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Characterization of Pigeon Paramyxoviruses (Newcastle disease virus) Isolated in Kazakhstan in 2005*

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Abstract: Isolates of Newcastle disease virus (NDV) from deceased wild and domestic pigeons in Kazakhstan were obtained from the Almaty region during 2005 and were genotypically analyzed by using reverse transcription polymerase chain reaction (RT-PCR) with primers specific to the viral fusion (F) protein gene. Part of the amplified F protein DNA product (nucleotide sequence 47–422) and the deduced amino acid sequences were compared phylogenetically with those from strains previously reported in other geographic regions. Phylogenetic analysis indicated that the Kazakhstanian pigeon paramyxovirus type 1 (PPMV-1) isolates belong to genotype VI or 4bii. To our knowledge, this is the first reported VI isolates that possess the sequences of ^{112}GKRQKR$^{116}{}_{*}$ F^{117} within the F0 protein. The information is fundamental to improving the efficiency of control strategies and vaccine development for NDV.

Key words: Newcastle disease virus; Paramyxovirus; Phylogenetic characterization; Pigeon

Avian paramyxovirus type I of pigeons (PPMV-1) is an antigenic variant of avian paramyxovirus type 1 (APMV-1; Newcastle disease virus) of chickens that is responsible for an autonomous Newcastle disease (ND)-like infectious disease of pigeons. Clinical symptoms pigeons and doves infection include paralysis wings. legs or stiff neck and excessive drinking, watery to haemorrhagic diarrhoea[3]. The APMV-I viruses, including PPMV-1, are members of the genus *Avulavirus* in the family *Paramyxoviridae*, order *Mononegavirales*[10]. NDV isolates have been divided into nine genotypes by phylogenetic analysis of the part of gene, including the F protein cleavage site, and were reclassified into six distinct lineages (1 to 6) by the using of monoclonal antibody binding method (mAb group P)[2, 16] PPMV-I viruses associated with the permanent panzootic in pigeons and doves were placed into sublineage 4b(VIb) of lineage 4(VI)

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which later were divided into two 4bi and 4bii groups. Each of subgroups, 4bi and 4bii were subdivided into some clades, a, b, c and d, e, f, g respectively [2,4,8,10,16,17]. In chickens, intracerebral pathogenicity (ICPI) values for PPMV-1 are typical of mesogenic Newcastle disease viruses but in most cases, PPMV-1 isolates have increased their virulence for chickens after passage, and therefore represent a threat to poultry production[2, 9]. Besides pigeons, doves and chickens, PPMV-1 viruses have also been isolated from kestrels, falcons, cockatoos, budgerigars, pheasants, swans and a robin[1, 2, 9, 10, 12].

Large die-offs in doves and pigeons have occasionally been reported in the Almaty region of Kazakhstan in 2005. In the present study, the phylogenetic relationships between 3 Kazakhstanian PPMV-1 viruses isolated from pigeons were investigated. We decided to describe these three strains of NDV due to the fact that since 1995, only a few strains of this monophyletic group of viruses isolated in Poland, Austria and Croatia were reported, and suddenly the same viruses were isolated in Kazakhstan.which was previously characterized by another group of these viruses.

MATERIALS AND METHODS

Viruses

Three NDV isolates were recovered from pigeons during the 2005 NDV outbreaks in Almaty, Kazakhstan. The observed clinical signs and high mortality were evidence of the severity of this Newcastle disease outbreak (NDV). NDV isolates were recovered from samples taken from dead birds. Initial isolation of the virus was performed in 9–10 day old embryonated chicken eggs (ECE). The type of the virus was determined in standard haemagglutination inhibition and neurarmnidase inhibition tests using specific antisera to the reference strains of paramyxovirus and influenza virus. Allantoic fluids were harvested from ECE inoculated with the viruses and used as a stock for sequence analysis. Methods for biological and molecular characterization were as described previously[5, 6].

