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Evaluation of the Viral Interference between Lentogenic Newcastle Disease Virus (Lasota) and Avian Influenza Virus (H9N2) using Real-Time Reverse Transcription Polymerase Chain Reaction in SPF Chicken

ABSTRACT

Lentogenic Newcastle disease virus (NDV) such as Lasota strain and low pathogenicity avian influenza such as H9N2 virus are two of the most economically important viruses affecting poultry worldwide, and little attention in recent years has been paid to simultaneous infections in chickens with these two viruses for the reason that co-infection do occur but are not easily detected. In the present study, chickens were inoculated with NDV (Lasota) and LPAIV (A/chicken/Tehran/ZMT-173/99(H9N2)) simultaneously or sequentially three days apart. Oropharyngeal and cloacal swabs were collected from chickens from 1 to 14 days after inoculation. RRT-PCR for AIV and NDV detection was performed. The rate of viral shedding was measured within 14 days. No clinical symptoms were observed during the experiment however the pattern of virus shed was different with co-infection, thus comparing the results obtained from viral shedding showed that AIV is a much stronger agent than NDV in the occurrence of viral interference. This is due to the fact that in simultaneous inoculation, AIV replication delayed and reduced NDV replication, while replication of Lasota in simultaneous or pre-inoculated inoculation could not significantly disrupt H9N2 virus replication. These findings indicate that the infection with one virus can interfere with the replication of another, modifying the pathogenesis of the viruses. So, infection of the host with both viral agents simultaneously causes higher shedding of LPAIV than NDV in OP and CL areas. In conclusion, co-infection with LPAIV in chickens did not impact clinical signs but affected the replication dynamics of these viruses.

INTRODUCTION

Avian Influenza (AI) and Newcastle Disease (ND) are two dangerous diseases and the biggest threat to poultry and other avian species recognized worldwide, Ge *et al.*, 2012, Costa-Hurtado *et al.*, 2014. Viruses of AI and ND cause heavy economic losses to the poultry industry, Monne *et al.*, 2008; Pantin-Jackwood *et al.*, 2015. The genome of both avian influenza virus (AIV) and Newcastle disease virus (NDV) is of single-stranded RNA with a negative antisense, Alexander & Senen., 2008; Costa-Hurtado *et al.*, 2014. Low pathogenic avian influenza virus (LPAIV) and lentogenic Newcastle disease virus (NDV) are transmitted from wild birds to domestic birds, and both cause a range of conditions from subclinical infections to diseases with high clinical signs, Wise *et al.*, 2004; Jinnan *et al.*, 2012; Pantin-Jackwood *et al.*, 2015.

AIV belongs to type A of *Orthomyxoviridae* family. Based on their differences in surface glycoproteins of hemagglutinin (HA) and neuraminidase (NA) in the cleavage site, these viruses are divided into two groups of low pathogenic (LP) and high pathogenic (HP), Suarez *et al.*, 2007; Alexander & Senen, 2008; Capua & Alexander., 2009; Ben



Shabat *et al.*, 2010; Ge *et al.*, 2012; Pantin-Jackwood *et al.*, 2015. So far, 17 subtypes of HA (H1-H17) and 9 subtypes of NA (N1-N9) have been identified, Sorrell & Perez, 2007; Alexander, 2007; Lee & Saif, 2009; Xishan *et al.*, 2012.

Birds are usually infected with both low pathogenicity AI (LPAIV) and high pathogenicity AI (HPAI); these groups have the potential to cause disease in susceptible birds, De Jong & Hien, 2006; Peiris *et al.*, 2007; Monne *et al.*, 2008; EL Bayoumi *et al.*, 2013. Viruses belonging to H9 subtypes are of LPAIV.

Infection with LPAIV has been a major threat to commercial poultry, especially in the Middle East, since 1998, and caused many cases of outbreaks in Iran, Pakistan, Saudi Arabia, and the United Arab Emirates, Naeem *et al.*, 1999; Nilli & Asasi, 2002; Alexander, 2003; Bano *et al.*, 2003; Capua & Alexander, 2004; Alexander, 2007; Monne *et al.*, 2008; Halvorson, 2008; Pazani *et al.*, 2008. It is noteworthy that the H9N2 strain of LPAIV is circulating in Iran; it caused a severe epidemic among birds in Tehran province in 1998 and imposed heavy economic losses to the poultry industry, Pazani *et al.*, 2008.

NDVs, known as Paramyxovirus 1 (AMPV-1), belongs to the genus *Avulavirus* of the *Paramyxoviridae* family, Wise *et al.*, 2004; Jang *et al.*, 2011; Ebrahimi *et al.*, 2012. Based on their ability to increase the severity of the disease, NDV strains are divided into three groups of lentogenic (low virulence), mesogenic (moderate), and velogenic (high virulence), Beard & Hanson, 1984; Pham *et al.*, 2005; De leeuw *et al.*, 2005; Jang *et al.*, 2011; Maclachlan & Dubovi, 2011; Ebrahimi *et al.*, 2012; Pantin-Jackwood *et al.*, 2015. Lasota and Hitchner B1 are lentogenic strains of NDV that are jointly used as /NDV live vaccines against ND outbreaks in the developed and developing countries, Alexander & Senen, 2008; Ebrahimi *et al.*, 2012; Pantin-Jackwood *et al.*, 2015. Both influenza and Newcastle are considered one of the major problems of wild birds in many countries, as they are listed in Class A of poultry diseases by the Office International des Epizootie (OIE), Wise *et al.*, 2004; Perk *et al.*, 2006; Ge *et al.*, 2012; OIE, 2012; Pantin-Jackwood *et al.*, 2015.

