P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com

Research Article

Effect of Avian Influenza (Subtype H9N2) on the Pathogenesis and Virulence of Velogenic Newcastle Disease Virus in Chicken under Experimental Co-Infection

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Article History: 19-676 Received: October 03, 2019 Revised: November 04, 2019 Accepted: November 09, 2019

ABSTRACT

In this study, the effect of coinfection of avian influenza (subtype H9N2) on the pathogenesis and virulence of vNDV was investigated. In co-infection experiment, one strain of H9N2 by titer 10^6 EID_{50} and one strain of vNDV by titer 10^3 EID_{50} were used. In experimental design, 70 specific pathogen free chicks (four weeks old) were grouped into 7 groups (G1-G7) ten birds/group. Group 1 was uninfected control group, group 2 and 3 were positive control for H9N2 and vNDV respectively, group 4 infected firstly with vNDV and after 3days it was infected by H9N2 and vice versa in group 5 while in group 6 two studied viruses were inoculated simultaneously. The birds in group 7 were infected firstly by H9N2 and after 14 days were infected with vNDV. The obtained results showed that the mortality due to vNDV infection (G 3) and in group 7 started from 6 Days post infection (DPI) and reached 100% at 9DPI, while the mortalities started at 2 DPI in G4 and at 4DPI in G5,6 also the mortalities were reached 100% at 6,7,8DPI in G4,5and 6. The vNDV shedding in control group (G3); G7; G6 and G4 started at 2DPI by titer $10^{2/}$/bird and it reached to 10^4/bird at 6DPI. While the vNDV shedding in group5 was reduced by 40% at 2DPI and at 4DPI the all tested birds were considered shedder by titer $5X10^5$/bird. This study concluded that H9N2 prior infection interfere with pathogenesis and virulence of second vNDV infection while no significant interference in post or simultaneous infection.

Key words: H9N2, Velogenic NDV, Coinfection, Pathogenesis, Virulence

INTRODUCTION

Avian influenza virus (AIV) and Newcastle disease virus (NDV) identified as the most important respiratory pathogens of chicken (Malik *et al*., 2004). Newcastle disease virus is one of Paramyxoviridae family, genus Avulavirus (Mayo, 2002). Although many vaccination programs were implemented in Middle East with live, attenuated and inactivated vaccines to control the NDV infection, Banet-Noach *et al*., 2007 was reported that in the presence of protective level of immunity in vaccinated flocks by NDV vaccines, there are some vaccinal breaks have been caused by vNDV were occurred and these clinically manifested by severe drop in egg production as well as increased mortality rates and that could be due to coinfection of investigated flocks with H9N2.

Mixed infections in poultry population with AIV and NDV were reported in Middle East since 2000's which supported by presence of H9N2 outbreaks (Davidson *et al*., 2014). Interference was reported in co-infection of embryonated eggs with AIV and NDV. The outcome of this interference was depended on virus related factors like (virus virulence and dose of virus infection) and host related factors, as well as other factors as time and alternatives of infection that resulted in different clinical and virological pictures (Ge *et al*., 2012).

It is important to evaluate the role of LPAIV in exaggerating or inhibition of vNDV infection in chickens. So, in this study, the effect of H9N2 infection on the pathogenicity and virulence of vNDV was investigated using selected Egyptian field circulating viruses of H9N2 and VNDV.

MATERIALS AND METHODS

Viruses

A low pathogenic avian influenza viruses LPAIVs H9N2 subtype A/chicken/Egypt/114940/2011 (JQ44037 3), A/chicken/Egypt/16194/2016 (MH762046), A/chicken/ Egypt/ME543V/2016 (ASM80129). Newcastle disease virus (vNDV) genotype VIId NDV-GZ-986F-2015 (KX686727) were isolated at Reference Laboratory of

Cite This Article as: Ibrahim DS, Elsanousi AA, ElDeeb AH, Arafa A and Selim AA, 2020. Effect of avian influenza (subtype H9N2) on the pathogenesis and virulence of velogenic Newcastle disease virus in chicken under experimental co-infection. Int J Vet Sci, 9(1): 136-140. www.ijvets.com

Veterinary Quality Control on Poultry Production (RLQP) Animal Health Research Institute (AHRI) Giza, EGYPT. The used viruses were prepared according to (OIE, 2012; OIE, 2015) briefly 0.2ml virus isolate was inoculated into allantoic sac of 10-day old SPF ECE obtained from kom-oushim, Fayoum SPF farm. The EID50 of harvested allantoic fluids were calculated using Reed and Muench method (Reed & Muench, 1938). The purity of the harvested viruses was tested according to WHO (WHO, 2002).

