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(Research Article)

Analysis of mutations in the fusion protein and hemagglutinin-neuraminidase in twelve strains of NDV isolated in Madagascar by bioinformatics methods

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Abstract

Many researchers have made efforts to generate genotype-matched and subunit vaccines for Newcastle disease (ND). During the design process, we found that they only considered certain characteristics of the virulent strains. As a result, the resulting vaccines are still poorly effective against new emerging strains of ND virus (NDV). This analysis suggests that consideration of various characteristics of virulent strains in the design is necessary to increase the efficacy of these new vaccines. Thus, analysis of mutations in viral proteins targeted in the design such as fusion proteins (F) and hemagglutinin-neuraminidase (HN) is essential. For this reason, the present study aims to analyze mutations in these two proteins in twelve NDV strains isolated in Madagascar.

To achieve this, we employed bioinformatics methods such as sequence alignment, molecular modeling, 3D structure comparison and mutation impact prediction via bioinformatics software and servers.

As results, we identified respectively 26 and 41 mutations in the F and HN proteins of the Madagascar isolates. All these mutations have an impact on the stability of the protein. However, only 6 mutations (D344N, G303V, P315S, E347K, P391S and E495S) have an impact on their 3D structures. Finally, the 3 mutations (A477T, G303V and P315S) also affect the functions of these two proteins.

In conclusion, we have identified 67 mutations that may affect the stability, structures, and functions of NDV F and HN proteins. Further studies are needed to know their effects on the antigenicity and immunogenicity of these two proteins.

Keywords: NDV; Fusion protein; HN protein; Mutation; Vaccine design; Bioinformatics

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1. Introduction

Newcastle disease (ND) is caused by a virus belonging to the family Paramyxoviridae and the genus Avian Orthoavulavirus-1 (AOAV-1). This virus is also commonly known as Newcastle Disease Virus (NDV) [1]. The viral genome is a single-stranded RNA of negative polarity, composed of approximately 15.200 nucleotides and encodes 6 structural proteins including RNA-dependent RNA polymerase (L), hemagglutinin-neuraminidase (HN), fusion proteins (F), matrix protein (M), phosphoprotein (P) and nucleoprotein (NP) [2]. In Madagascar, 14 strains of NDV (named MG isolates) were isolated between 1992 and 2011 [2,3]. According to Maminiaina, these strains belong to genotype XI, which derive from the older genotype IV [2].

Vaccination is the best option to protect poultry against ND. Several types of vaccines are available on the market. Most of them are generated with non-virulent strains of NDV, belonging to genotypes I to III. Currently, the efficacy of these vaccines is disputed due to the multiplicity of newly identified strains [4]. Therefore, research has been conducted for the development of a new vaccine, capable of protecting poultry against the newly emerging strains. Genotype-matched vaccines are being developed and proposed by several researchers. They are usually made by manipulation of the F and/or HN protein of a virulent strain and its expression in a non-virulent strain [5,6]. However, a single virulent strain cannot represent all virulent strains circulating in a ND endemic terrain. In other words, each virulent strain has specific characteristics, which can be identified and must be incorporated into the vaccine design. As a result, genotype-matched vaccines face a certain flaw in target selection for design. This flaw has diminished their efficacy against emerging strains, which have characteristics distinct from those of the strains used in the design [7]. Faced with this situation, research is again directed towards the design of subunit vaccines, based on antigenic peptides or epitopes of many virulent strains [8,9]. Unfortunately, vaccine development in this new platform has only considered conserved epitopes or antigenic peptides in the target proteins. This means that non-conserved regions, which differentiate virulent strains, have also not been considered in the design. Although this vaccine platform is still under development, a question must be asked: "Do mutations in the non-conserved regions of the target proteins have no effect on their structure, function and antigenicity? »

To address this question, this study aims to analyze mutations present in the F and HN proteins of 12 MG isolates using bioinformatics methods and tools. We chose these two proteins because they are all potential targets in NDV vaccine development [5,6]. To achieve this goal, we first identified by multiple sequence alignment the mutations present in these two viral proteins. Then, we modeled the 3D structures of these F and HN proteins and evaluated the effect of mutations on their structures. We also analyzed the effects of mutations on their stability, as well as on their global structure and functions. The information obtained from this study should be applied to optimize target selection for ND vaccine design.

