*10^5$	$10*10^5$	$10*10^5$
96	$18*10^5$	$2*10^5$	$2*10^5$	$3.8*10^5$	$12*10^5$	$12*10^5$

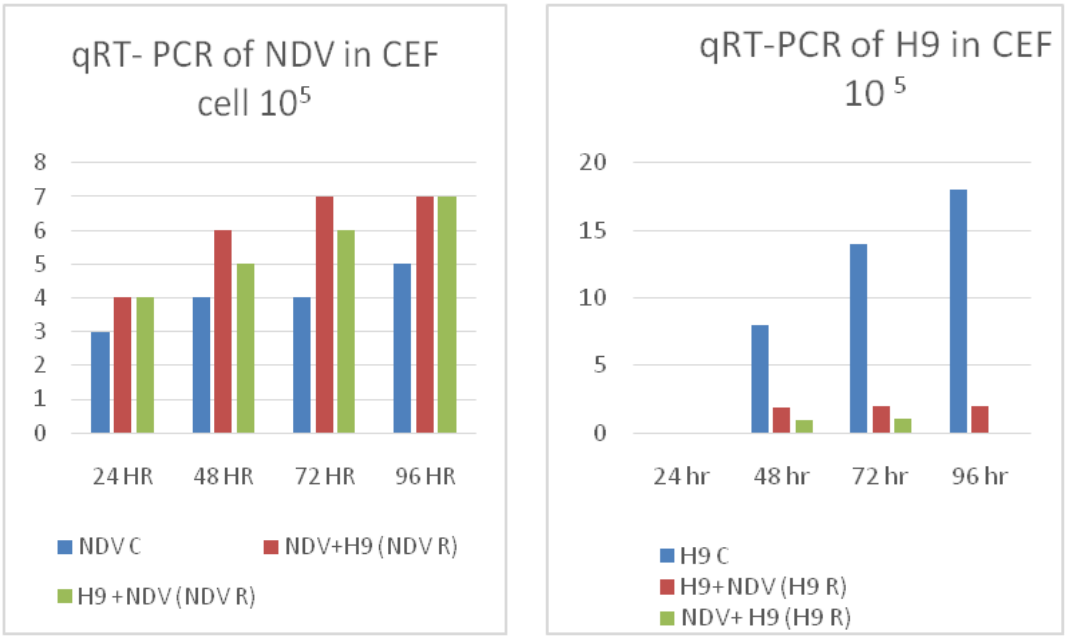

Fig. (3): qRT-PCR results of (CEF) cell line for detection of No. of viruses copies

The results from tables (4&5) and fig (2&3) showed that in case of ECE the No. of H9N2 in (H9N2 +VNDV) was decreased at 48 HPI and remain constant at the same growth level for 96 HPI while VNDV showed increasing in No. of progeny copies at 24 HPI and continued increasing till reached 3fold the control one then remain to increase by time.

In case of ECE the No. of H9N2 in (VNDV +H9N2) clarified that the growth of such virus was lesser than that yield from (H9N2 +VNDV) and behaved as the same manner till the end of the experiment at 96 HPI. But in VNDV was increased 4 fold at 48 HPI and remained increasing till the end of the experiment at 96 HPI.
Results obtained from ECE were the same which obtained from CEF cell line but decreased by one log.

Discussion

Avian influenza (AI) and Newcastle disease virus (NDV) are two of the most severe diseases of poultry. In the Middle East, current epizootic of low pathogenic avian influenza (LPAI) of the H9N2subtype has been encourage the existence of NDV-AI mixed infections in backyard and commercial farms of layers, breeders, broilers and turkeys, since the year 2000 **(Davidson *et al.*, 2014).**

Leading to slight respiratory disease syndrome and reduce egg production, which make huge economic losses and mixed infection with other pathogens **(Li *et al.*, 2003 & Nagarajan *et al.*, 2009).**

In case of co-infection occur with VNDV viral interference was done so we applied this study to detect the amount of viral interference occurred on both viruses when sequentially entered the ECE&CEF cell line with interval 24h then measure the number of viral copies yield till 96 (HPI) for both viruses.