Intravenous Pathogenicity Index (IVPI) according to OIE, 2015.

The Pathogenicity of all LPAI viruses were evaluated in BSL3 lab animal facility unit at RLQP, Animal health Research institute, Giza, Egypt, according to OIE, 2015. Briefly all inoculated birds were examined daily for 10 days post infection. The intravenous pathogenicity index was calculated according to (OIE, 2015). The tested viruses were considered low pathogenic if the IVPI<1.2.

Birds

Seventy SPF chicks four weeks old were obtained from Nile SPF Kom-oushim farm, Fayoum, Egypt. Blood samples and tracheal swabs were collected from all examined birds and tested by hemagglutination inhibition (HI) and RT PCR respectively to ensure that all tested the birds were negative for NDV and AIV. The chickens were reared in poultry isolators at RLQP (Bioflex B50, Bell isolation system, England). The all birds were supplied with feed and water ad-libitum as well as continuous lighting for all experiment period.

Experimental infections

Avian influenza (H9N2) A/chicken/Egypt/ME543V/2016 (ASM80129) and Newcastle disease virus NDV-GZ-986F-2015 (KX686727) were used in coinfection trial. 70 birds of 28-day old SPF chickens were divided into 7 groups, 10 birds in each. The experimental design and different approach of each group shown in Tab1. The birds were infected via intranasal route table1. Birds were monitored daily for estimation of morbidity and mortality rates; the tracheal swabs were collected from all examined birds starting at 2nd day post challenge with interval 2 day apart till 8th day post challenge for quantification of viral shedding.

Virus detection and quantification

The vNDV shedding was estimated by real time-PCR. Briefly, viral genomic RNAs were extracted from all collected samples according to manufacturer's instructions using QIAamp Viral RNA Mini kit (Qiagen GmbH, Hilden, Germany),rRT-PCR was carried out using a commercial kit Quantitect Probe rRT-PCR kit (Qiagen, Inc., Valencia CA). Primers used for NDV F+4839 TCCGGAGGATACAAGGGTCT, F-4939 AGCTGTTGC AACCCCAAG and probe F+4894 [FAM]AAGCGTTTC TGTCTCCTTCCTCCA[TAMRA]; Metabion; Germany (Ben Shabat *et al*., 2010). The QRT-PCR reaction was performed using Stratagene 3005P MXpro Real-Time PCR System (Stratagene, USA).

Statistical analyses

Statistical analysis done using Prism v.8.0.2 software (GraphPad Software Inc., La Jolla, CA, USA).

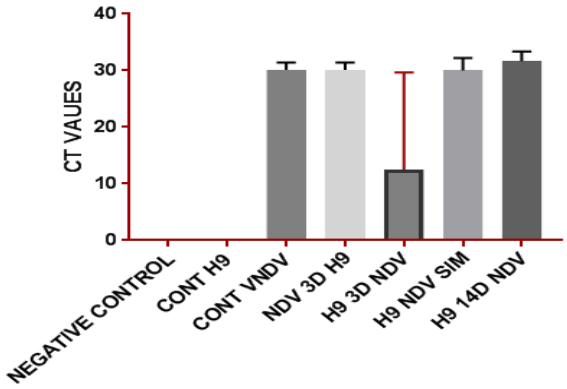

Fig. 1: Comparative shedding of VNDV 2DPI.

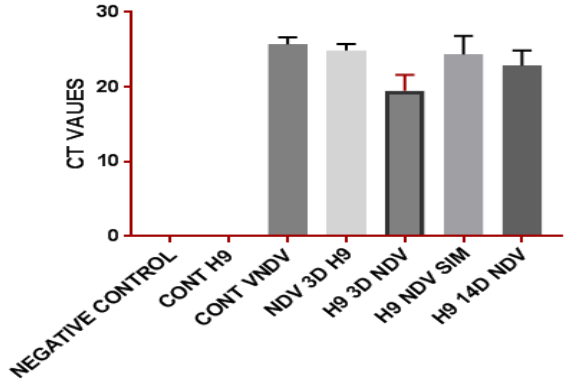

Fig. 2: Comparative shedding of VNDV 4DPI.