Co-infection of birds with both /NDV and LPAIV has been reported in domestic and waterfowl birds, Halvorson, 2008; Jahangir *et al.*, 2009; Molia *et al.*, 2011; Couacy-Hymann *et al.*, 2012; França *et al.*, 2014; Pantin-Jackwood *et al.*, 2015. Hence, the infection of a host with a pathogen, including viruses or bacteria, and subsequent exposure to another pathogen lead to a co-infection. In relation to viruses, this phenomenon

is called viral interference, Dianzani, 1975; Dapalma *et al.*, 2010; Costa-Hurtado *et al.*, 2014; Pantin-Jackwood *et al.*, 2015. From 1949 till now, only six studies have been done on the interference between AIV and NDV. These studies were done by Bang F.B. 1949, Carr J.H. 1960, Florman AL 1984, Kennedy F 1983, Wenbo Liu 2003 and Ge 2012. [Ge *et al.*, 2012]. The results of these studies indicated that LPAIV subtype caused severe interference in the replication of NDV, Ge *et al.*, 2012.

Therefore, co-infection of birds with LPAIV and /NDV can create a complex picture of clinical signs which makes it more difficult and time-consuming to detect and identify both of these viruses, El Zowalaty *et al.*, 2011; Pan *et al.*, 2012; Stipkovits *et al.*, 2012. Measurable differences may include changes in tissue permissiveness or tropism, viral replication, patterns of virus progeny production and release, latency, pathology including immunological responses and immunopathology, Dapalma *et al.*, 2010; Costa-Hurtado *et al.*, 2014. Nevertheless, there is little information on the interaction of these two viruses when they simultaneously infect chicken species, Ge *et al.*, 2012; Pantin-Jackwood *et al.*, 2015. However, the aim of the present study was to obtain information on the effects of viral interference between Lasota (/NDV) strain and LPAIV (A/chicken/Tehran/ZMT-173/99 (H9N2)) on specific pathogen free (SPF) chickens.

MATERIALS AND METHODS

Virus strains

In the present study, A/chicken/Tehran/ZMT-173/99 (H9N2), which is an isolate obtained from infected birds in Iran, provided by Razi Vaccine and Serum Research Institute- Southern Iran Branch (Shiraz), and Lasota (avirulent NDV strains of chicken) were used. These viruses were propagated in SPF embryonated chicken eggs (ECE) by inoculating 100µl of each virus. According to the predesigned pattern of the experiment, these viruses were inoculated individually or in combination on specified days (Table 1). SPF eggs were purchased from SPF Chicken Centre of Venky, India.

EID₅₀

Before each experiment, 50% egg infectious dose (EID₅₀) of each virus stock was determined. To calculate the mean embryo infectious dose (EID₅₀), AIV and ND viruses were titrated by preparing 10-fold dilutions over the range 10⁻¹ to 10⁻¹⁰ in PBS and inoculating each



of five 9-day-old embryonated SPF chickens eggs with 100µl dilution each and incubated at a temperature of 37°C with humidity of 60-65%. All ECE after 5 days were tested for the presence of haemagglutinin activity. Titres of virus in 0.1 ml inoculated material, expressed as median egg infectious doses (EID₅₀), were calculated by Reed and Munch method.

Birds

Although the Maternal Derived Antibodies (MDA) are known as passive immunity in young chickens and primary barriers of antigen-specific protection against pathogens, chickens may still be at risk of infection. According to previous studies, 28-day-old chickens which have the lowest MDA levels are stated to be at higher risk of viral infection, Hamal *et al.*, 2006; Sasipreeyajan *et al.*, 2012. Ninety 28-day-old birds SPF white leghorn chickens were used in this study. They were assigned to 6 groups (A to F) of 15, and each group was separately kept in isolator under standard conditions. Feed and water were provided *ad libitum*.

Table1 – Experimental design

Group	Age (days)	Number of birds	Viral Strains		weigh	Days of sampling	
			Day0	Day3		OP and CI swaps	serology
A	28	15	Negative Control		0,3,6	1,6,14	14
B	28	15	INDV	-	0,3	1,2,3,6,8,10	14
C	28	15	LPAIV	-	0,3	1,2,3,6,8,10	14
D	28	15	INDV+LPAIV	-	0,3	1,2,3,4,5,6,8,10	14
E	28	15	LPAIV	INDV	0,3,6	1,2,3,4,5,6,8,10	14
F	28	15	INDV	LPAIV	0,3,6	1,2,3,4,5,6,8,10	14

RNA extraction

OP and CL swabs were collected from all chickens from the first to the tenth day after inoculation for the study of viral shedding. These samples were kept in the liquid medium of PBS-BSA containing antibiotics of different concentrations, such as Penicillin G (2000 units/ml) and Gentamicin (200 µg/ml), in a freezer at -70°C until the end of the experiment. For extraction of virus RNA from collected samples, High Pure Viral RNA Kit (Roche Applied Science, Mannheim, Germany) was used according to the manufacturer's recommended protocol. This stage of the experiment was conducted in the Institute Zooprofilattico Sperimentale delle Venezie (IZSVE), Italy, which is a reference laboratory for influenza and Newcastle.

Probes and primers design

Nucleotide sequences of Matrix (M) gene for influenza and L gene for Newcastle virus were considered for the identifica-

Experimental design

Chickens were assigned to 6 groups including a control (group A) and 5 groups (B to F) for virus inoculation. Each group consisted of 15 SPF chickens with an age of 4 weeks. Chickens in the control group received 50 µl of normal saline and those in the other groups were inoculated with 50 µl with 10⁶EID₅₀/100µl (single bird inoculation dose) of viruses by the intraocular and intranasal choral cleft routes. Viruses were inoculated separately, simultaneously or alternately (secondary inoculation 3 days after the initial inoculation). Chickens of all groups were daily examined for the observation and control of disease symptoms. In addition, their weight was measured on the first day and third day after the inoculation (dpi). On the fourteenth day, blood sampling was done for serological tests. Swabs of oropharyngeal (OP) and cloacal (CL) were collected from all chickens from the first to the tenth day after inoculation for the study of viral shedding (Table 1).

tion of H9N2 and Lasota during a molecular process. Thus, specific primers and probes were developed for the detection of Gene M of H9N2 and Gene L of Lasota. Oligonucleotide sequences of specific primers and probes have been shown below. Primers and probes for the detection of NDV were developed using the European reference center/AHVCA (WEIBRIAGE UK), and those for the identification of AIV were extracted from studies of Spackman (2002) and Monne (2008) (Table 2).

