```
                    10        20        30        40        50        60
                    |    |    |    |    |    |    |    |    |    |    |
Consensus   CAAGGGTCTGTGTCCACATCGGGAGGAAGGAGACAGAGACGCTTTATAGGTGCCGTTATC
MK7         ........................................T...................
MK12        ........................................T...................
MK13        ..............G....A..........G.....G..............A.....
MK14        ..................A.........................T...................
Krd76       ........................................T...................
Kasra97     ........................................T...................
GH77        ........................................T...................
KH2/78      ........................................T...................
ES1/99      ........................................T...................
TexasGB     ....A......AA.T.....T.....G..........A............C...A....T
ITA45       ....A......AA.T.....T.....G..........A............C...A....T
Herts/33    ....A.......A.T..T..C.....................................A.....
A.Victoria  ....A.......A.T.....C.................A................TA.....
Ulster      ....A.......A.T.....T......G...A....G.....C.......C...A.....
LaSota      ....A.......A.T.....T.....GG........G.G...C.......C...A....T
MIY/51      ....A.......A.T.....C.....................................A.....
AF2240      ............A.T.....T.....G..........A................T.....T
VOL95       ........................................T...........T.......

                    70        80        90        100       110       120
                    |    |    |    |    |    |    |    |    |    |    |
Consensus   GGCAGTGTAGCTCTTGGGGTTGCAACAGCGGCACAGATAACAGCAGCTGCGGCTCTAATA
MK7         ............................................................
MK12        ............................................................
MK13        ...G..........C.....C..G...T..............G.....A..C.......
MK14        ............................................................
Krd76       ............................................................
Kasra97     ............................................................
GH77        ............................................................
KH2/78      ............................................................
ES1/99      ............................................................
TexasGB     ...G....G.................T..T.....A........G..C..A.....G...
ITA45       ...G....G.................T..T.....A........G..C..A.....G...
Herts/33    ..........................T...........G.....CT....C..G...
A.Victoria  ..........................T...............CT....C..G...
Ulster      ...G...C......C...........C..T...................T.......G...
LaSota      ...G....G.................T..C.....A........G..C..A.....G...
MIY/51      ..T.......................T...............CT.......G...
AF2240      ..........................T...............C........G...
VOL95       ............................................................

                    130       140       150       160       170
                    |    |    |    |    |    |    |    |    |    |    |
Consensus   CAAGCCAACCAGAATGCTGCCAACATCCTCCGGCTTAAAGAGAGCATTGCTGCAACCAA
MK7         ..................T.....................................
MK12        ..................T.....................................
MK13        .....T..A.............T...........G..G...................
MK14        ..................T.....................................-
Krd76       ..................T.....................................-
Kasra97     ..................T.....................................
GH77        ..................T.....................................
KH2/78      ..................T.....................................
ES1/99      ..................T.....................................
TexasGB     ........A..A.....................A.................C........
ITA45       ........A..A.....................A.................C........
Herts/33    ..................................G.....................
A.Victoria  ........T.........................G.........A..........
Ulster      .TT........A.....................................
LaSota      ........A..A.....................A.................C........
MIY/51      ..................................G.....................
AF2240      ..G.....G........C.....................G.....................
VOL95       ..................T.....................T..............
```

Fig. 1
Nucleotide sequence of F protein gene (nts 351–531) of the Iranian and other NDV isolates

different regions of Iran were used in this study. They were isolated at the Department of Poultry Diseases Diagnosis, Razi Institute, Teheran, Iran from ND outbreaks in 1995–1999.

Virus propagation and purification. The stock of each isolate was propagated in 9-day-old SPF embryonated eggs that were obtained from the Department of Poultry Vaccines, Razi Institute, Teheran, Iran. The inoculated allantoic fluid (AF) was purified by ultracentrifugation as described by Hamaguchi *et al.* (1983).

Extraction of viral RNAs from purified viruses was performed using the Trizol LS reagent (Gibco BRL) as described by the manufacturer.