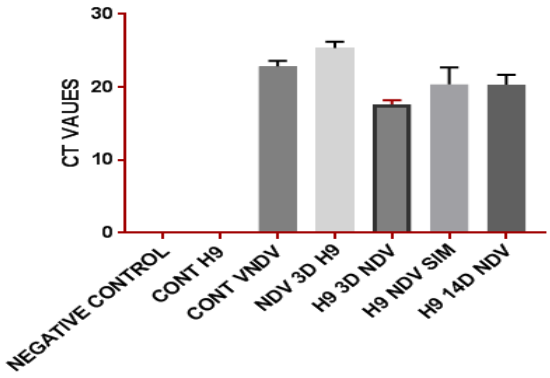

Fig. 3: Comparative shedding of VNDV 6DPI.

Viral shedding in swabs analyzed by one-way ANOVA with Bonferroni multiple comparison analysis. The negative swabs were supposed to be $10^{1.4}$ EID50/mL for NDV. Which was the lowest detectable value of virus according to used method.

RESULTS

Pathogenicity and infectivity titration

The IVPI of H9N2 viruses were <1.2 and The EID50 of VNDv was $10^{7.9}$/ml (Table 2).

Mortality rates

Mortalities as showed in (table 3) were recorded after second infection either by AI or vNDV. In group 3 the mortalities were started from 6 days post infection (DPI) and reached to 100% 9 DPI. In group 4 mortality started from

Table 1: Experimental design.

Group NO./ Name (10 birds/gp)	Days of inoculation		
	Day 0	Day 3	Day 14
Uninfected control (group 1)	----------	----------	----------
Control Pos H9N2 (group 2)	H9N2*	---------	-------
Control Pos VNDV (group 3)	VNDV**	--------	--------
VNDV 3d H9N2 (group 4)	VNDV**	H9N2*	
H9N2 3d VNDV (group 5)	H9N2*	VNDV**	--------
H9N2 VNDV SIM (group 6)	H9N2* VNDV**	-------	--------
H9N2 14d VNDV (group 7)	H9N2*	--------	VNDV**

*H9N2 dose in infection was 10^6 EID_{50}/100µl, **VNDV dose in challenge was 10^3 EID_{50}/100µl.

Table 2: Pathogenicity index and infectivity titration

	Isolate identification	Pathogenicity index	EID_{50}/ml
H9N2	A/chicken/Egypt/114940/2011	0	8.2
	A/chicken/Egypt/16194/2016	0	7.9
	A/chicken/Egypt/ME543V/2016	0	8.5

Table 3: Mortalities post second infection

Group	Days post last challenge	Number of dead birds/total birds
1	Along experimental infection	No deaths
2		
3	6	4/10
	7	3/6
	8	2/3
	9	1/1
4	2	2/10
	3	4/8
	4	1/4
	6	3/3
5	1	1/10
	4	2/9
	5	4/7
	6	2/6
	7	4/4
6	4	1/10
	6	4/9
	7	1/5
	8	4/4
7	5	3/10
	7	2/7
	8	2/5
	9	3/3

2 days post second infection (DPSI) and reached to 100% at 6 DPSI, while in group 5 mortality started at 4 DPSI and it reached to 100% at 7 DPSI. On other hand mortality rate in group 6 mortality started from 4 DPI and it continued to 100% at 8 DSPI. The mortality in group 7 was the like the mortalities in group 3 (VNDV control) as mortality began on 5 DPSI and it reached to 100% at 9 DPSI. The survival rates lack significance with P value 0.3723.

vNDV shedding

vNDV shedding in tracheal swabs was examined by qRT-PCR and as shown (Table.4), the virus detected in all birds infected as positive control for vNDV (group3) from 2DPI. Also, in birds infected with H9N2 VNDV simultaneously (group 6), birds infected with VNDV 3 days prior to H9N2 (group 4) and birds infected with H9N2 14days prior to VNDV infection (group 7). On other hand the birds infected with H9N2 3 days prior to VNDV infection (group 5), the shedding was 2/5 at 2DPI and 3DPI while all swabs were positive at 4DPI. Comparing the qRT-PCR VNDV shedding in Tracheal swabs at points of time 2DPI (Table 5, Fig. 1), 4DPI (Table 6, Fig. 2), 6DPI (Table 7, Fig. 3).

DISCUSSION

Combined respiratory viral infections of poultry have been reported with high incidence especially in Middle East. H9N2 and NDV had an important role in the recorded co infections. Many studies were performed on separate viral while co-infections with both Avian Influenza Virus (AIV) and NDV need more investigations.

