Biological and Molecular Characterization of Newcastle Disease Virus Isolated from Iran

M. Kianizadeh*[1], A. Ideris[1], M.S. Shahrabadi[2], R. Kargar[3], S.A. Pourbakhsh[3], A.R. Omar[1] and K. Yusoff[1]

1.Faculty of Veterinary Medicine, Putra University, 43400 UPM Serdang, Selangor, Malaysia

2.Department of Virology, Iran Medical Science University, Tehran, Iran

3. Razi Vaccine & Serum Research Institute, P.O.Box 11365-1558,Tehran, Iran

Summary

A total of twelve Newcastle disease virus isolates recovered from different outbreaks in Iran were analyzed for *in vivo* pathogenicity, biological property and amplification of F cleavage site gene. Intracerebral pathogenicity index varied from 1.7 to 1.96 while the intravenous pathogenicity index was more than 2.0. The mean death time value for all the isolates ranged from 39.6 to 68.8 hours. All the isolates except 2, hemagglutinated bovine erythrocytes whereas only one of the isolates agglutinated the equine erythrocytes. Polymerase chain reaction was used to amplify the F cleavage site gene of all isolates. Based on mean death time the Newcastle disease virus isolates could be placed in mesogenic or velogenic group.

Key words**:** F protein, cleavage site, PCR, Newcastle disease, pathogenicity index

Archive of SID

Introduction

Newcastle disease (ND) is still considered as an economically important disease which is highly contagious infection for many avian species. The Newcastle disease virus (NDV) is a member of the Paramyxoviridae family, genus paramyxovirus. It may be a member of Rubula genus or a new genus of the Paramyxovirinae subfamily (Alexander *et al* 1997, Deleeuw *et al* 1999, Lomniczi *et al* 1998).

NDV causes a disease that varies in clinical severity and transmissibility depending on the pathotype involved. NDV can be divided into 3 groups based on their virulence such as lentogenic, mesogenic and velogenic. Lentogenic strains, especially in adult chickens may cause minimal or no clinical sign. However, the disease produced by mesogenic strains may cause mortality that can reach 25% and those by in velogenic strains maybe reach up to100% (Claudia Marin *et al* 1996).

www.SID.ir

The virulence of NDV strains may be assessed by the conventional methods including mean death time (MDT) in chick embryos, interacerebral pathogenicity index (ICPI) in day-old specific pathogen free (SPF) chick, and intravenous pathogenecity index (IVPI) in 6 wk-old chick (King & Seal 1997).

The genome of NDV possesses a negative sense, single stranded RNA with a molecular weight of 5.2-5.6x10^6 Dalton. (Seal *et al* 1995). The genome contains 6 genes in order as $3^{/}$-NP,P,M,F,HN,L-$5^{/}$ that encodes for 6 major polypeptides including nucleoprotein, phosphoprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase and polymerase (Claudia *et al* 1996). The membrane of the virus particle contains HN and F glycoproteins, which form the spike structures on the virion surface and play crucial roles in the initiation of infection (McGinnes & Morrison 1986). The ability of NDV to agglutinate red blood cells (RBCs) is due to the binding of the HN protein to receptors on the surface of the RBCs. Chicken RBCs are usually used in HA tests but NDV will cause agglutination of all amphibian, reptilian and avian cells. The ability of NDV strains to agglutinate cattle, goat, sheep, swine and horse RBCs varied among various strains (Alexander 1991).

The fusion protein is responsible for membrane fusion required in the first stage of infection (McGinnes *et al* 1986). Furthermore, if inserted into the host cell it can mediate fusion between the plasma membrane of the two cells. The molecular basis for NDV pathogenicity depends on the fusion protein cleavage site amino acid sequence (Seal *et al* 1995). The fusion protein is synthesized as a precursor F_0 (M_r 68000) which is proteolytically cleaved into 2 disulfide-link subunits F_1 and F_2 that is necessary for the infectivity of paramyxovirus (Le *et al* 1988). The objective of this study was to obtain primary information about the local NDV strains by using conventional virological methods and molecular techniques.

Archive of SID

Materials and Methods

Viruses. All viruses were isolated from ND outbreaks during 1995 to 1999 in different provinces of Iran. A listing of viruses, local of origin and the years of isolation are presented in table 1.