The results of **tables (1, 2 and 3)** and **fig. (1)** stated that in case of VNDV the HA titer was increased in case of H9+NDV 2 log titer at 48 HPI while NDV+H9 showed 3 log titer at 72 HPI. When daily deaths of ECE showed that NDV+H9 inoculated die 2 out of 5 in 48HPI while NDV+H9 inoculated die 2 out of 5 but in 72HPI.

We find in both ECE&CEF cell line that the amount of viral growth in ECE is higher than CEF cell line almost one log **as shown in all results** due to there were Many factors effect on ECE and cell culture system for virus isolation such as molecular genetics properties of the virus, receptor binding properties of the host cell and some other host related factors

lead to successful and efficient propagation **(Azab *et al.*, 2017).**

In case of H9N2 + VNDV **as shown in tables (4 &5) and fig. (2&3)** the amount of H9 virus yield is decreased markedly in comparison to control but in case of NDV there was increasing in the amount of VNDV yield ranged from 2-4 log in comparison to control and these result explained by **Bonfante *et al.*, (2017)** as in vitro and in vivo studies supposed that H9N2 may act as an immune-suppressive agent, hindering vaccination responses, leading to degeneration of lymphatic organs **(Qiang & Youxiang, 2011),** causing apoptotic processes indifferent types of cells **(Xing *et al.*, 2011).** Transient dysfunction in the host's immune response may occurred led to increase the capability of NDV to replicate in host cell leading to high virus copies yield.

From the obtained results **as shown in tables (4 &5) and fig. (2&3)** appeared that in case of VNDV+H9N2 with interval 24h the H9N2 virus yield was very decreased due to VNDV interfere the growth as there was competition on the same host cell but in case of VNDV the results showed that the amount of virus copies yield was increased by approximately 2 fold than control and these finding was matched with by **Ellakany *et al*. (2018)** who concluded that co-infection of VNDV with LPAI (H9N2) can affect the viral replication dynamics and the disease caused by these viruses in chickens, but this effect will depend on the virulence of the viruses involved, the challenge titer of the viruses and the timing of the co-infections. So VNDV had the chance to replicate as it very virulent strain but H9N2 was low virulent and furthermore time of co-infection occurred play good role for VNDV growth on behave of H9N2.

Finally, we concluded that in case of challenging with VNDV following LPAI-H9N2 with 24 hours there is increasing in the amount of VNDV progeny yield, but when challenging with VNDV before LPAI-H9N2 with 24 hours interference phenomena occurred indicated by the adverse effect of VNDV onLPAI-H9N2 growth.

References

Azab, A.A.N; Shalaby, A.G. and Sliem, A. (2017). Effect of Mutations in Highly Pathogenic Avian Influenza H5N1 Viruses on the Isolation in Different Cell Culture in comparison with Newly Circulated H5N8 Viruses in Egypt. Clin Microbiol 6: 298. doi: 10. 4172/2327-5073.1000298**.**

Banet-Noach, C.; Perk, S.; Simanov, L.; Grebenyuk, N.; Rozenblut, E.; Pokamunski, S.; Pirak, M.; Tendler, Y. and Panshin, A. (2007). H9N2 influenza viruses from Israeli poultry: a five-year outbreak. Avian Diseases, 51, 290–296.

Ben Shabat, M.; Meir, R.; Haddas, R.; Lapin, E.; Shkoda, I.; Raibstein, I.; Perk, S. and Davidson, I. (2010). Development of a real-time TaqMan RT-PCR assay for the detection of H9N2 avian influenza viruses. J Virolog Methods. 2010; 168(1–2): 72–7.

Bonfante, Francesco; Giovanni, Cattoli; Sofia, Leardini; Angela, Salomoni; Eva, Mazzetto; Irit, Davidson; Ruth, Haddas and Calogero, Terregino (2017). Synergy or interferenceof a H9N2 avian influenza virus with a velogenic Newcastle disease virus in chickens is dosedependent, Avian Pathology, 46:5, 488-496, DOI: 10. 1080 /03079457.2017.1319904

Ellakany, Hany F.; Ahmed, R. Gado; Ahmed, R. Elbestawy; Hatem, S. Abd El-Hamid; Hafez, M. Hafez; Mohamed, E. Abd El-Hack; Ayman, A. Swelum; Abdullah, Al-Owaimer and Islam, M. Saadeldin (2018). Interaction between avian influenza subtype H9N2 and Newcastle disease virus vaccine strain (LaSota) in chickens BMC Veterinary Research(2018)14:358https://doi.org/10.1186/s12917-018-1689-4.