2. Material and methods

2.1. Multiple sequence retrieval and alignment

Twelve F and HN protein sequences from MG isolates were extracted from the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein) in FASTA format. The information about these sequences is summarized in Table 1. The retrieved sequences were aligned in BioEdit version 7.0.5.3 [10], using the ClustalW algorithm [11]. MG-1992 is considered as the reference sequence.

2.2. Molecular modeling and comparison of tertiary (3D) structures

The 3D structures of the F and HN proteins were modeled and minimized using the SwissModel server (www.swissmodel.expasy.org) and the Swiss pdb Viewer software respectively[12,13]. The obtained 3D structures were compared by superposition using the online server TM-Align (https://zhanggroup.org/TM-align/)[14]. This server provides a TM score and root mean square deviation (RMSD) value by comparing two 3D structures of proteins [14]. The TM score value between 0.0 and 0.3 and an RMSD value ≥ 5.0 Å indicate structural similarity[15]. On the other hand, a higher RMSD value indicates a higher variation between the superimposed structures[16]. These are visualized in Discovery studio version 2017 or D.S v2017 software (http://accelrys.com/products/collaborative-science/biovia-discovery) [17].

2.3. Predictions of mutation effects on the stability, structures, and functions of F and HN proteins

The servers MUpro (http://mupro.proteomics.ics.uci.edu/) [18], Mutpred2 (http://mutpred.mutdb.org/) [19] and PROVEAN (http://provean.jcvi.org/) [20] were used to evaluate the impacts of mutation on the stability, structure, and function of F and HN proteins.

MUpro predicted the change in stability of protein structures, by calculating the thermodynamic free energy value ($\Delta\Delta G$) after a single mutation: the value of $\Delta\Delta G > 0$ indicates an increase in stability while the value of $\Delta\Delta G < 0$ indicates a decrease in structural stability [21]. In addition, the Mutpred2 server was used with a threshold value of 0.5 to predict the effects of mutation on global protein structures. Finally, the PROVEAN server was used to predict the effects of the mutation on global protein functions, using the threshold score of -2.5: scores above this threshold value were considered "deleterious" while scores below this threshold value were considered "neutral".

Table 1 F and HN protein sequences used in this study

Viral strains	Accession number		Collection date	Collection regions	References
	F protein	HN protein			
MG-1992	ADQ64395	ADQ64396	1992	Antananarivo	[2]
MG-MEOLA	ADQ64398	ADQ64399	2008	Antananarivo	[2]
MG-39	ADQ64401	ADQ64402	2008	Antananarivo	[2]
MGMNJ	AGC23353	AGW43230	2009	Antananarivo	[3]
MG-725	ADQ64389	ADQ64390	2010	Antananarivo	[2]
MGF003C	AGC23347	AGW43224	2010	Alaotra Mangoro	[3]
MGF082T	AGC23349	AGW43226	2010	Alaotra Mangoro	[3]
MGF015C	AGC23348	AGW43225	2011	Alaotra Mangoro	[3]
MGF120T	AGC23350	AGW43227	2011	Alaotra Mangoro	[3]
MGF166	AGC23351	AGW43228	2011	Alaotra Mangoro	[3]
MGF192C	AGC23352	AGW43229	2011	Alaotra Mangoro	[3]
MGS1130T	AGC23354	AGW43231	2011	Alaotra Mangoro	[3]

3. Results

3.1. Mutations in the F and HN proteins of MG isolates

Referring to MG-1992, multiple sequence alignment with BioEdit v7.0.5.3 revealed 26 mutations in F proteins and 41 mutations in HN proteins (Table 2). 11 of the 26 mutations found in the F protein are located in all five functional regions, including the N-terminal (N-T) or signal peptide region, heptadic regions 1 and 2 (HR1 and HR2), transmembrane region (T.M), and C-terminal or C.T region (Figure 1A). In contrast, 5 of the 41 mutations found in the HN protein are localized in the T.M region and the HR1 region (Figure 1B). The rest of the mutations are dispersed along the linear sequence of these two proteins (Supplementary Figure 1 and 2).