All probes were labelled at the 5' end with the 6-carboxyfluorescein (FAM) reporter dye and at the 3'

Table 2 – PCR primers and hydrolysis probes sequences

Target	Primer/probe	Sequence
Influenza A virus	M +25	5'- AGA TGA GTC TTC TAA CCG AGG TCG -3'
	M -124	5'- TGC AAA AAC ATC TTC AAG TCT CTG - 3'
	M +64	[FAM] 5'- TCA GGC CCC CTC AAA GCC GA - 3'[TAMRA]
Newcastle virus	NDF	5' - GAG CTA ATG AAC ATT CTT TC - 3'
	NDR	5' - AAT AGG CGG ACC ACA TCT G - 3'
	L pro MGB	[6 FAM] 5' - CCA ATC AAC TTC CC - 3' [MGBNFQ]
	L pro MGB2	[VIC] 5'- AAT AGT GTA TGA CAA CAC - 3' [MGBNFQ]



end with the 6-carboxytetramethylrhodamine (TAMRA) quencher dye, Spackman *et al.*, 2002.

Real-Time RT-PCR

The reagents contained in a QuantiTect multiplex RT-PCR kit (Qiagen, Hilden, Germany) were used for Real-Time RT-PCRs (RRT-PCR). The primers targeting the M gene of AIV were applied to the PCR at the optimized concentration of 300nM each. The exceptions were the L gene of NDV primers, which were used at a concentration of 500 nM. Specific fluorescently labeled probes were used at a final concentration of 100 nM for AIV and 200 nM for NDV. The RRT-PCR took place in a final volume of 25µl using a Rotor Gene 6000 apparatus. Each PCR tube contained a single primer/probe set for AIV and NDV. The identical thermal profile was adapted in order to detect both the distinct viruses within the same run. The following protocols were used for M gene primers/probes set: 20 min at 50°C and 15 min at 95°C, followed by 40 cycles at 94°C for 45 sec and 60°C for 45 sec. Also protocol were used for L gene primers/probes set: 20 min at 50°C and 15 min at 95°C, followed by 40 cycles at 94°C for 45 sec and 50°C for 45 sec.

Statistical analyses

Data were analyzed using IBM Statistical Package for the Social Sciences (SPSS) v.21 software. One-way ANOVA was used to analyse HI titres and body weights. Two-way ANOVA with Bonferroni multiple comparison analysis was used to evaluate virus titres in CL and OP swabs. Statistical significance was set at $p < 0.05$ unless otherwise stated.

RESULTS

Clinical signs

During the experiment, no clinical sign was observed in the groups under study (groups which were treated with Newcastle or influenza viruses individually or in combination). The results of weighting the chickens showed that there was no significant difference between the groups and all chickens grew naturally (Table 3).

Table 3 – Body weight results show that all chickens grew naturally

Groups	Body weight		Percent growth
	Day 1	Day 3	
B (NDV)	236.46	259.09	9.57
C (LPAIV)	262.06	277.26	5.80
D (NDV+LPAIV)	298.33	323.80	8.53
E (LPAIV 3days before /NDV)	319.53	394.73	13.53
F (NDV 3 days before LPAIV)	346.33	353.46	2.05
Total	292.54	321.86	11.02

Serology

Blood samples taken from all groups were examined in terms of antibody titres against AI and ND viruses. Hemagglutination inhibition (HI) assay was used to quantify antibody against NDV and AIV viruses. The results indicated that the antibody titre was negative in the control group, while it was positive in all groups to which the secondary virus was administered simultaneously or with an interval of 3 days. The results also demonstrated that in the group that the influenza virus was inoculated individually, the antibody titre of influenza and Newcastle was 4 and 5 Log₂ after 14 days, respectively (Figure 1).