Sequencing and Phylogenetic Analysis

Sequence analysis of PCR products was completed using an "fmol DNA Sequencing System" (Promega, USA) or ABI PRISM BigDye™ Terminator cycle sequencing reaction kit (Applied Biosystem, USA).

After sequencing, assembly of sequences, removal of low quality sequence data and nucleotide sequence translation into protein sequence, additional multiple sequence alignments and processing were performed with the Lasergene package and the BioEdit software version 7.1.3 with an engine based on the Clustal W version 2.0 algorithm. Phylogenetic analysis, based on the comparison of the nucleotide sequences of F gene fragments (from 47 to 421), was conducted with the Molecular Evolutionary Genetics Analysis (*MEGA*, version 5.0) software package using maximum likehood method to infer evolutionary trees and conduct the bootstrap test for nucleotide and amino acid alignments[13-15].

In addition to the 3 NDV strains isolated in Kazakhstan, 136 previously described NDV sequences representing different NDV genotype groups were used for comparison (on-line Supplementary Table S1). The nucleotide sequences determined in this study are available in GenBank under accession numbers JN806235, JN806236, JN806237.

Supplementary Table S1. List of representative NDV strains taken from GenBank database for phylogenetic analysis

Isolate identification	Genotype or subgroup	Accession number
PUKPI86239	VI	AY471854
PUKPI183299	VI	AY471852
PUKPI183264	VI	AY471851
PITPI84354	VI	AY471850
PUKCK84263	VI	AY471858
PHKPI86358	VI	AY471856
PBEPI84352	VI	AY471849
PUKPI84260	VI	AY471848
PUKPI84259	VI	AY471847
PITDO00289	VI	AY471846
PUKPI84342	VI	AY471855
EG-3-87	VI	AY150111
EG-6-87	VI	AY150112
NO-1-85	VI	AY150105
GB-1168-84(Vib)	VI	AF109885
SE-2-83	VI	AY150100
Pi-Japan-Ehime-93	VI	AB070416
SE-8-00	VI	AY150158
Pi-Japan-Kumamoto-95	VI	AB070417
Pi-Japan-Tokachi-91	VI	AB070413
China-Sh-2-98	VI	AF458017
China-XJ-1-97	VI	AF458021
SZb9803	VI	AY390293
SZA9803	VI	AY390292
NP9904	VI	AY390290
YZ9712	VI	AY390288
NC9701	VI	AY390289
China-XJ-3-97	VI	AF458019
China-ZhJ-2-86	VI	AF458016
SE-3-90	VI	AY150126
ZC94	VI	AY390295
Z32	VI	AY390296
Pi-Japan-Gunna-2000	VI	AB070434
Astr-74	VI	NDI243391
Iraq AG68	VI	AF001108
Israel 70	VI	AF001111
Kuwait 256	VI	AF001109
Vh448-06	VI	EU240578
JS-35-07PI	VI	GQ281085
PIQPI178442	VI	AY471857
PUKPI02434	VI	EF030957
PUKPI02431	VI	AY471755
PUKPI01426	VI	AY471753
PUKPI02435	VI	AY471754
PAEKE99364	VI	AY471785
YZ-21-07-Pi	VI	AB070434
PTRBU95211	VI	AY471773
Warwick	VI	NDVWAWRFG
PDEPI94216	VI	AY471763
ES-3-92	VI	AY150132
DOZA05N247	VI	EF030954
DOZA05N417	VI	EF030955
OZA06N549	VI	EF030957
PDKPI93202	VI	AY471761
Pi-Japan-Tochigi-95	VI	Ab070419
Pi-Japan-Fukushima-96	VI	Ab070423
Pi-Japan-Saitama-97	VI	Ef030957
Pi-Japan-Shiga-96	VI	Ab070422
PFRPI98372	VI	AY471765
Pi-Japan-FK-1-84	VI	AB070434
Pi-Japan-Niigata-88	VI	AB070434
FR-99299	VI	AB070434
STP96	VI	AB070434
Vh 357-06	VI	EU240576
HR-3-02	VI	AY150165
HR-111-01	VI	AY150162
DOZA05AM68313	VI	EF030952
DOZA05N240	VI	EF030953
PATPI00323	VI	AY471789
WX-10-07-Pi	VI	GQ281086
DOZA06N591	VI	EF030959
Vh 536-06	VI	EU240582
Vh 529-06	VI	EU240580
Vh 538-06	VI	EU240583
Gxp40	VI	AY635815
SD-15-08-Pi	VI	GQ245801
Pi-Dnipro-2007	VI	EF030957
PI-PL-H2-10	VI	HM627541
BG-99-82	VI	AF402131
CA-28-89	VI	AY150119
Chicken-Japan-Chiba-69	VI	AB070387
DE-60-93	VI	AY150134
DE-61-93	VI	AY150135
HR-65-95	VI	AY150144
HR-155-01	VI	AY150163
HU-61-83	VI	AY150099
HU-238-84	VI	AY150103
JAEFA96038	VI	AY175738
JN08	VI	HM776583
Lebanon 70	VI	AF001110
NDV05-027	VI	DQ439884
Pigeon-NY-US-1984	VI	EF520716
SD-2-75	VI	AY151384
BG-29-86	VI	AF402135
BG-72-74	VI	AF402116
BG-48695	VI	AF402138
BG-99-82	VI	AF402131
GR-12-68	VI	EU604251
KR-5-99	VI	EU665683
CH-1-95	VI	AF001132
DK-1-95	VI	AF001129
PB9601	VI	AY390291
China-98-1	VI	AF358785
China-JC-2-98-Go(Vlb)	VI	AF456439
TW-06-223	VI	DQ898521
Chicken-Japan-Chiba-81	VI	AB070388

