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Trial for Chemical Attenuation of Chinese VIId Newcastle Disease Virus Egyptian Isolate Using Nitrous Acid

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Abstract: In the present study, chemical attenuation of local NDV genotype VIId designated as NDV-F278-RLQP-CH-EG circulating in Egypt was carried out. The virus was propagated in 9-11 day specific pathogen free (S.P.F) eggs via allantoic cavity and was serially passaged for 35 passages. Then, nitrous acid was used for attenuation and the treated virus was inoculated into S.P.F eggs. After incubation at 26°C for 4-6 days, the harvested allantoic fluid revealed negative hemagglutination even after five passages of such harvest. Assaying of the allantoic fluid harvested from fifth passage by real time RT-PCR revealed positive result indicating that nitrous acid affects the HA protein of the virus. Groups of S.P.F chickens (ten each) were used in inoculation of treated, non treated original viruses and other group non inoculated as negative control. After ten days of observation period, mortalities began on the chemically treated group virus and HA property of the virus was recovered again. The virus was successfully isolated in S.P.F eggs indicating failure of attenuation. The study reports that one cycle of chemical attenuation using nitrous acid was not enough to complete attenuating of NDV genotype VIId. Further study using other method of attenuation of NDV will be addressed.

Key words: NDV genotype VIId · Chemical attenuation and chicken experiment

INTRODUCTION

Newcastle disease (ND) is one of the most devastating diseases of poultry with global distribution. It is caused by Newcastle disease virus (NDV), also known as avian paramyxovirus serotype 1 (APMV-1) and is classified as List A notifiable disease by the World Animal Health Organization (Office International des Epizooties, OIE) because it is highly contagious and responsible for severe disease and high mortality in susceptible birds [1]. NDV is a member of the genus *Avulavirus* within the *Paramyxoviridae* family [2]. It is a negative sense, single stranded, non segmented, enveloped RNA virus [3]. The genome is composed of six genes and encodes six structural proteins; nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN) and the RNA polymerase (L) [4].

Although NDV has only one serotype but strains cause wide variation of disease in chickens. Based on severity of disease, all NDV isolates are divided into five pathotypes in chickens which are viscerotropic velogenic (highly pathogenic), neurotropic velogenic, mesogenic (moderately pathogenic), lentogenic (lowly pathogenic) or respiratory and asymptomatic enteric type [5].

Based on the analysis of the nucleotide sequence of the F gene NDV is classified into two major classes, Class I viruses: These viruses are of single genotype and mainly comprised of viruses that have been isolated from waterfowl, shorebirds and occasionally from live bird markets and captured wild birds. Class II viruses: These viruses comprise 19 genotypes (I-XIX) which were involved in five main global streams of infection in the history of ND panzootics in chickens and other bird species [6, 7].

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In Egypt, the first record of isolation and molecular characterization of NDV genotype VII was in year 2011 and the phylogenetic analysis revealed that it is velogenic isolate clustered within genotype VII sub genotype d [8].Co-infection between NDV genotype VIId and H5N1 was reported in year 2011 in both vaccinated and unvaccinated poultry farms in Egypt [9].

Chemical attenuation depends on induction of virus mutants which have less virulence than wild type strain. Chemical attenuation using nitrous acid was studied by [10] and succeeded to induce plaque type mutant's. Also [11] succeeded to isolate mutants of the virulent NDV Essex 70 strain to be used as vaccine using nitrous acid.

In the present study, trial for attenuation of NDV genotype VIId was carried out using one cycle of chemical attenuation by nitrous acid. HA and pathogenecity of treated virus was studied.

MATERIALS AND METHODS

Virus Propagation: The well characterized NDV genotype VIId designated as NDV-F278-RLQP-CH-EG was diluted 1:1 in phosphate buffer saline (P.B.S) then propagated by inoculation of 0.2 ml in 9-11 day S.P.F eggs via allantoic cavity. The eggs were then incubated at 37°C with 40-60% humidity for 3-5 days according to Allan [12].