Birds and SPF embryonated egg. SPF embryonated eggs (Lohmann), day-old chicks and 6-wk old SPF chickens raised on special conditions were provided by Poultry Vaccine Dept. of Razi Institute, Iran.

Birds and SPF embryonated egg. SPF embryonated eggs (Lohmann), day-old chicks and 6-wk old SPF chickens raised on special conditions were provided by Poultry Vaccine Dept. of Razi Institute, Iran.

www.SID.ir

Pathogenicity Indices. Pathogenicity indices were carried out as described by Allan *et al* (1987).

Table 1. *Some epizootiological data on the Newcastle disease outbreaks in Iran*

Isolate	Isolation date	Location	Type of birds	Age of birds
MK5	Apr 1995	Kh. Mashhad	Ind.	3mon.
MK6	Aug 1995	Kh. Mashhad	Bro.	30days
MK7	Oct 1995	Kh. Mashhad	Ind.	90days
MK11	Mar 1996	Kh.Nishabour	Bro./lay	12mon.
MK12	Apr 1996	Kh. Nishabour	Bro.	45days
MK13	Apr 1996	Kh.Torbat-Jam	Ind.	60days
MK14	Jun 1996	Mashhad	Ind.	40days
Krd76	Dec1997	Tehran, Kradj.	Bro.	36days
Kasra97	Dec1997	Kermanshah	Bro.	24days
GH77	May1998	Qazvin	Bro.	25days
KH2/78	May1999	Khozestan	Bro.	17days
ES1/99	Jun 1999	Isfahan	Bro.	28days

Note: Ind (Indigenous)
Bro (broiler)
Kh (Khorasan province)

MDT was performed in 9-10 day-old SPF embryonated egg. Serial tenfold dilutions of allantoic fluid (AF) were prepared and 0.1ml of the last 3 dilutions (10^{-7}, 10^{-8},10^{-9}) was inoculated into the allantoic cavity using a minimum 10 eggs per dilution. The highest dilution at which all embryos died soon was considered as mean lethal dose (MLD) and the MDT is the average time at which the eggs inoculated with MLD were died. Shorter the killing time was considered as the more virulent strain.

ICPI was carried out in day-old SPF chicks. Briefly, fresh allantoic fluid (AF) of each isolates was 10-fold diluted and 0.05ml was injected intracerebrally into 10 day-old SPF chicks. The birds were observed for 8 days postinoculation. The results scored cumulatively for every day of test. The results were recorded as healthy birds scored as zero, sick birds as 1 and dead bird as 2. The sum obtained was divided by number of observations.

IVPI was carried out in 6 wk-old chickens. The birds were inoculated intravenously with 0.1ml of 10^{-1} AF dilution and then were observed for 10 days. The results were scored as healthy birds as zero, sick birds as 1, paralyzed birds as 2 and dead birds as 3. The sum obtained is divided by 100.

Virus purification. In order to purify NDV isolates, fresh AF harvested from 9-10 day-old SPF embryonated eggs inoculated with 10^{-5} virus dilution, was clarified by

Archive of SID

www.SID.ir

centrifugation at 8000 rpm for 30 min (Sorval OTD-T865). Virus was then concentrated at 18000 rpm for 2h (Sorval OTD, SW50L). The pelleted virus was suspended in TNE buffer (0.01M Tris HCl [pH 7.4], 0.1M NaCl, 0.001M EDTA). One ml of suspended virus was layered onto 30-60% sucrose density gradient and centrifuged at 18000 rpm for 4h (Beckman, 41Ti). The virus band was taken out and diluted with equal volume of TNE buffer and pelleted at 18000 rpm for 2h (Beckman, 41Ti), followed by suspension in 2 ml of TNE buffer.