In the present study, the effect of Egyptian H9N2 on the pathogenesis and virulence VNDV were evaluated. The low pathogenicity of the used avian influenza virus (subtype H9N2) was confirmed using IVPI. All tested isolates were having the same IVPI so the avian influenza isolate (A/chicken/Egypt/ME543V/2016(H9N2) (ASM80 129) was selected for experimental trial of this study for its low pathogenicity. Velogenic NDV strains are highly virulent as it was caused mortality rate up to 100% in inoculated chickens (Beard *et al*., 1981), so the vNDV virus titer 10^3 EID_{50} was used for infective trial as shown by (Davidson *et al*. ,2018) that the morbidity and mortality rates of birds were observed on 5DPI compared to 2DPI in case of 10^6 EID_{50}.

The clinical data of infected groups reveled that no deaths recorded in group infected with H9N2 separately while group infected with VNDV separately deaths started 5DPI reached 100% at 9DPI. In group infected with H9N2 3days prior to VNDV infection the deaths started from 2 DSPI and it reached to 100% 6 DSPI that indicate that infection firstly with H9N2 increased the mortalities and lowering the death time when it compared with single infection with vNDV. Meanwhile this clear difference in deaths, it lacks significance with p value 0.3723 which also seen in (Bonfante *et al*., 2018) where no statistical significance had been shown in comparison of death time of co infected group H9/NDV-10^3 compared with the other NDV groups. In their study they suggested that the H9N2 virus infection act as synergistic pathogen subsequent vNDV infection that showed in increased mortality rates of challenged birds and confirm vNDV multiplcation delaying by the previous H9N2 replication.

NDV shedding was affected by 3-day prior infection with H9N2 where it reduced to 40% shedder birds compared with other groups shedding 100% at 2DPI, so the presence of H9N2 antagonist the replication of NDV. This interference may be related to that on 1st Day of LPAI infection expression of peak level of IFNα and IFNβ mRNA (Cornelissen *et al*., 2012) and drop from 2nd day that may be related to virus replication and a mechanism of interferon inhibitor (Xing *et al*., 2009). Also, it may be related to interference phenomena (Kimura *et al*., 1976), as cell infected with one virus prevent the replication of a second super infectant virus (Dianzani, 1975).

Although the shedding reduced in prior infection with H9N2 2DPI it reached to 100% from 4th DPI and the shedding mean was 5.04x105/bird compared to 6.136x103/bird in control group which accelerate the death in this group to 6DPI compared to 9DPI in control group.

Table 4: Number of birds positive for vNDV in tracheal swabs in single and co-infected groups.

First Inoculation	Second Inoculation		Day post Inoculation [a]							
0 DAY	DAY 3	DAY 14	2	3	4	5	6	7	8	9
VND POS control			10/10[b]	NC	10/10	NC	6/6	NC	3/3	1/1
H9 POS control			0/10	NC	0/10	NC	0/10	NC	0/10	NC
VND	H9N2		5/5	5/5	5/5	3/3	D	D	D	D
H9N2	VND		2/5	2/5	5/5	NC	5/5	4/4	D	D
VND+H9N2			5/5	5/5	5/5	5/5	5/5	NC	4/4	D
H9N2		VND	5/5	NC	5/5	NC	5/5	NC	2/2	1/1

[a]days post VNDV challenge, [b]Number of positive birds/total number of birds sampled, NC no collected samples at these points of time, D: all birds were dead.

Table 5: Comparative shedding and virus titer of vNDV 2DPI.

Item	Groups						
	NEG	POS H9	Pos NDV	VNDV 3d H9	H9 3d VNDV	H9 VNDV SIM	H9 14d VNDV
Positivity[a]	0/5	0/5	5/5	5/5	2/5	5/5	5/5
CT values	ND	ND	28.79	28.79	31.44	28.74	29.67
			31.66	31.66	31.19	32.75	33.6
			28.95	28.95	ND	27.03	31.52
			30.15	30.15	ND	30.94	33.10
			31.11	31.11	ND	30.6	30.5
Conc[b]	ND	ND	$6.821X10^2$	$6.821X10^2$	1.200×10^2	7.049×10^2	3.830×10^2
			$1.039X10^2$	$1.039X10^2$	1.414×10^2	5.081×10^1	2.910×10^1
			$6.142X10^2$	$6.142X10^2$	ND	2.163×10^3	1.138×10^2
			$2.749X10^2$	$2.749X10^2$	ND	1.665×10^2	$2.954x10^1$
			$1.490X10^2$	$1.490X10^2$	ND	2.081×10^2	2.222×10^2

[a] Number of positive birds/total number of birds sampled, [b] concentration of the virus EID_{50}/ml, ND; nondetectable level of virus.