Table 2 Mutations observed in the F and HN proteins of MG isolates

Protein	Mutation and position in linear sequence
F	K4Q S12F I17V A20V L28S V63I K73R A79S T108I G110E V121I A149T I202V N258S/R Y337H N344D P357S T371M H422D I426V Q445R T465A A477T K490R V516M T550N
HN	K3H S6G N12R N19Y A28S I36T T43A V57A P60S S64T R65 KA73T S75R V80I Y112V A117V N120T V142I I208L L220F I254V G303V P315S N323S P325L E347K G362E K377R T380A G383S M387V P391S T437A T443A I452T I453V E495G A497S S510A S520G I571T

No mutations are found in the cleavage site or C.S ([112]*RRRRRRF*[117]) and in the heptadic region 3 or HR3 of F proteins (Figure 1A). Similarly, the intervening region or IR, the HR2 region, and the sialic acid binding site (S.A.B.S) of HN proteins were not mutated (Figure 1B). In addition, the 25 cysteines residues involved in disulfide bond formation and the 12 asparagine residues involved in structuring the glycosylation site of F and HN proteins are also conserved (Supplementary Figures 1and 2).

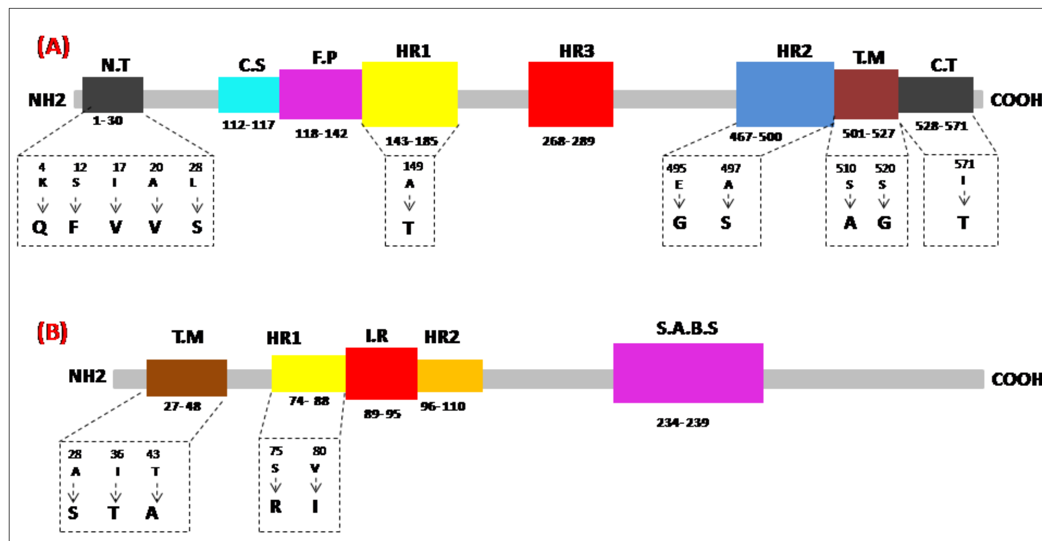

Figure 1 Schematic diagram showing the mutations observed in the different domains of the F and HN protein: (A) mutations found in the 5 domains of the F protein. No mutations are observed in the cleavage site (C.S), the fusion peptide (F.P) and the heptad repeat 3 (HR3). (B) Mutations observed in the different domains of the HN protein. No mutations are observed in the intervening region (I.R) and in the Sialic Acid Binding Site (S.A.B.S)

3.2. Molecular modeling and visualization of mutation positions

3.2.1. 3D structure of F proteins and mutation positions

After modeling, we obtained homotrimeric 3D structures for F proteins of MG isolates. They are composed of a globular head and a spirally rolled stalk (Figure 2A). The monomers forming these trimers have six active domains including the C.S, F.P, HR1, HR2 and HR3 domains (Figure 2B). The N-T, T.M, and C.T regions were not modeled because they were not present in the model structure (pdb ID= *2b9b*).