Table 1 (continue)

Kr-12A-8	VI	AY630414
Kr-102-89	VI	GQ507801
Kr-163-90	VI	AY630416
Kr-M-88	VI	AY630413
SNU88139	VI	AF257749
BOR74	I	Y16049
Ulstr	I	NDVULSTFG
Komarov	II	AY170137
ZA-405-01	II	AF532748
H-Ph-02	III	AY170136
Muktesvak1	III	AY117022
Pok-70	IV	NDI243388
Simf-64	IV	NDV19017
YU(VO)-C-91	V	AY117003
IT-129-88	V	AF218131
NL-1-93	VII	AF001124
TR-8-97	VII	AF136785
ZA-5-68	VIII	AF136762
ZA-10-74	VIII	AF136763
B-14-93	VII	AF001121
BG-109-84	VII	AF402133
China-T02	VII	AY390307
China-YLF3	VII	AY390312
D-83-95	VII	AF001118
MZ 46-95	VII	AF136778
FIN-1-96	VII	AF091623
RI-1-88	VII	AF001134
ZA-32-93	VII	AF136771
ZA-549-99	VII	AY210500

RESULTS AND DISCUSSION

Estimation of NDV Isolates Pathotype

The biological properties of pigeon NDV strains isolated in Kazakhstan during 2005 are presented in Table 2. NDV isolates were characterized as mesogenic based on their mean death time (MDT), which ranged from 60,8 to 73 h and intracerebral pathogenicity index (ICPI) that ranged from 0,51 to 1.06. The deduced amino acid sequence of F protein cleavage site for all of these strains was determined to be^{112} GKRQKR^{116}* F^{117}and was characteristic of mesogenic NDV strains.