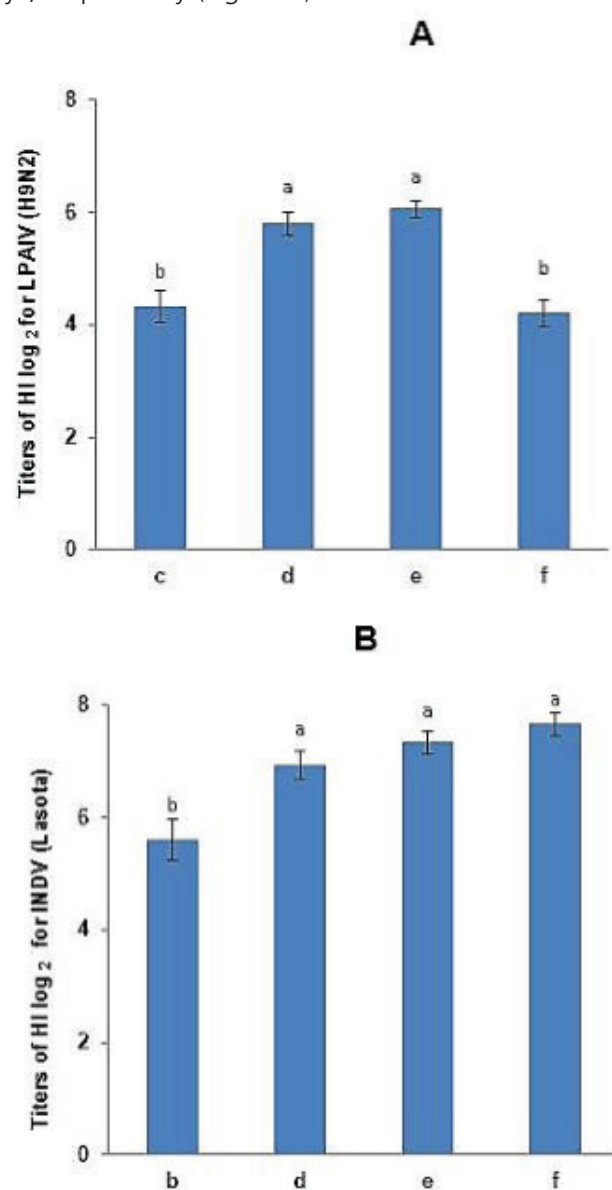


Figure 1 – Mean HI titers (log₂) in SPF chickens. Serum samples were taken at 14 days after inoculation with LPAIV and /NDV. Horizontal axes are the names of groups in both charts as (b: /NDV, c: LPAIV, d: LPAIV+/NDV, e: LPAIV 3 days before /NDV, f: /NDV 3 days before LPAIV). A: this chart shows the titers of HI associated with LPAIV and B: shows the titers of HI associated with /NDV. The highest rate was reported when both viruses were received commonly.



Infection and viral shedding

OP and CL viral shedding were measured using RRT-PCR technique. In all samples of OP and CL swabs, the gene M of matrix area and the gene L were examined for the detection of H9N2 and Lasota viruses, respectively. The results obtained from this technique showed that there was a difference between the groups in terms of viral shedding, while the main route of shedding for LPAIV was detected in both OP and CL areas ($p < 0.05$). In addition, the main route of shedding

for /NDV was mostly observed in OP area ($p < 0.05$). In Group F, in which chickens were infected with /NDV and then LPAIV, the number of positive samples in OP and CL areas was higher for LPAIV. This suggested that the shedding rate of influenza in OP and CL areas will be more when the inoculation of NDV precedes the inoculation of LPAIV, compared to the case that influenza virus is inoculated alone. It is noteworthy that the main route of shedding was detected for both /NDV and LPAIV (Figure 2).

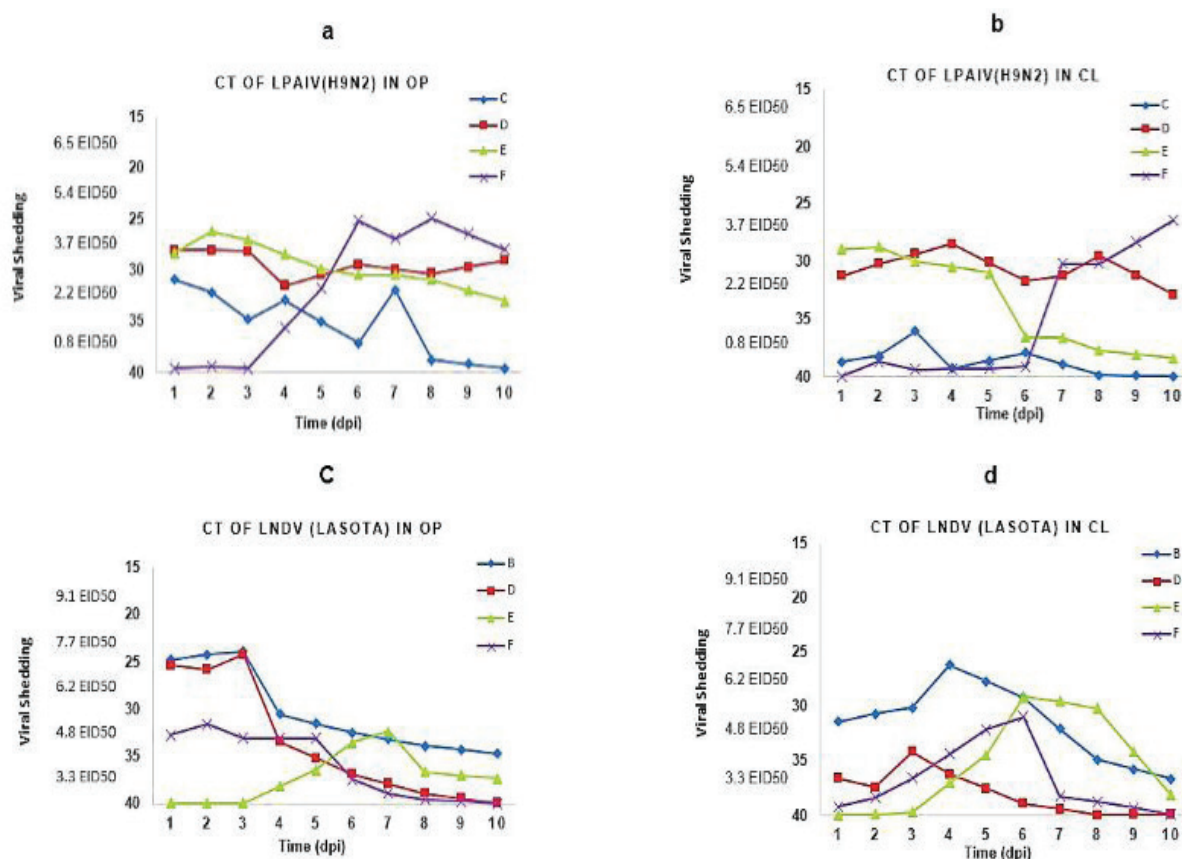


Figure 2 – LPAIV and /NDV shedding in SPF chickens. Mean Ct value of real time RT-PCR and equivalent EID50/mL of AIV and NDV are detected in OP and CL swabs at different time points after inoculation. Detection of LPAIV in OP (a) and CL (b) swabs also detect /NDV in OP(c) and CL (d). Different time points show the viral shedding so that the blue square is related to single infected birds (groups : B and C), the red square is related to co-infected birds with both viruses (group D), the green triangle is related to sequential infected birds (group E) and the purple multiplication is related to group F.