The locations of the 26 mutations identified in the F proteins are shown in Figure 2C. In which, 17 mutations are located in the globular head of the protein and 2 mutations are found in the stalk. The 7 mutations located in the missing regions (N-T, T.M and C.T) are not presented.

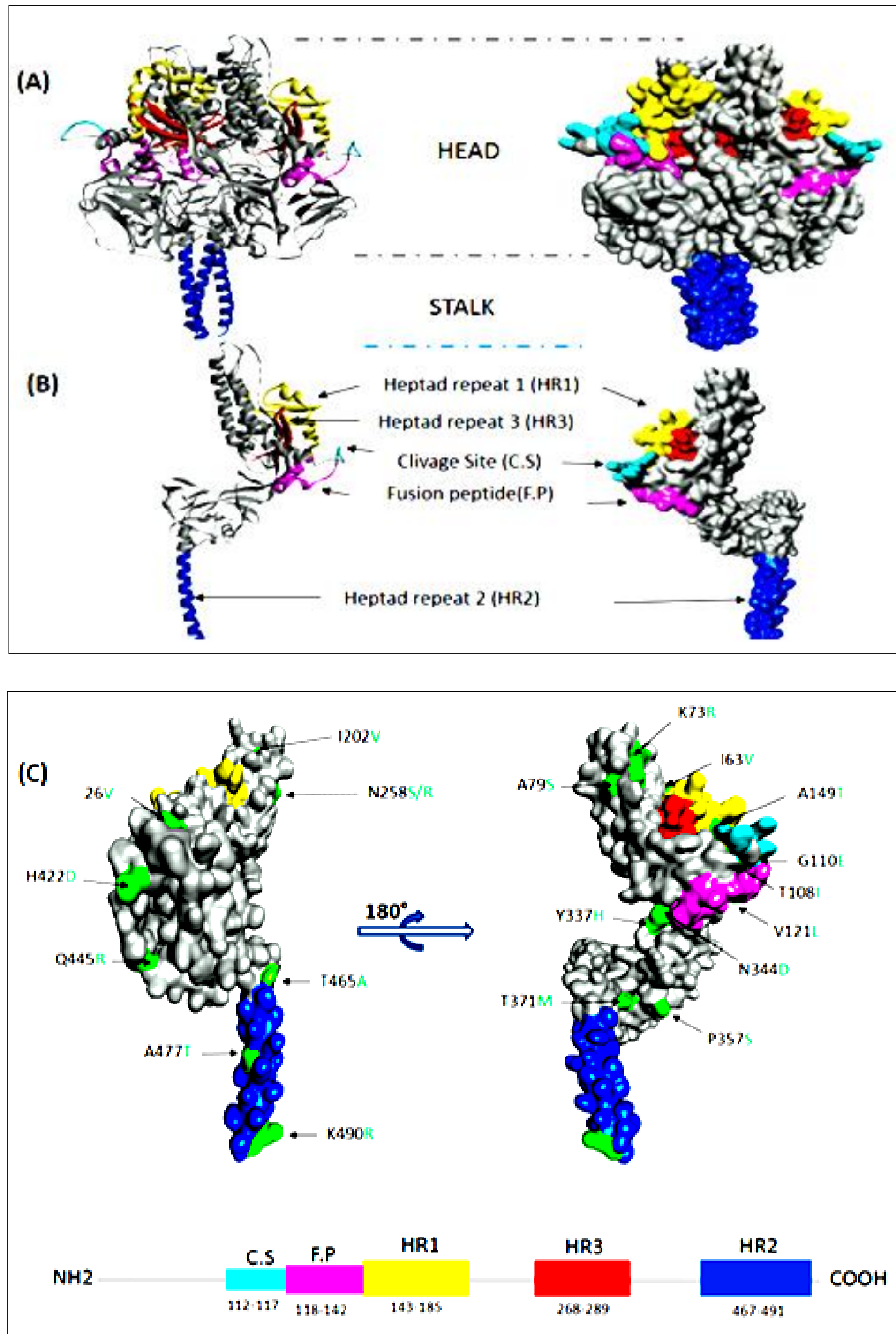

Figure 2 3D model of the F protein and mutation locations: The 3D structure was generated in SWISS-Model and visualized in D.S v2017. The pre-fusion structure of Parainfluenza Virus-5 protein F (pdb ID= *2b9b*) was chosen as a homologous model. (A) Representation of trimeric structure (ribbon and soft solid): the structure includes a globular head and a spirally rolled stalk. (B) Representation of a single monomer (in ribbon and soft solid): the different domains are shown with different colors: C.S (light green), F.P (purple), HR1 (yellow), HR2 (blue), HR3 (red) and the rest of the regions are colored in gray. (C) Positions of mutations in the F protein: the mutated amino acid residues are colored in green

3.2.2. 3D structure of HN proteins and mutation positions

The HN proteins of MG isolates are homotetramer, containing a globular head and a spirally rolled stalk (Figure 3A). The peptide fragments, flanked by amino acids at positions 1-79 and 116-122, are absent in the model structure (pdb ID: *3t1e*) and were not modeled.

The 41 mutations in the HN proteins are generally localized in its globular head. However, two mutations are still found in the stalk (Figure 3B). Of the mutations in the head, four mutations (G303V, P315S, P391S, and T443A) are not exposed on the surface but reside inside the structure. The 15 mutations in the missing fragments of the structure were not presented here.