The harvested allantoic fluid was tested for HA and HI tests [13].

Attenuation of the Propagated Virus by Passage in S.P.F Eggs: The attenuation of the propagated virus was carried out by repeated passages (serial passage) for 35 times.

Titration of NDV Infectivity in Embryonated Chicken Eggs: The infectivity of NDV in the harvested allantoic fluids was titrated in 9-11 day S.P.F eggs [14] and the end point EID50 was estimated according to Reed and Muench [15].

Chemical Attenuation of the Propagated Virus (35th Passage): The harvested allantoic fluid from the 35th passage was subjected to chemical treatment using nitrous as described by Granof [10] where in the reaction tube contained two parts of virus, one part of 4 molar sodium nitrite and one part of Molar acetate buffer.

The reaction was carried out at room temperature (25°C) for five minutes at pH 4.2 and then diluted 1:100 in PBS to stop the reaction. Trial for isolation of virus mutant's survivors (attenuated) was carried out Gits and Zygraich [16]. Briefly, the treated suspension was inoculated into ten 9-11 day old SPF eggs and incubated at 26°C for 4-6 days. The harvested undiluted allantoic fluid was submitted first to three further passages in the same conditions and thereafter to three other passages at 26°C using allantoic fluid diluted from 10^{-3} to 10^{-6} (each dilution was inoculated into five eggs). The virus harvested from the seventh passage is incubated at 26° C by diluting the allantoic fluid to such degree that one egg out of ten gives a positive response. The controls were consisted of 2ml virus and 1ml acetate buffer in which was inoculated into five 9-11days S.P.F eggs and incubated at 26°C for 4-6 days. This protocol was applied till fifth passage (fifth dilution).

Characterization of the Attenuated Virus Post Chemical Treatment: The harvested allantoic fluid from the first passage till the fifth passage (fifth dilution) after chemical treatment was tested by both rapid slide and plate HA test as mentioned by OIE [13].

Real Time RT-PCR and Conventional RT-PCR: According to the method described by Wise [17] primers, probes utilized in real–time RT-PCR and primers [18] utilized for amplification of 300 bp of NDV F- gene were as follow:

Primer/probe	Genomic target	Sequence 5'-3'
NDV-F	F+4839	TCCGGAGGATACAAGGGTCT
NDV-R	F-4939	AGCTGTTGCAACCCCAAG
Probe (VFP-1)	F+4894	(FAM) AAGCGTTTCTGTCTCCTTCCTCCA (TAMRA)
F1 sense	F gene	TACACCTCATCC-CAGACAGG
F2 antisense	F gene	AGTCGGAGGATGTTGGCAGC

Sequencing of PCR Product: The PCR products were purified with Qiagen PCR purification kit and used as template for sequencing on genetic analyzer ABI PRISM 310 automated DNA sequencer in Korea by MACROGEN Company.

The purified PCR products were sequencing from forward direction using forward primer (F1).

Inoculation of Chemically Treated Virus in SPF Chickens: The treated virus was inoculated in 10 SPF three weeks old chickens with a dose of 0.5 ml/s.c and/or 100 µL oculonasal /bird [11], non treated virus (35th passage) containing 10^{7} EID50 / ml received the same dose and ten non inoculated negative control group [19]. Each group was kept in a separate isolator and chickens were observed for 10 days to detect any symptoms which may arise.

Organs from dead chickens were collected from both groups for trial of virus isolation [20] with confirmation by RT-PCR and sequencing on treated group and non treated group as mentioned above.

RESULTS

Results of Virus Propagation: By candling of eggs the second day post inoculation, all embryos of S.P.F eggs died. The allantoic fluid collected from S.P.F propagated virus revealing positive agglutination by rapid slide HA test.

Results of Virus Attenuation by Serial Passage in S.P.F Eggs: The harvested allantoic fluid from 35th passages were found to cause deaths of embryos of inoculated eggs in the second day of inoculation.