RNA extraction. Trizol LS reagent (Boehringer-Mannheim) was added to the purified virus in 2:1 ratio followed by incubation at room temperature for 5 min. Then 350ul chloroform (Merck) was added to the mixture and vortexes gently and kept at bench for 15 min, followed by cold centrifugation (4C) at 13000 rpm for 15 min (Eppendorf, 5417R). The aqueous phase was transferred into a clean microtube and cold isopropanol (Merck) was added as half volume of Trizol. The mixture was placed at -70C overnight. Then it was left at room temperature for 5 min, followed by spinning down at 12000 rpm for 10 min, and the supernatant was carefully removed. The RNA was washed with cold 75% Ethanol (Merck) mixed well and centrifuged at 7500 rpm for 5 min at 4C. The precipitated RNA is usually invisible. The supernatant was discarded carefully and the pelleted RNA was dried at room temperature or in speed vac (Eppendorf 5301) and suspended in 10µl distilled water. The RNA was stored at -20C.

Polymerase chain reaction (PCR) reagents. Reverse transcriptase AMV (Boehringer) was used for making cDNA. PCR core kit, Taq DNA polymerase and RNase inhibitor (Boehringer) were used. Primers were synthesized by Genosys. Forward primer (BK1) was a 20-mer oligonucleotide localized at position 289-307 genome RNA. Reverse primer (BK2) was a 19-mer oligonucleotide localized at position 512-530 genome RNA (Table 2). DNA molecular weight markers 50 &100 bp (Gibco, Promega, and Boehringer) was used. For purification of PCR products, high pure PCR product purification kit (Boehringer) was used.

Table 2. *Primers used for RT-PCR*

Primers	Sequences(5'→ 3')	Position
BK1	GGGAGGCATACAACAGGACA	289 – 307
BK2	TGGTTGCAGCAATGCTCTC	512- 530

Data from: Ling,K.C.(1997).

Archive of SID

www.SID.ir

cDNA synthesis. In this study, RT-PCR was done in 2 steps. For cDNA synthesis, a reaction mixture contained dNTPs, RNA, reverse transcriptase (AMV) and RNase prepared followed by a profile as 94C for 2 min., 42C for 45 min in master cycler gradient (Eppendorf). This reaction mixture was then used for RT-PCR using Taq DNA polymerase.

RT-PCR product. For a positive RT-PCR test, modifying $MgCl_2$ concentration, annealing temperature and AMV activity temperature were optimized the protocol. Two oligonucleotide RT-PCR primers were designed to amplify a region of fusion protein gene including its cleavage site. The PCR consisted of 40 cycles of 30 sec at 94C, 30 sec at 55C and 1 min at 72C, followed by final extention at 72C for 10 min.

Analysis of PCR product. Samples were analyzed in 1.5% agarose gel electrophoresis stained with Ethidium bromide. The amplified segment was about 242 bp.

Results

Characterization and pathogenicity evaluation of NDV isolates. The results of MDT, ICPI and IVPI (Table 3) demonstrated that all isolates were highly virulent. MDT of less than 60h. ICPI of more than 1.4 and IVPI 0.5, fulfill all criteria of virulent strains. However, two isolates, MK5 and MK6, showed a higher MDT value than that of the others which matched a mesogenic strain MDT, but they showed virulent characteristics according to ICPI and IVPI indices. Three isolates, (Kasra97, Krd76, GH77) showed shorter MDT value and higher ICPI and IVPI value in comparison to that of the other isolates. All the isolates caused death of inoculated chickens in IVPI test after 72h postinoculation but isolate Mk14 caused death by 72h postinoculation.

Table 3. *Results of Pathogenicity tests on the Newcastle disease virus isolates*

	Isolates									
Index	MK7	MK11	MK12	MK13	MK14	Krd76	Kasra97	GH77	KH2/78	ES1/99
MDT	56	59.2	44.2	47.4	45.6	40.6	41.4	39.6	49	47.4
ICPI	1.8	1.83	1.91	1.77	1.96	1.92	1.96	1.9	1.97	1.93
IVPI	2.33	2.21	2.37	2.32	2.46	2.46	2.34	2.55	2.55	2.55

Note: MDT (Mean Death Time of chick embryos in hours)
ICPI (Intracerebral pathogenicity index)
IVPI (Intravenous pathogenicity index)

Hemagglutination of mammalian erythrocytes. The entire field isolates and La-Sota vaccine strain agglutinated bovine erythrocytes but 3 isolates (GH77, KH2/78

Archive of SID

www.SID.ir

and ES1/99) did not. Most field isolates (except for MK13 isolate) and La Sota vaccine strain could not agglutinate equine erythrocytes (Table 4).