Reverse transcription–polymerase chain reaction (RT-PCR) of the F protein gene encompassing the cleavage site was carried out using specific primers. The forward primer BK1 (nts 289–307, 5'-GGGAGGCATACAACAGGACA-3') and the reverse primer BK2 (nts 512–530, 5'-TGGTTGCAGCAATGCTCTC-3') were designed on the basis of the sequence of the F protein gene of a Malaysian velogenic NDV isolate (Salih *et al.*, 2000). The viral RNA was reversibly transcribed in a reaction mixture (20 µl) containing a PCR buffer, 25 mmol/l $MgCl_2$, 25 mmol/l dNTPs, 1.5 mmol/l BK1 primer, 1.5 mmol/l BK2 primer, 2U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim), 10 U of RNase inhibitor and 10 µl of viral RNA (100 ng/ml). The RT conditions were 42°C for 45 mins and 94°C for 5 mins. The samples were either cooled to 4°C and subjected immediately to PCR or stored at -80°C. The PCR was run in a 100 µl volume containing 20 µl of RT mixture, 10x PCR buffer, 25 mmol/l $MgCl_2$, and 1.2 U of *Taq* DNA polymerase. Except for the RT mixture all the components originated from the PCR Core Kit (Boehringer Mannheim). The PCR conditions were as follows: 40 cycles of 94°C for 30 secs, 55°C for 30 secs and 72°C for 1 min. The last cycle was followed by 72°C for 10 mins and cooling to 4°C.

Sequencing of PCR amplified DNA. PCR products were subjected to agarose gel electrophoresis, isolated and purified by using the PCR Product Purification Kit (Boehringer Mannheim) according to the manufacturer's instructions and used as templates in the sequencing reaction. The latter was performed using the ABI PRISM® Big Dye™ Terminator in an automated sequencer (ABI PRISM® 377) following the method described by the manufacturer. The cycle sequencing was conducted in GeneAmp PCR System 9600 (Perkin Elmer) under the following conditions: 25 cycles of 96°C for 10 secs, 50°C for 5 secs, and 60°C for 4 mins. At least 2 independent PCR products for each isolate were used for sequence analysis and each fragment was sequenced in both directions.

Sequence alignment and phylogenetic analysis. The nucleotides and deduced amino acid sequences of the NDV isolates were assembled and analysed using the Bio-edit Package Version 3.57c. The isolates, Genbank Acc. Nos. and references of sequences used for sequence alignment and phylogenetic analysis were as follows: TexasGB (U22293) (Seal *et al.*, 1995), ITA45 (M24703) (Toyoda *et al.*, 1989), Herts/33 (U22275.1) (Seal *et al.*, 1995), A. Victoria (M21881) (McGinnes and Morrison, 1986), Ulster (D00243) (Millar *et al.*, 1988), LaSota (AF077761) (de Leeuw and Peeters, 1999), MIY/51 (M24701) (Toyoda *et al.*, 1989), AF2240 (AF048763) (Salih *et al.*, 2000) and VOL95 (Y16169). The phylogenetic analysis was carried out based on amino acids 103–163 using the Genetics Computer Group (Madison, Wisconsin, USA). Phylogenetical trees were constructed by the UPGMA method. The sequences of the nine Iranian NDV isolates were deposited at GenBank under Acc. Nos. AY036960, AY036961, AY036962, AY036963, AY036964, AY036965, AY036966, AY036967 and AY036968.