Influenza viral shedding

By comparing the number of viral shedding particles using RRT-PCR technique at different times after the inoculation, the shedding pattern in OP area showed that the type of infection and exposure of the virus to the host tissue play a major role in virus replication. Based on the results obtained from OP area, the same rate of viral shedding was observed on the first and second day after the inoculation in chickens of groups C (LPAIV alone), D (/NDV and LPAIV simultaneously), and E (LPAIV three days before /NDV) (Figure 2a). However, viral shedding was reported to be distinctly

low in the early days in chickens of group F (LPAIV three days after /NDV) and the highest rate of AIV shedding was observed on the fifth day after the secondary inoculation, i.e. on the eighth day after the inoculation (Figure 2a). In group C, where H9N2 was inoculated individually, viral shedding showed a reduction in the fourth to sixth days after the inoculation while such a reduction was not observed in other groups which had been treated with both viruses simultaneously (Figure 2a). The influenza virus shedding in the cloacal area was studied in all groups and the results indicated that the highest shedding rate was related to groups C, D,



and E in the second to fourth days after the inoculation, while this peak in group F was found on the tenth day after the inoculation, i.e. 7 days after the secondary inoculation (Figure 2b).

Newcastle viral shedding

According to the results of this study, there was a difference between the groups regarding the shedding rate of /NDV (Figure 2c). Shedding of /NDV was alternatively observed in OP area. As the results indicated, shedding was observed on the second and third day after the inoculation in the group in which /NDV was administered alone (group B) and in the groups where the virus was inoculated simultaneously or with an interval of 3 days (groups D and F). The highest rate of /NDV shedding in OP area was found in groups B and D on the third day and in group F on the second day after the inoculation. On the other hand, the lowest shedding rate was observed in group E where /NDV was injected three days after the administration of LPAIV, as the highest virus titre was measured on the fourth day after the secondary inoculation (the seventh day) (Figure 2c). The /NDV shedding in CL area was recorded from group B on the fourth to the sixth day (three days after the secondary inoculation) while the highest shedding rate in groups E and F was observed only on the sixth day and it had a downward trend gradually until the tenth day in group D (Figure 2d). All control groups were reported to be negative in terms of the presence of the virus in the swab sample of OP and CL.

DISCUSSION

The main objective of the present research was to study the effects of viral interference between /NDV and LPAIV on SPF chickens. These viruses are circulating in poultry in many parts of the world, especially in Iran, and chickens are susceptible tanks of them. Co-infection with NDV and AIV has already been reported *in vitro* using chicken embryo and the viral interference between /NDV and LPAIV has been demonstrated to prevent the growth of the other virus, Roussan *et al.*, 2008; Pawar *et al.*, 2012; Costa-Hurdato *et al.*, 2014.

It was mentioned that in co-infection with these viruses, one virus prevents the growth of the other, Ge *et al.*, 2012; Pantin-Jackwood *et al.*, 2015. Previous studies have shown that chickens inoculated with both /NDV NDV and LPAIV cause co-infection in other chickens, Ge *et al.*, 2012; Costa-Hurdato *et al.*, 2014; França *et al.*, 2014; Pantin-Jackwood *et al.*, 2015. Co-infection and viral interference depend on factors such

as bird species, type of virus, timing inoculation, and virus inoculation type, also tissue tropism, latency, dose, virulence and biological properties of pathogens and altered immune response are important, Pantin-Jackwood *et al.*, 2015; Zarkov, 2017.

Despite the severity, natural co-infections of INDV and AIV are anticipated to occur in poultry, and the effects of such co-infections on several host responses such as viral shedding dynamic, seroconversion and clinical signs are not fully known in chickens, Costa-Hurdato *et al.*, 2014. Even with the differences in the type of virus replication, co-infection of chickens with /NDV and LPAIV has not had significant effects on the increase or decrease of clinical symptoms, Pantin-Jackwood *et al.*, 2015. The clinical signs in birds depend on the species, age and virus type, para OIE, 2015. The main clinical signs are nasal discharge, cough, nervous signs, diarrhea, inappetance in laying birds, para OIE, 2015. Therefore, the host species is a factor that can influence the severity of clinical signs and amount of virus replication in such virus co-infections, Costa-Hurtado *et al.*, 2014. Costa-Hurtado *et al* infected chickens and turkeys with /NDV vaccine strain (Lasota) and H7N2 LPAIV simultaneously or sequentially, they reported that none of the chickens infected with LPAIV and /NDV showed clinical signs, while all turkeys exposed with both viruses presented mild clinical symptoms, Costa-Hurtado *et al.*, 2014. According to the previous studies, no effect on clinical signs of co-infection with the two viruses was found in the present study. For the first time in Iran, a report of viral interference between /NDV and LPAIV in SPF chickens using RRT-PCR technique is provided in the present study.

All chickens were infected with /NDV (Lasota) and LPAIV (A/chicken/Tehran/ZMT-173/99 (H9N2)) simultaneously or sequentially. The results clearly showed that viral titre on the OP route was distinctly more than viral titre on the CL route, these results are consistent with a previous study by Costa-Hurtado, 2014 that stated that LPAIV viral shedding is mainly in the OP route, Costa-Hurtado *et al.*, 2014. In addition, shedding was lower in groups which were treated with viruses individually. Similarly to the previous studies, the findings of the present study prove that LPAIV is more pathogenic for chickens, as the presence of AIV in the host could delay or prohibit the replication of NDV.