Results of Chemical Attenuation of the NDV Harvested from 35th Passage: The harvested allantoic fluid from controls (non treated virus) caused embryos deaths by the end of incubation period with positive agglutination by rapid slide HA test. The isolation of mutant virus was not succeeded till the fifth dilution of fifth passage after chemical treatment in which the eggs survive with no HA activity.

Results of Real Time RT-PCR: The harvested allantoic fluid from the fifth dilution of the fifth passage obtained from chemically treated virus revealed positive amplification curve by real time PCR as shown in Figure (1).

Result of RT-PCR: The harvested allantoic fluid from the fifth dilution of the fifth passage obtained from chemically treated virus revealed positive band at expected site (300 bp) by RT- PCR as shown in Figure (2).

Result of Sequencing: BLAST analysis of the obtained sequence of the isolated virus from allantoic fluid which had no HA activity revealed that the virus is closely related to original virus (Accession No.KM28862).

Results of Inoculation of Chemically Treated Virus in SPF Chickens: The third day post inoculation in chickens, mortalities were started for both treated group and non treated positive control group with appearance of NDV clinical signs such as depression, nervous manifestation, ruffled feather, sternal recumbancy and by the end of observation period all chickens from both treated and non treated group died.

Table 1: Summarize the obtained results of the virus titration after serial passages in eggs

Passage	Deaths	HA	HI	Titration
0	All eggs (five eggs)	7 $\log_2$	12 $\log_2$	10^9 EID50/ml
35	All eggs (five eggs)	9 $\log_2$	12 $\log_2$	10^7 EID50/ml

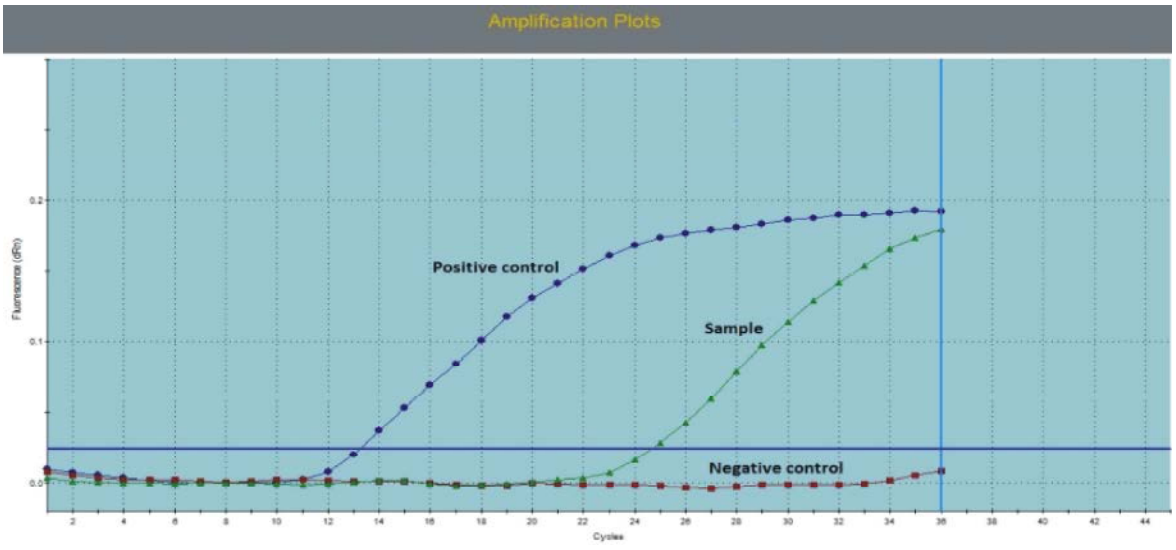

Fig. 1: qRT-PCR curve showing detection of NDV RNA from allantoic fluid of fifth passage (Fifth dilution) (sample) with cycle threshold (c.t) 26 and positive control (infected allantoic fluid) with c.t 14 while negative control (non infected allantoic fluid) shows no c.t