Results and Discussion

The RT-PCR amplified a product of 242 bp (289–530 bp) which contained the cleavage site and the F_1 N-terminal sequence of hydrophobic fusogenic region (aa 82–162). Nucleotide sequences of the amplified segments originating from the nine Iranian NDV isolates revealed some differences. Seven isolates showed a complete identity, while 20 and 1 nucleotides changes in the isolates MK13 and MK14, respectively, were observed (Fig. 1). Nucleotides changes in MK13 resulted in 5 amino acid substitutions as compared to other local isolates, of which 3 substitutions occurred in the F_1 N-terminus and included isoleucine (I) for valine (V), glycine (G) for serine (S) and S for alanine (A) at positions 121, 124 and 132, respectively (Fig. 2). These substitutions have been reported for several velogenic NDV isolates such as: Herts/33, Texas GB, ITA45 (Seal *et al.*, 1995), A. Victoria (McGinnes and Morrison, 1986), Miyadera (Toyoda *et al.*, 1989) and AF2240 (Salih *et al.*, 2000), indicating that they are common for velogenic NDV.

The other two substitutions A and S at positions 108 and 132, respectively, have not been reported for the isolates considered in this study. All the five substitutions did not change the cleavage site motif. The deduced amino acids of the cleavage site for all the Iranian isolates are similar to those of velogenic strains containing 2 pairs of basic amino acids (arginine) at positions 112, 113, 115 and 116 followed by a phenylalanine (F) at position 117, forming the motif ^{112}RRQRRF^{117} (Fig. 2). The obtained results showed that the cleavage-activation site in virulent isolates consists of double set of two dibasic amino acids with an intervening glutamine (^{112}RRQR/KR^{116}) in the C terminus of the F_2 protein and F at the position117, while the corresponding region in avirulent isolates such as LaSota (Le *et al.*, 1988), Ulster (Millar *et al.*, 1988), B1, D26 and QUE (Toyoda *et al.*, 1989) consists of single pairs of basic amino acids (^{112}GK/RQGR^{116}) and L at the position 117.

The phylogenetical tree based on the distance matrix values for amino acid sequences is shown in Fig. 3. Three distinct groups were generated in the tree. Group A consists of avirulent isolate Ulster. Isolates MK13, LaSota, ITA45 and TexasGB create together the B group. The C group consists of 2 subgroups. The isolates A.Victoria, MIY51 and Herts33 which showing close relationships form one subgroup. The distance matrices of amino acids (PROdist) value between Herts33 and MIY51 was 0.0, while the value

```
                    10        20        30        40        50        60
                ....|....|....|....|....|....|....|....|....|....|....|....
Consensus       QGSVSTSGGRRQRRFIGAVIGSVALGVATAAQITAAAALIQANQNAANILRLKESIAAT
MK7             ............................................................
MK12            ............................................................
MK13            .....A.............I..G.......S............K................
MK14            ............................................................
Krd76           ............................................................
Kasra97         ............................................................
GH77            ............................................................
KH2/78          ............................................................
ES1/99          ............................................................
TexasGB         .E..T.......K.....I..G....................K................
ITA45           .E..T.......K.....I..G....................K................
Herts/33        .E..T.............I.....................S....................
A.Victoria      .E..T.......K.....I.....................S...................T..
Ulster          .E..T....GK.G.L...I..GA..............S...L..................
LaSota          .E..T....G..G.L...I..G....................K................
MIY/51          .E..T.............I.....................S....................
AF2240          ....T.......K..............................K................
VOL95           ............................................................
```

Fig. 2
Deduced amino acid sequence of F protein gene (aa 103–163) of the Iranian and other NDV isolates
Basic amino acids and phenylalanine at cleavage site are bold and underlined.