Phylogenetic Relationships among NDV Isolates

Phylogenetic analysis of pigeon NDV strains isolated in Kazakhstan and study of their phylogenetic relationships with other NDV worldwide isolates was completed based on sequence analysis of the variable region of the F gene (47-421) (Fig. 1 and Fig. 2). Although the bootstrap values supporting the phylogenetic relationships within lineages 4bi and 4bii are low[1,2], amino acid sequences support the phylogenetic relationships: lineage 4bi clade (c) is distinguished from clades (a) and (b) by T3, L10 and R27 substitutions, and lineage 4bii clade (d) differs from clade (e) by a P36→S substitution. The low level of bootstrap analysis is connected with continuing evolution of this group of viruses from regular outbreaks

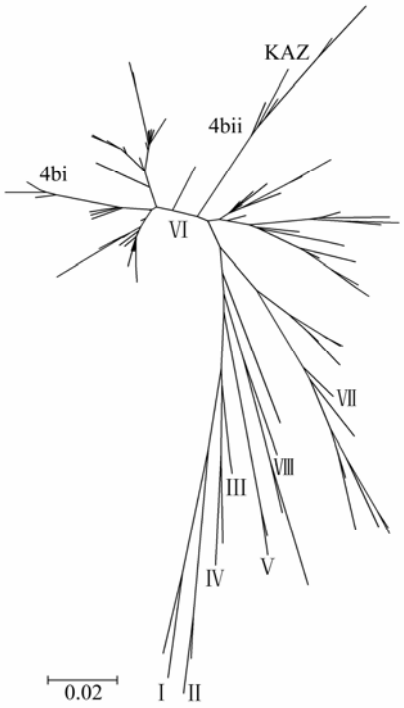

Fig. 1. Unrooted maximum likelihood radial phylogram based on nucleotide sequence data from 139 APMV-1 isolates, including 115 PPMV-1 isolates and 24 representative of the other genetic lineages [2]. The region analysed was a 374 base pair fragment (47 to 422) at the 3' end of the fusion protein gene. Branch lengths represent the predicted number of substitutions and are proportional to the differences between the isolates. The individual names of each PPMV-1 isolate included in this phylogram are not included. Bar=0.02 μm.

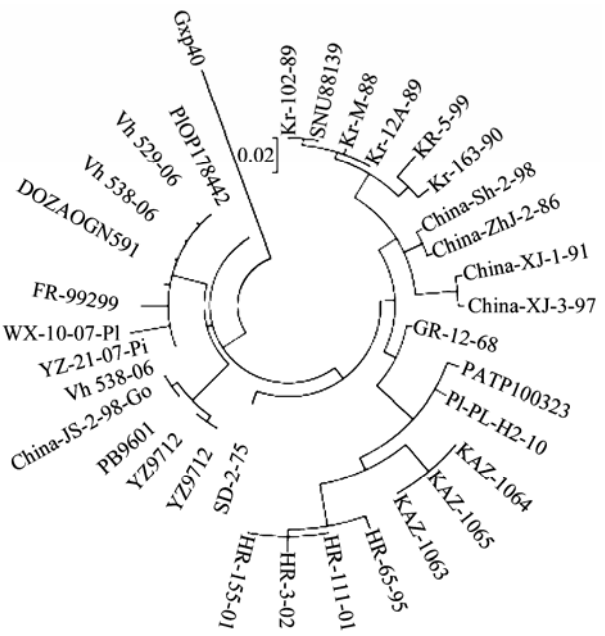

Fig. 2. Unrooted maximum likelihood circular phylogram based on nucleotide sequence data from 39 PPMV-1 4bii isolates. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [14]. The tree with the highest log likelihood (-987.0619) is shown. Initial tree(s) for the heuristic search were obtained automatically as follows. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 35 nucleotide sequences. Evolutionary analyses were conducted in MEGA5 [15].