Table 6: Comparative shedding and virus titer of vNDV 4DPI.

Item	Groups						
	NEG	POS H9	Pos NDV	VNDV 3d H9	H9 3d VNDV	H9 VNDV SIM	H9 14d VNDV
Positivity[a]	0/5	0/5	5/5	5/5	5/5	5/5	5/5
CT values	Nd	Nd	24.28	25.89	17.87	22.74	26.38
			25.63	24.84	19.2	25.59	21.77
			26.44	23.76	19.71	20.89	21.63
			26.7	24.47	17.7	25.85	21.63
			25.41	25.51	22.96	26.78	22.76
Conc [b]	Nd	Nd	$1.313X10^4$	$4.569X10^3$	8.792×10^5	3.606×10^4	$2.857x10^3$
			$5.419X10^3$	$9.097X10^4$	3.675×10^5	5.563×10^3	6.812×10^4
			$3.186X10^3$	$1.847X10^4$	2.630×10^5	1.213×10^5	7.467×10^4
			$2.686X10^3$	$1.160X10^3$	9.829×10^5	4.691×10^3	7.467×10^4
			$6.260X10^3$	$4.637X10^3$	3.121×10^4	2.549×10^3	$3.149x\ 10^4$

[a] Number of positive birds/total number of birds sampled, [b] concentration of virus EID_{50}/ml, Nd no detectable level of virus.

Table 7: Cogfrr5rmparative shedding and virus titer of vNDV 6 DPI.

Item	Group						
	NEG	POS H9	Pos NDV	VNDV 3d H9	H9 3d VNDV	H9 VNDV SIM	H9 14d VNDV
Positivity[a]	0/5	0/5	5/5	5/5	5/5	5/5	5/5
CT values	Nd	Nd	22.51	25.01	16.73	17.86	19.63
			23.85	24.73	17.98	21.17	21.73
			23.18	26.68	17.54	17.99	19.76
			22.87	24.56	17.96	21.87	21.78
			21.72	25.83	17.99	22.96	18.76
Conc [b]	Nd	Nd	$4.193X10^4$	$4.725X10^3$	1.857×10^6	8.850×10^5	2.772×10^5
			$1.741X10^4$	$4.714X10^3$	8.180×10^5	1.010×10^5	6.993×10^4
			$2.702X10^4$	$2.722X10^3$	$1.092X10^6$	8.127×10^5	2.546×10^5
			$3.311X10^4$	$4.804X10^3$	8.288×10^5	6.380×10^4	$6.809x10^4$
			$7.039X10^4$	$4.580X10^3$	$8.127X10^5$	$3.121x10^4$	4.905×10^5

[a] Number of positive birds/total number of birds sampled, [b] concentration of virus EID_{50}/ml, Nd no detectable level of virus.

That's mean prior infection with H9N2 is temporarily antagonist and delay the replication of vNDV (for 2 days) then it exaggerates the replication and the load of shedding virus is higher than vNDV separate infection.

In previous study it had shown that the mixed infection of chickens with LPAIV were significantly affected lentogenic NDV virus shedding and it showed the same pattern of the present study with velogenic NDV at the early time of shedding, co-infected birds presented lower titer of viral shedding than birds infected with lento genic NDV only. At 6 dpi significantly higher amounts of virus shed was seen in co-infected chickens, and also at 8 dpi for sequentially infected birds, compared with birds only infected with NDV (Costa-Hurtado *et al*., 2014). In simultaneous coinfection and post infection with H9N2, the H9N2 had no significant effect on VNDV pathogenesis or

virulence, Although, competition at chicken respiratory tract receptors as both viruses bind to sialic acid receptors (Bonfante *et al*., 2018; Davidson *et al*., 2018) that indicate that the simultaneous infection of chicken with H9N2 and VNDV did not affect the opportunities of VNDV replication. Meanwhile it was showed that AIVs have replicative privilege over NDVs in SPF egg chicken embryos and differs according the strain of NDV used (Ge *et al*., 2012).

Conclusions

Mixed infection of chicken with velogenic Newcastle disease virus and H9N2 interfere with the replication of VNDV in prior infection with H9N2 while simultaneous infection or post infection with H9N2 had no significant effect on the pathogenesis and virulence of VNDV.

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