Table 4. *Agglutination of mammalian erythrocytes by NDV isolates*

Mamm. Erythr.	**Isolates**									
	MK7	MK11	MK12	MK13	MK14	Krd76	Kasra97	GH77	KH2/78	ES1/99
Bovine	+	+	+	+	+	+	+	+	-	-
Equine	-	-	-	+	-	-	-	-	-	-

Lane 1: Marker, 50/150/300/500/750/1000(G3161)
Lane 2: Sample1
Lane 3: Negative Sample
Lane 4: Sample 2

Figure 1. *Gel electrophoresis of PCR product*

Discussion

Regardless of the virulence or source, ND viruses had been considered to be an antigenically homogeneous group by conventional serological methods and were thus placed in the APMV-1 serogroup. The ability of NDV to agglutinate mammalian RBCs varies with the strains. Among the field isolates tested, differences in hemagglutination patterns were observed which could not be correlated to the virulence of the NDV isolates. The agglutination pattern of bovine erythrocytes, by all field isolates (except for one isolate), and LaSota vaccine strain were similar.

Archive of SID

Pathotyping of ND viruses has been based on virulence value not on antigenic differences. Based on the virulence value, the ND viruses may be placed in one of the pathotypes velogenic (highly virulent), mesogenic (medium virulent) or lentogenic (non-virulent). The virulence of NDV are expressed in pathogenicity indices in which velogenic strains have MDT value of <60 h ICPI & IVPI >1.4 and 2-3, respectively. These values for mesogenic strains are 60-90 h (MDT), 1-1.4 (ICPI) and 0.5 (IVPI). For lentogenic strains the value are >90h for MDT, for ICPI 0-0.2, and 0.0 for IVPI (Alexander 1989). The range of values of pathogenicity index for some of the NDV strains is presented in table 5.

Table 5. *The range of pathogenicity indices for each pathotype of ND viruses*

	Range of values			
Pathotype	MDT	ICPI	IVPI	Example
Velogenic	<60	1.5-2	2.0-3.0	Herts33, Milano, Essex70, Texas GB
Mesogenic	60-90	1-1.5	0.0-0.5	Roakin, Komarov, Mukteswar
Lentogenic	>90	0.2-0.5	0.0	HitchnerB1, Lasota, Clone30

Data from: Alexander,D.J.(1989).

Using BK1 and BK2 primers in RT-PCR for all isolates resulted in 242 bp amplified segment.

Based on MDT, ICPI and IVPI, our results indicated that all the local isolates had virulent strain characteristics except for 2 isolates. Isolates MK5 and MK6 showed MDT value higher than 60h presenting a mesogenic strain characteristic but other indices indicated velogenic characteristic. Similar mismatch of the indices in one isolate has been reported previously (Pearson *et al* 1987). About some isolate from pigeon that showed ICPI value between 1.2 -1.5 and a range of IVPI values of 0-1.3 suggesting the viruses were at least mesogenic, however MDT value recorded was 98h a characteristic of lentogenic virus. Thus there are some drawbacks to the conventional tests, which result in difficulties in the interpretation of results. The conventional methods are also not able to distinguish between vaccine, enzootic and epizootic virus during outbreaks. The applications of conventional methods therefore face some problems. In addition, virus isolation and determination of the index value are laborious and time consuming. In disease outbreaks, in order to prevent further

Archive of SID

www.SID.ir

spread of the disease, rapid diagnostic methods, which can differentiate between virulent and non-virulent NDV strains, are required (kant *et al* 1997). Recently it has been reported that the virulence of ND viruses is related to the F protein cleavability, which in turn arise from the amino acid sequence of the cleavage site. The molecular methods such as PCR have provided the ease of study on the genetic characteristics of the organisms.

The RT-PCR is a reliable method, which allows rapid identification of many samples. Based molecular and virological tests, it is proposed that the molecular technique may be used as a routine laboratory procedure. The aim of this study was to obtain primary information about the local ND viruses using conventional methods and detection of Newcastle disease through the amplification of F cleavage site using primers against conserved regions of F protein. Further studies are required to complete information of local NDV strains.