of pigeons in the various regions of the globe. The phylogenetic analysis indicated that the 3 Kazakhstanian PPMV-1 strains cluster together with viral isolates from Poland, Austria and the Croatia. The nucleotide substitutions characteristic for this group of Kazakhstan viruses were found in positions 18 (T changed to C), 32 (T changed to C), 43 (C changed to T), 111 (T changed to C), and 187 (G changed to A). Calculation of the synonymous and non-synonymous substitution rate demonstrate that all isolates have a rate ranging between 0.081 - 0.264; a value less than one demonstrates the presence of only purifying (negative) selection, despite the most variable portion of the F gene between nucleotide positions 47-421 was used in the analysis. Phylogenetic analysis based on the amino acid NDV fusion protein exhibited similar properties (Fig. 3). According to the phylogenetic tree, all the isolates belong to the genotype to the genotype VI or 4bii—of sublineage 4b. A further analysis on the amino acid sequence of the protease cleavage site reveals that the F1 protein of all the recent Kazakhstanian isolates contain a phenylalanine (F) on the residue 117 on the N-terminus and four basic amino acids in the motif. ^{112}GKRQKR^{116}* F^{117}, which indicates they are velogenic strains[2, 3]. Based on amino acids analysis, unique amino acid substitutions (V_{11} to A_{11}, C_{27} to R_{27}, V_{63} to I_{63}, T_{90} to A_{90}) in all strains of this monophyletic group were found.

Although these results demonstrated some sequence similarity between the isolated strains and strains responsible for outbreaks of Newcastle disease in Europe, it is the region of isolation of this monophyletic group of viruses that is unusual. The results indicate that the exchange of breeding birds by owners of pigeon lofts may become a serious factor in

Table 2. Biological and molecular characteristics of NDV isolates recovered from pigeons in Kazakhstan during 2005

Isolate identification	Strain abbreviation	ICPI	MDT	Fusion protein cleavage site (molecular pathotyping)
PPMV- 1/pigeon/Алматы/1063/05	KAZ-1063-05	0,51	60,8	^{112}GKRQKR^{116}* F^{117}
PPMV- 1/pigeon/Алматы/1064/05	KAZ-1064-05	1,06	73,0	^{112}GKRQKR^{116}* F^{117}
PPMV- 1/pigeon/Алматы/1065/05	KAZ-1065-05	0,81	60,8	^{112}GKRQKR^{116}* F^{117}

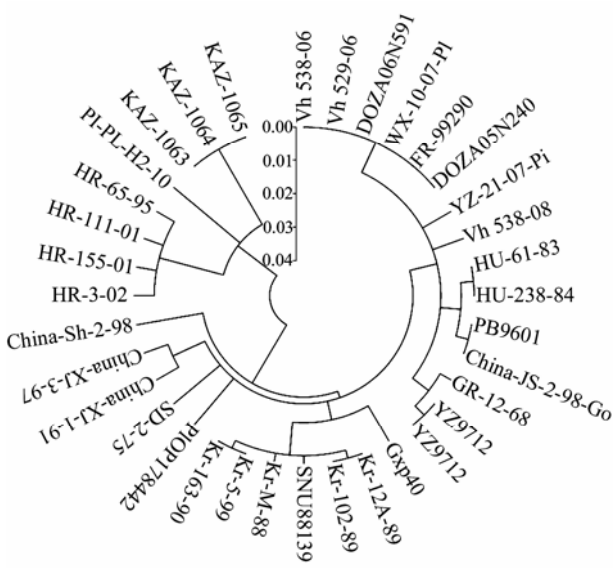

Fig. 3. Unrooted maximum likelihood circular phylogram based on amino acids sequence data from 39 PPMV-1 4bii isolates. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model [7]. The tree with the highest log likelihood (-445.5714) is shown. Initial tree(s) for the heuristic search were obtained automatically as follows. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA5[15].

the spread of Newcastle disease virus because, at the time of this outbreak, the National Exhibition of Racing Pigeons took place, organized by the Polish Association of Racing Pigeon Breeders, the main institution which deals with pigeon breeding. Another possibility may be introduction of new NDV strains from migrating birds during their direct contact with chickens in primarily open poultry farms in Kazakhstan. Further characterization of NDV strains circulating in Kazakhstan and other Central Asia countries is important for better control and understanding of NDV epizootics in the Central Asia region.

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