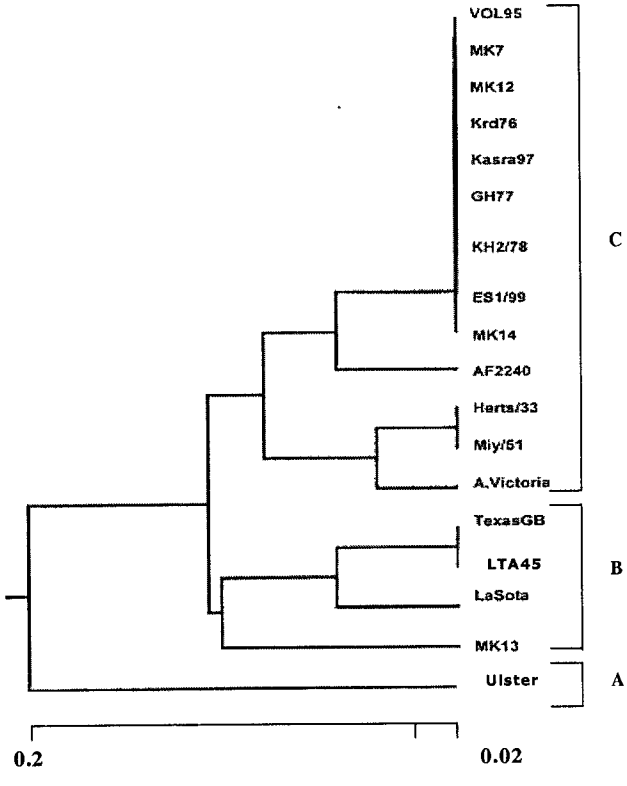

Fig. 3
Phylogenetic tree of Iranian and other NDV isolates based on the deduced amino acid sequences of F protein gene (aa 103–163)

for A.Victoria in this group was 0.03472. The second subgroup consists of VOL95, AF2240 and all the Iranian isolates except for MK13. The PRODist value for the Iranian isolates in this group was 0.0. Meanwhile, the differences in the values for MK13 and AF2240 as compared to the Iranian isolates were 0.09012 and 0.05273, respectively. The MK13 had a PRODist value of 0.09012–0.12949 as compared to other velogenic isolates (Herts/33, TexasGB, ITA45 and VOL95) and also had a difference of 0.17102 as compared with the Australian velogenic strain A. Victoria. The classification of NDV isolates into 3 groups has been described earlier by Toyoda *et al.* (1989). In that study, groups A and B consisted of a mixture of avirulent (e.g. Ulster) and virulent strains (e.g. TexasGB, ITA45 and LaSota) isolates, respectively. Other studies have also shown that avirulent strains such as Ulster, D26 and Que76 may form the A group, while group B may consist of isolates of both avirulent and virulent pathotype (King and Seal, 1997; Sakaguchi *et al.*, 1989; Toyoda *et al.*, 1989). Unlike groups A and B, the C group may consist exclusively of velogenic pathotypes (e.g. the isolates Herts33, MIY51, ITA45, AF2240 and A.Victoria (Fig. 3). This group may also contain of all the nine Iranian isolates except for MK13.

None of the Iranian isolates matched with MK13, indicating that this isolate originated from an indigenous flock in rural area and did not circulate among the poultry population across the country for four years during this study. Among the published NDV strains, only the Russian isolate

VOL95 showed the same amino acid sequence as the Iranian isolates except for MK13 (Fig. 2). However, the nucleotide sequence of VOL95 was different from that of the Iranian isolates, indicating that the additional nucleotide substitutions in VOL95 did not contribute to the amino acid variation (Fig. 1). Based on the topology of the tree and PROdist values, all the Iranian isolates except MK13 showed a close relationship to AF2240 and VOL95 (Fig. 3). Thus the isolates/strains belonging to the C group have circulated across the world and have caused ND outbreaks in many countries. The close relationship between the Russian isolate VOL95 from 1995 and the Iranian isolates except for MK13 is worth mentioning. Based on the amino acid sequence results so far available VOL95 is the only reported isolate that showed the same sequence as the Iranian isolates except for MK13. Thus, there may be assumed a common origin. The mode of transmission of the viruses is not known, but it may be associated with (i) vehicle traffic and human migration caused by common border trade between Iran and Russia and (ii) the winter climate condition in northern Iran which attracts birds from Siberia to spend the winter in northern and central Iran. Further studies on the mode of transmission of ND outbreaks are required in order to implement effective control programs for ND in Iran.

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