References

Alexander, D.J., Manvell, R.J., Lowings, J.P., Frost, K.M., Collins, M.S., Russel, P.H. and Smith, J.E.(1997). Antigenical diversity and similarity detected in avian paramyxovirus type1 (NDV) isolates using monoclonal antibodies. *Avian Pathology* 26: 399-418.

Alexander, D.J.(1991). Newcastle disease and other paramyxovirus infection, In: B.W. Calnek(Ed.), *Diseases of Poultry* (9th edn.).Pp: 496-519. Iowa State Press. Ames.

Alexander, D.J., Parson, G.(1986). Pathogenicity for chickens of avian paramyxovirus type 1 isolates obtained from pigeon in Great Britain during 1983-86. *Avian Pathology* 15: 487-493.

Alexander, D.J.(1989). Newcastle disease. In: H. Graham., L.H. Arp., C.H. Domermuth and J.E. Pearson.(Eds), *A laboratora y manual for the isolation and identification of avian pathogens* .Pp: 114-120. Pennsylvania. Kendall/Hunt Pubs Co.

Allan, W.H., Lancaster, J.E.(1987). Newcastle Disease Vaccines, Their Production and Use. *FAO. Rome*. 74-79.

ClaudiaMarin, M., Villegas, P., Bennet, J.D. and Seal, B.S.(1996). Virus characterization and sequence of the fusion protein gene cleavage site of recent Newcastle disease virus field isolates from the Southeastern United States and Puerto Rico. *Avian Disease* 40: 382-390.

Archive of SID

www.SID.ir

Deleeuw, O., Peeters, B.(1999). Complete nucleotide sequence of Newcastle disease virus: evidence for existing of a new genus within the subfamily paramyxovirinea. *Journal of General Virology* 80 (pt 1): 131-136.

Kant, A., Koch, G., Van Roozelaar, D.J, Balk, F. and Terhuurne, A.(1997). Differentiation of virulent and non-virulent strains of Newcastle disease virus within 24 h by polymerase chain reaction. *Avian Pathology* 26: 837-849

King, D.J., Seal, B.S.(1997). Biological and molecular characterization of Newcastle disease virus isolates from surveillance of live Bird Markets in the Northeastern United States. *Avian Disease* 4: 683-689.

Le, L., Brasseur, R., Wemers, C., Meulemans, G and Burny, A.(1988). Fusion (F) protein gene of Newcastle disease virus: sequence and hydrophobicity comparative analysis between virulent and avirulent strains. *Virus Genes* 1(4): 333-350.

Ling, K.C.(1997). Nucleotide Sequence Analysis of the F Cleavage Sites in Velogenic NDV Isolation. M.Sc. thesis. University Putra Malaysia. Serdang, Selangor.

Lomniczi, B., Wehmann, E., Herczeg, J., Ballagi-Pordany, A., Kaleta, E.F., Meulemans, O., Capca, I. and Damosery, J.(1998). Newcastle disease outbreaks in recent years in Western Europe were caused by an old (VI) and novel genotype (VII). *Archives Virology* 143: 49-64.

McGinnes, L.W., Morison, T.G.(1986). Nucleotide sequence of the gene encoding the Newcastle disease virus fusion protein and comparison of paramyxovirus fusion protein sequence. *Virus Research* 5: 343-356.

Pearson, J.E., Senne, D.A., Alexander, D.J., Taylor, W.D., Peterson, L.A. and Russel, P.H.(1987). Characterization of Newcastle disease virus (avian paramyxovirus-1) isolated from pigeons. *Avian Disease* 31: 105-111

Seal, B.S., King, D.J. and Bennet, J.D.(1995). Chracterization of Newcastle disease virus isolates by Reverse transcription PCR coupled to direct nucleotide sequencing and development of sequence database for pathotype prediction and molecular epidemiological analysis. *Journal of Clinical Microbiology* 2624-2630.

Waterson, A.P., Pennington, T.H. and Allan, W.H.(1967). Virulence in Newcastle disease virus, a preliminary studies. *British Medicine Bulletin* 138-143.

Archive of SID

www.SID.ir