Chu, J.; Zhang, Q.; Zhang, T.; Han, E.; Zhao, P.; Khan, A.; He, C. and Wu, Y. (2016). Chlamydia pittance infection increases mortality of avian influenza virus H9N2 by suppressing host immune response. Scientific Reports, 6, 29421.

Davidson, I.; Fusaro, A.; Heidari, A.; Monne, I. and Cattoli, G. (2014). Molecular evolution of H9N2 avian influenza viruses in Israel. Virus Genes, 48, 457–463.

Gharaibeh, S. (2008). Pathogenicity of an avian influenza virus serotype H9N2 in chickens. Avian Diseases, 52, 106–110.

Haghighat-Jahromi, M.; Asasi, K.; Nili, H.; Dadras, H. and Shooshtari, A.H. (2008). Co-infection of avian influenza virus (H9N2 subtype) with infectious bronchitis live vaccine. Archives of Virology, 153, 651–655.

Kishida, N.; Sakoda, Y.; Eto, M.; Sunaga, Y. and Kida, H. (2004). Co-infection of Staphylococcus aureus or Haemophilusparagallinarum exacerbates H9N2 influenza A virus infection in chickens. Archives of Virology, 149, 2095–2104.

Kwon, J.S.; Lee, H.J.; Lee, D.H.; Lee, Y.J.; Mo, I.P.; Nahm, S.S.; Kim, M.J.; Lee, J.B.; Park, S.Y.; Choi, I.S. and Song, C.S. (2008). Immune responses and pathogenesis in immune-compromised chickens in response to infection with the H9N2 low pathogenic avian influenza virus.Virus Research, 133, 187–194.

Li, C.; Yu, K.; Tian, G.; Yu, D..; Liu, L. and Jing, B. (2003). Evolution of H9N2 influenza viruses from domestic poultry in Mainland China. Virology 2003;340:70–83.

Mosleh, N.; Dadras, H. and Mohammadi, A. (2009). Evaluation of H9N2 avian influenza virus dissemination in various organs of experimentally infected broiler chickens using RT-PCR. Iranian Journal of Veterinary Research, 10, 12–20.

Nagarajan, S.; Rajukumar, K.; Tosh, C.; Ramaswam, V.; Purohit, K. and Saxena, G. (2009). Isolation and patho-typing of H9N2 avian influenza viruses in Indian poultry. Vet Microbiol., 2009; 133: 154–63.

OIE (2015). Avian influenza, Chapter 2.3.4.

Qiang, F. and Youxiang, D. (2011). The effects of H9N2 influenza A on the immune system of broiler chickens in the Shandong Province. Trans-boundary and Emerging Diseases, 58, 145–151.

Wise, M.G.; Suarez, D.L.; Seal, B.S.; Pedersen, J.C.; Senne, D.A.; King, DJ.; Kapczynski, D.R. and Erica Spackman, E. (2004). Development of a Real-Time Reverse-Transcription PCR for Detection of Newcastle Disease Virus RNA in Clinical Samples. Journal of Clinical Microbiology, Vol. 42, No. 1, p. 329–338**.**

Xing, Z.; Cardona, C.J.; Adams, S.; Yang, Z.; Li, J.; Perez, D. and Woolcock, P.R. (2009). Differential regulation of antiviral and pro-inflammatory cytokines and suppression of Fas-mediated apoptosis by NS1 of H9N2 avian influenza virus in chicken macrophages. Journal of General Virology, 90, 1109–1118.

Xing, Z.; Harper, R.; Anunciacion, J.; Yang, Z.; Gao, W.; Qu, B.; Guan, Y. and Cardona, C.J. (2011). Host immune and apoptotic responses to avian influenza virus H9N2 in human trachea-bronchial epithelial cells. American Journal of Respiratory Cell and Molecular Biology, 44, 24–33.