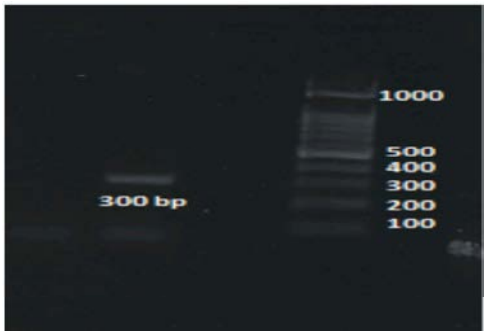

Fig. 2: PCR product with 300 bp molecular size of sample (fifth passage – fifth dilution) along with the 1000 bp molecular weight marker, gives band at 300bp

Virus was successfully isolated from both treated and non treated group after inoculation of tissue organs homogenate (trachea, spleen, brain, kidney and cecal tonsils) in 9-11 day S.P.F eggs via allantoic cavity and incubation period 3-5 days. All isolated viruses showed HA titer of 8 $\log_2$ and HI titer of 11 $\log_2$ for non treated group while in treated group, HA titer was 7 $\log_2$ and HI titer of 11 $\log_2$. Also, RT-PCR and sequencing confirmed the isolation of NDV genotype VII from treated and non treated chicken groups.

DISCUSSION

In the present study, we utilized chemical attenuation using nitrous acid for induction of NDV mutants of NDV genotype VII circulating in Egypt. Previously, Granof [10] studied the mutagenic effect of nitrous acid on several NDV strains and succeeded to induce plaque type mutants which escape from the inactivation process. For example, the frequency of small plaque type mutants in stocks of NDV RO+ strain was between 3 and 7%. Also [11] succeeded to isolate mutants of the Essex 70 Strain of NDV to be used as vaccine using nitrous acid. The approximate percentage of infectious virus particles that survived after exposure to nitrous acid for 5, 6, 7, 8, 9 and 10 minutes was 0.1, 0.03, 0.01, 0.001, 0.0001 and 0.00001% respectively when the reaction was allowed to proceed at room temperature. Similarly, Thiry [21] stated that in vitro treatment of wild type NDV with nitrous acid produced three distinct mutations: the red plaque, the small plaque and the minute plaque. The mechanism of action of nitrous acid in chemical attenuation is the deamination of cytosine, guanine and adenine, into uracil, xanthine and hypoxanthine; respectively. Cytosine is the only base which is transformed by nitrous acid into another normal base and it has therefore been considered as the best candidate for being the site of a non lethal point mutation. Nitrous acid failed to induce back mutations toward the wild type [21].

However, this study didn't support the chemical attenuation using one cycle of treatment with nitrous acid for NDV genotype VII because the virus mutants didn't obtain. During the chemical attenuation, nitrous acid broke HN protein of NDV genotype VIId from the first passage in 9-11 day S.P.F eggs till reaching the fifth passage as illustrated in Table (2) with no HA survival although egg embryos remain alive for 4 days at 26°C with suitable humidity. These observations dissimilar to those of Gits and Zygraich [16] who found that these mutants had a titer of 10^2 EID50/ml after the end of chemical treatment. When the allantoic fluid (chemically treated) harvested from fifth passage (fifth dilution) tested for real time RT-PCR and/or conventional RT-PCR (F-gene) and sequencing confirmed the presence of NDV genotype VII. When this treated virus with no HA activity inoculated in S.P.F three weeks old chickens and kept in a separate isolator for 10 days to detect any symptoms which may arise, the mutants attached to R.B.Cs sialic acid receptors, began to increase in population causing viremia and signs of NDV started to appear indicating that these mutants are of mixed populations. In addition, mortalities began in the 3rd day post inoculation and by the end of observation period all chickens died. Virus was successfully isolated from inoculated chickens and demonstrates HA property. In deed, Chemical attenuation by nitrous acid was not enough for complete attenuation of such virus and further methods need to be addressed. In conclusion the study reports the failure of one cycle nitrous acid treatment of genotype VIId NDV to attenuate the virus.

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