Therefore, it is recommended that the severity and degree of interference depend on the amount and pathogenicity of the virus strain, Spackman *et al.*, 2002. The present study also showed that /NDV cannot



suppress the shedding of LPAIV. Hence, infection with NDV could not always prevent subsequent infections by AIV even if Lasota and H9N2 are simultaneously injected. This suggests that Lasota, if inoculated individually or simultaneously, cannot interfere with the replication and shedding of H9N2 but can only slow down the process of viral replication.

In this study, it was observed that the shedding pattern of both H9N2 and Lasota was strongly influenced by each other, as the viral shedding of LPAIV in OP and CL areas was distinctly delayed in chickens that were infected with NDV and successively exposed to LPAIV. In other words, due to the inoculation of Lasota before H9N2, the replication of the influenza virus had a slower process and the viral shedding was lower. These values were evaluated in the sixth to the tenth day after the inoculation (Figure 2a, b). By comparing the results of this study with qualitative achievements of previous studies, it can be stated that AIV virus is a more powerful agent than NDV in the interference between these two viruses.

Viral interference is a very important phenomenon in which one cell is infected with a virus, as the virus can prevent the replication of secondary viruses. In other words, it can suppress the shedding of a virus of the homologous or heterologous type which enters the cell, Dianzani, 1975.

Several important mechanisms have been proposed to explain the viral interference: Competing by attachment interference. In this mechanism, receptors for the superinfecting virus are reduced or blocked, competing intracellular for replication of the host machinery, virus-induced interferon interference, Kimura *et al.*, 1976.

NDV and LPAIV are replicated in the upper area of the respiratory system and epithelial cells of the intestinal tract, where there are trypsin-like enzymes. NDV binds through the HN glycoprotein to sialic acid receptors on the cell surface, the same as HA glycoprotein does for LPAIV. Therefore, superficial cell receptors for AIV are glycoconjugates containing sialic acid, whereas these receptors for NDV are recommended to be ganglioside and N-glycoprotein, Rott, 1979; Ferreira *et al.*, 2004; Ge *et al.*, 2012; Swayne *et al.*, 2013. These findings suggest that both viruses may compete for the same target cell as Lasota can connect to the HN glycoprotein, LPAIV can connect to HA glycoprotein compounds because both of these compounds contain sialic acid, Costa-Hurtado *et al.*, 2014.

Previous studies have explained that an inactivated particle of influenza can interfere with the replication

of a live virus that later enters the cell. Through the interferon system, these results led to the discovery of interference phenomenon, Ziegler & Horsfall, 1944; Henle, 1950. Other studies have also stated that the production of interferon can prevent or suppress the replication of many viruses, particularly AIV and NDV, Issacs & Lindenmann, 1987. Replication of a virus following the activation of anti-viral immune responses, including immunomodulators or the use of immune cells, can influence the replication of other viruses in a similar area, Ge *et al.*, 2012. Stimulation of local interferon generation by NDV may interfere with the replication of LPAIV because Lasota is known as a poor inducer of interferon, Costa-Hurtado *et al.*, 2014.

As mentioned in the previous studies, Ge *et al.* (2012) found that if the host is infected with NDV prior to, H9N2 replication can be suppressed. However, in another study, Costa (2014) stated that high replication of LPAIV in turkeys could prevent the replication of NDV, Costa-Hurtado *et al.*, 2014. França (2014) reported that co-infection of poultry and wild birds with NDV and AIV *in vivo* showed that differences in the pattern of viral shedding depend on the time of co-infection, and that the delay in the peak of viral shedding of LPAIV occurs when NDV is already inoculated [França *et al.*, 2014]. According to França's study, although co-infection of chickens with both viruses was observed, no reduction was observed in humoral immune response. This is consistent with the findings of Gelb (2007) in relation to the co-infection of broilers with IBV and NDV [Gelb *et al.*, 2007; França *et al.*, 2014].

In the present study, the reduction in antibody titre against LPAIV, compared with NDV, was partially observed (Figure 1). Therefore, in confirmation of the previous studies, it is recommended that humoral response against co-infection may not be much effective in antibody titre courses. In summary, SPF chickens received 50 µl viruses with 10^6 EID₅₀/ml from Lasota and H9N2 for the study of co-infection and interference between LPAIV and NDV. The data showed that infection of the host with both viral agents simultaneously caused higher shedding of LPAIV than NDV in OP and CL areas.

In general, effects of viral interference depend on the adaptation of viruses to a host species, pathogenicity of viruses, time of co-infection, and environmental factors. Assessment and identification of the factors affecting the viral interference or understanding the factors that cause delay in the replication and infection of viruses will help us to better find the path



of pathogenicity and transmission of these viruses in chickens. That understanding could also provide new plans of virus control programs, including new tools for identification and more improved protocols of vaccination.

CONCLUSION

Birds are often infected with more than one infectious agents. The co-infection of birds with LPAIV and /NDV is due to the fact that they compete for the same receptors in susceptible cells. AIV have a negative impact on NDV growth if they are inoculated simultaneously or sequentially. According to the results obtained from this study, co-infection of LPAIV and /NDV had no effect on the severity of clinical signs.

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REFERENCES

- Alexander DJ, Senen DA. Newcastle disease. Newcastle disease, other avian paramyxoviruses and pneumovirus infections. In: Saif YM, Fadly AM, Glisson JR, McDougald LR, Nolan LK, Swayne DE, editors. Diseases of poultry. 12th ed. Ames: Blackwell Publishing; 2008. p.75–100.
- Alexander DJ. An overview of the epidemiology of avian influenza. *Vaccine* 2007;25(30):5637–5644.
- Alexander DJ. Report on avian influenza in the eastern hemisphere during 1997–2002. *Avian Disease* 2003;47(s3):792–797.
- Bano S, Naeem K, Malik SA. Evaluation of pathogenic potential of avian influenza virus serotype H9N2 in chickens. *Avian Disease* 2003;47(s3):817–822.
- Beard CW, Hanson RP. Newcastle disease. In: Hofstad MS, Barnes HJ, Calnek BW, Reid WM, Yoder HW, editors. Diseases of poultry. 8th ed. Ames: Iowa State University; 1984. p.452–470.
- Ben Shabat M, Meir R, Haddas R, Lapin E, Shkoda I, Raibstein I, et al. Development of a real-time TaqMan RT-PCR assay for the detection of H9N2 avian influenza viruses. *Journal of Virological Methods* 2010;168(1-2):72–77.
- Capua I, Alexander DJ. Avian influenza and Newcastle disease: a field and laboratory manual. Rome: Springer Verlag; 2009.
- Capua I, Alexander DJ. Avian influenza: recent developments. *Avian Pathology* 2004;33(4):393–404.
- Costa-Hurtado M, Afonso CL, Miller PJ, Spackman E, Kapczynski DR, Swayne DE, et al. Virus interference between H7N2 low pathogenic avian influenza virus and lentogenic Newcastle disease virus in experimental co-infections in chickens and turkeys. *Veterinary Research* 2014;45:1.
- Couacy-Hymann E, Kouakou V A., Aplogan GL., Awoume F, Kouakou CK, Kakpo L, et al. Surveillance for influenza viruses in poultry and Swine, West Africa, 2006–2008. *Emerging Infectious Diseases* 2012;18(9):1446–1452.
- Dapalma T1, Doonan BP, Trager NM, Kasman LM. A systematic approach to virus-virus interactions. *Virus Research* 2010;149(1):1–9.
- De Jong MD, Hien TT. Avian influenza A (H5N1). *Journal of Clinical Virology* 2006;35:2–13.
- De Leeuw OS, Koch G, Hartog L, Ravenshorst N, Peeters BPH. Virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the haemagglutinin-neuraminidase protein. *Journal of General Virology* 2005;86(Pt 6):1759–1769.
- Dianzani F. Viral interference and interferon. *La Ricerca in Clinica e in Laboratorio* 1975;5(3):196–213.
- Ebrahimi MM1, Shahsavandi S, Moazenijula G, Shamsara M. Phylogeny and evolution of Newcastle disease virus genotypes isolated in Asia during 2008–2011. *Virus Genes* 2012;45(1):63–68.
- ELbayoumi KM, Mahgoub K, Mekky HM, Kutkat MA. Molecular detection of H5N1, H9N2 and Newcastle disease viruses isolated from chicken in mixed infection in Egypt. *World Applied Sciences Journal* 2013;27(1):44–50.
- Ferreira L, Villar E, Muñoz-Barroso I. Gangliosides and N-glycoproteins function as Newcastle disease virus receptors. *The International Journal of Biochemistry & Cell Biology* 2004;36(11):2344–2356.
- França M, Howerth EW, Carter D, Byas A, Poulson R, Afonso CL, et al. Co-infection of mallards with low-virulence Newcastle disease virus and low-pathogenic avian influenza virus. *Avian Pathology* 2014;43(1):96–104.
- Ge S, Zheng D, Zhao Y, Liu H, Liu W, Sun Q, et al. Evaluating viral interference between Influenza virus and Newcastle disease virus using real-time reverse transcription-polymerase chain reaction in chicken eggs. *Virology Journal* 2012;9:128.
- Gelb J Jr, Ladman BS, Licata MJ, Shapiro MH, Campion LR. Evaluating viral interference between infectious bronchitis virus and Newcastle disease virus vaccine strains using quantitative reverse transcription-polymerase chain reaction. *Avian Disease* 2007;51(4):924–934.
- Halvorson DA. Control of low pathogenicity avian influenza. In: Swayne DE, editor. Avian influenza. Ames: Blackwell Publishing; 2008. p.513–536.
- Hamal KR, Burgess SC, Pevzner IY, Erf GF. Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens. *Poultry Science* 2006;85(8):1364–1372.
- Henle W. Interference phenomena between animal viruses: a review. *Journal of Immunology* 1950;64(3):203–236.
- Isaacs A, Lindenmann J. Virus interference. I. The interferon. *Journal of Interferon Research* 1987;7(5):429–438.
- Jahangir A, Ruenphet S, Ueda S, Ueno Y, Shoham D, Shindo J, et al. Avian influenza and Newcastle disease viruses from northern pintail in Japan: isolation, characterization and inter-annual comparisons during 2006–2008. *Virus Research* 2009;143(1):44–52.



- Jang J, Hong SH, Kim IH. Validation of a real-time RT-PCR method to quantify Newcastle disease virus (NDV) titer and comparison with other quantifiable methods. *Journal of Microbiology and Biotechnology* 2011;21(1):100–108.
- Jinnan Li, Haixia H, Qingzhong Y, Diego GD, De-shan L, Patti JM. Generation and characterization of a recombinant Newcastle disease virus expressing the red fluorescent protein for use in co-infection studies. *Virology Journal* 2012;9:227.
- Kimura Y, Norrby E, Nagata I, Ito Y, Shimokata K. Homologous interference induced by a temperature-sensitive mutant derived from an H5N1 (Sendai virus) carrier culture. *Journal of General Virology* 1976;33(2):333–343.
- Lee CW, Saif YM. Avian influenza virus. *Comparative Immunology, Microbiology & Infectious Diseases* 2009;32(4):301–310.
- Maclachlan NJ, Dubovi EJ. Paramyxoviridae. In: Maclachlan NJ, Dubovi EJ, editors. *Fenner's Veterinary virology*. 4th ed. London: Academic Press; 2011. p.299–235.
- Molia S, Samaké K, Diarra A, Sidibé MS, Doumbia L, Camara S, et al. Avian influenza and Newcastle disease in three risk areas for H5N1 highly pathogenic avian influenza in Mali, 2007–2008. *Avian Disease* 2011;55(4):650–658.
- Monne I, Ormelli S, Salviato A, De Battisti C, Bettini F, Salomoni A, et al. Development and validation of a one-step real-time PCR assay for simultaneous detection of subtype H5, H7, and H9 avian influenza viruses. *Journal of Clinical Microbiology* 2008;46(5):1769–1773.
- Naeem K, Ullah A, Manvell RJ, Alexander DJ. Avian influenza A subtype H9N2 in poultry in Pakistan. *Veterinary Record* 1999;145(19):560.
- Nili H, Asasi K. Natural cases and an experimental study of H9N2 avian influenza in commercial broiler chickens of Iran. *Avian Pathology* 2002;31(3):247–252.
- OIE - World Organisation For Animal Health. Avian influenza: infection with avian influenza viruses [terrestrial manual]. Paris; 2015. chapter 2.3.4, p.1–23.
- OIE - World Organisation For Animal Health. Manual of diagnostic test and vaccines for terrestrial animals: mammals, birds and bees. Paris; 2012.
- Pan Q, Liu A, Zhang F, Ling Y, Ou C, Hou N, et al. Co-infection of broilers with *Ornithobacterium rhinotracheale* and H9N2 avian influenza virus. *BMC Veterinary Research* 2012;8:104.
- Pantin-Jackwood MJ, Costa-Hurtado M, Miller PJ, Afonso CL, Spackman E, et al. Experimental co-infections of domestic ducks with a virulent Newcastle disease virus and low or highly pathogenic avian influenza viruses. *Veterinary Microbiology* 2015;177(1–2):7–17.
- Pawar SD, Kale SD, Rawankar AS, Koratkar SS, Raut CG, Pande SA, et al. Avian influenza surveillance reveals presence of low pathogenic avian influenza viruses in poultry during 2009–2011 in the West Bengal State, India. *Virology Journal* 2012;9:151.
- Pazani J, Marandi MV, Ashrafihelan J, Marjanmehr SH, Ghods F. Pathological studies of A/Chicken/Tehran/ZMT-173/99 (H9N2) influenza virus in commercial broiler chickens of Iran. *International Journal of Poultry Science* 2008;7:502–510.
- Peiris JS, de Jong MD, Guan Y. Avian influenza virus (H5N1): a threat to human health. *Clinical Microbiology Reviews* 2007;20(2):243–267.
- Perk S, Panshin A, Shihmanter E, Gissin I, Pokamunski S, Pirak M, et al. Ecology and molecular epidemiology of H9N2 avian influenza viruses isolated in Israel during 2000–2004 epizootic. *Developmental Biology* 2006;124:201–209.
- Pham HM, Konnai S, Usui T, Chang KS, Murata S, Mase M, et al. Rapid detection and differentiation of Newcastle disease virus by real-time PCR with melting-curve analysis. *Archives of Virology* 2005;150(12):2429–2438.
- Rott, R. Molecular basis of infectivity and pathogenicity of myxovirus. *Archives of Virology* 1979;59(4):285–298.
- Roussan DA, Haddad R, Khawaldeh G. Molecular survey of avian respiratory pathogens in commercial broiler chicken flocks with respiratory diseases in Jordan. *Poultry Science* 2008;87(3):444–448.
- Sasipreeyajan J, Pohuang T, Sirikobkul N. Efficacy of different vaccination programs against Newcastle disease virus challenge in broiler chickens. *The Thai Journal of Veterinary Medicine* 2012;42:431–437.
- Sorrell EM, Perez DR. Adaptation of influenza A/Mallard/Potsdam/178-4/83 H2N2 virus in Japanese quail leads to infection and transmission in chickens. *Avian Diseases* 2007;264–268.
- Spackman E, Dennis AS, Myers TJ, Bulaga LL, Garber LP, Perdue ML, et al. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *Journal of Clinical Microbiology* 2002;40(9):3256–3260.
- Stipkovits L, Egyed L, Palfi V, Beres A, Pitlik E, Somogyi M, et al. Effect of low-pathogenicity influenza virus H3N8 infection on *Mycoplasma gallisepticum* infection of chickens. *Avian Pathology* 2012;41(1):51–57.
- Suarez DL, Das A, Ellis E. Review of rapid molecular diagnostic tools for avian influenza virus. *Avian Diseases* 2007; (1 Suppl):201–208.
- Swayne DE, Suarez DL, Sims LD. Influenza. In: Swayne DE, Glisson JR, McDougald LR, Nair V, Nolan LK, Suarez DL, editors. *Diseases of poultry*. 13th ed. Ames: Willey-Blackwell; 2013. p.181–218.
- Wise MG, Suarez DL, Seal BS, Pedersen JC, Senne DA, King DJ, et al. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. *Journal of Clinical Microbiology* 2004;42(1):329–338.
- Xishan Lu, Shi Yi, Gao F, Xiao H, Wang M, Qi J, et al. Insights into avian influenza virus pathogenicity: the hemagglutinin precursor HA0 of subtype H16 has an alpha-helix structure in its cleavage site with inefficient HA1/HA2 cleavage. *Journal of Virology* 2012;86(23):12861–12870.
- Zarkov IV. Avian influenza A virus and Newcastle disease virus mono and co-infections in birds. *Trakia Journal of Sciences* 2017;2:182–191.
- Ziegler JE, Horsfall FL. Interference between the influenza viruses, I. the effect of active virus upon the multiplication of influenza viruses in the chick embryo. *Journal of Experimental Medicine* 1944;79(4):361–377.