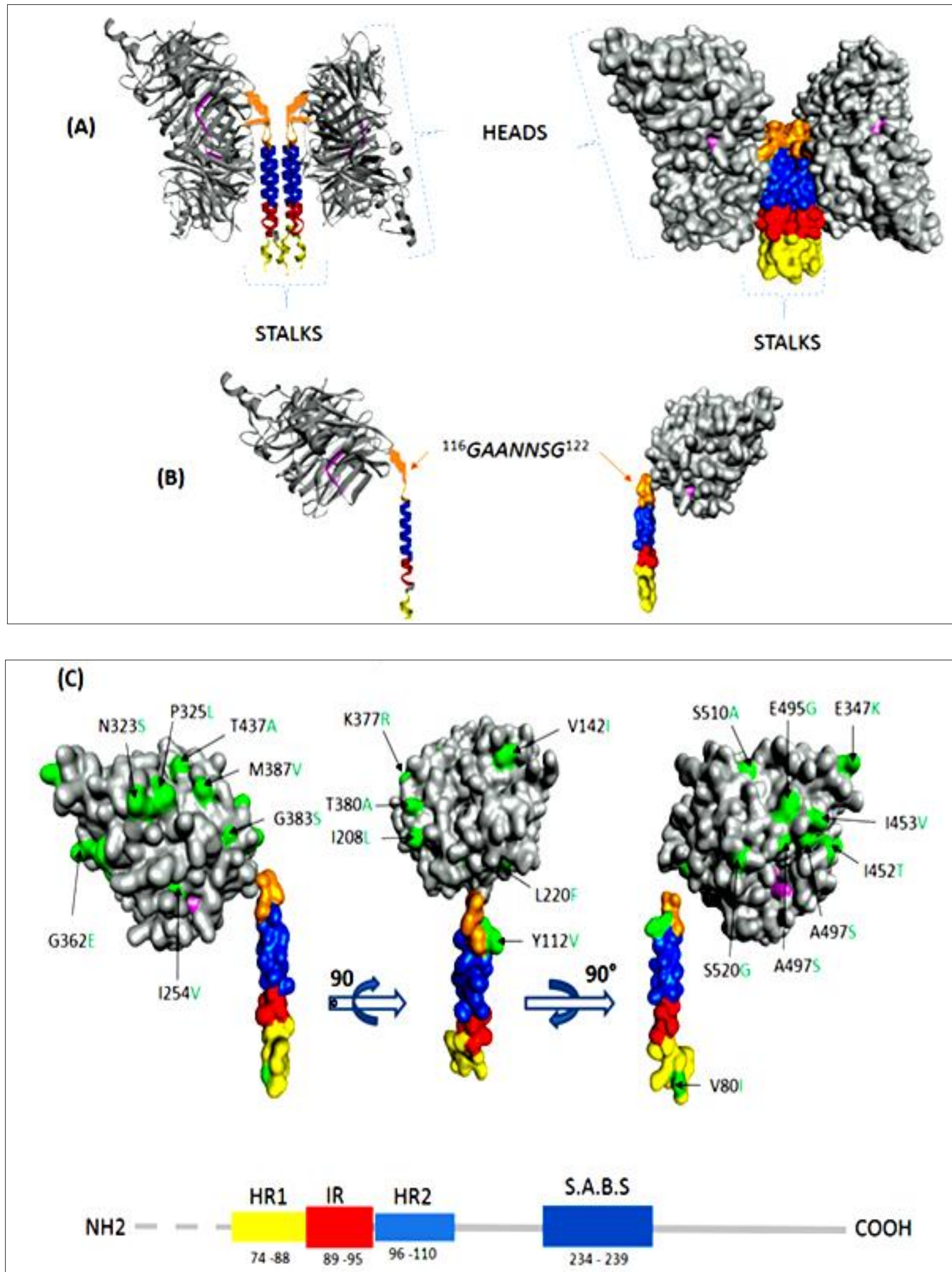

Figure 3 3D structure of the HN protein and position of mutations: (A) the structure is presented as a ribbon and soft solid. It consists of a globular head (amino acids at position 127-571) and a spirally wound stem (amino acids at position 49-126). (B) Representation of a ribbon and soft solid monomer. The missing fragment connecting the head and stalk is shown in yellow. Regions of the structure are shown with different colors. (C) Positions of mutations in the 3D structure of the HN protein: mutations in missing regions are not exposed.

3.3. Comparison of 3D structures of proteins

TM-Align was used to compare the 3D structures of F proteins and HN proteins of MG isolates. This server provided TM scores between 0.99963 and 0.99999 for F proteins and TM scores between 0.40613 and 0.99987 for HN proteins (Table 3). It also gave RMSD values between 0.02 to 0.15Å and between 0.015 to 0.30Å for F and HN proteins, respectively (Table 3). These results indicate that the global structures of the F and HN proteins of MG isolates are not similar. However, the variation is minimal because the RMSD values are less than 5Å.

Table 3 Values of TM and RMSD scores obtained after comparison of F and HN protein of MG isolates

Strains	F protein		HN protein	
	TM align score	RMSD (Å)	TM align score	RMSD score
MGF-MEOLA	0.99997	0.04	0.99975	0.10
MG-39	0.99997	0.05	0.40613	0.13
MGMNJ	0.99997	0.15	0.99987	0.09
MGF-725	0.99963	0.15	0.99987	0.09
MGF003C	0.99999	0.02	0.99975	0.12
MGF082T	0.99999	0.02	0.99987	0.09
MGF015C	0.,99999	0.02	0.99539	0.30
MGF120T	0.99999	0.02	0.99978	0.11
MGF166	0.99995	0.06	0.99987	0.09
MGF192C	0.99999	0.02	0.99987	0.09
MGS1130T	0.99999	0.02	0.99987	0.09

We also visualized the 3D structures of the F and HN proteins superimposed in D.S v2017. Thus, we found that 6 mutations in the F proteins and 15 mutations in the HN proteins resulted in a visible deviation on the superimposed 3D structures (Figure 4A and B). The changes caused by the other mutations are less visible and we considered them as "not significant".

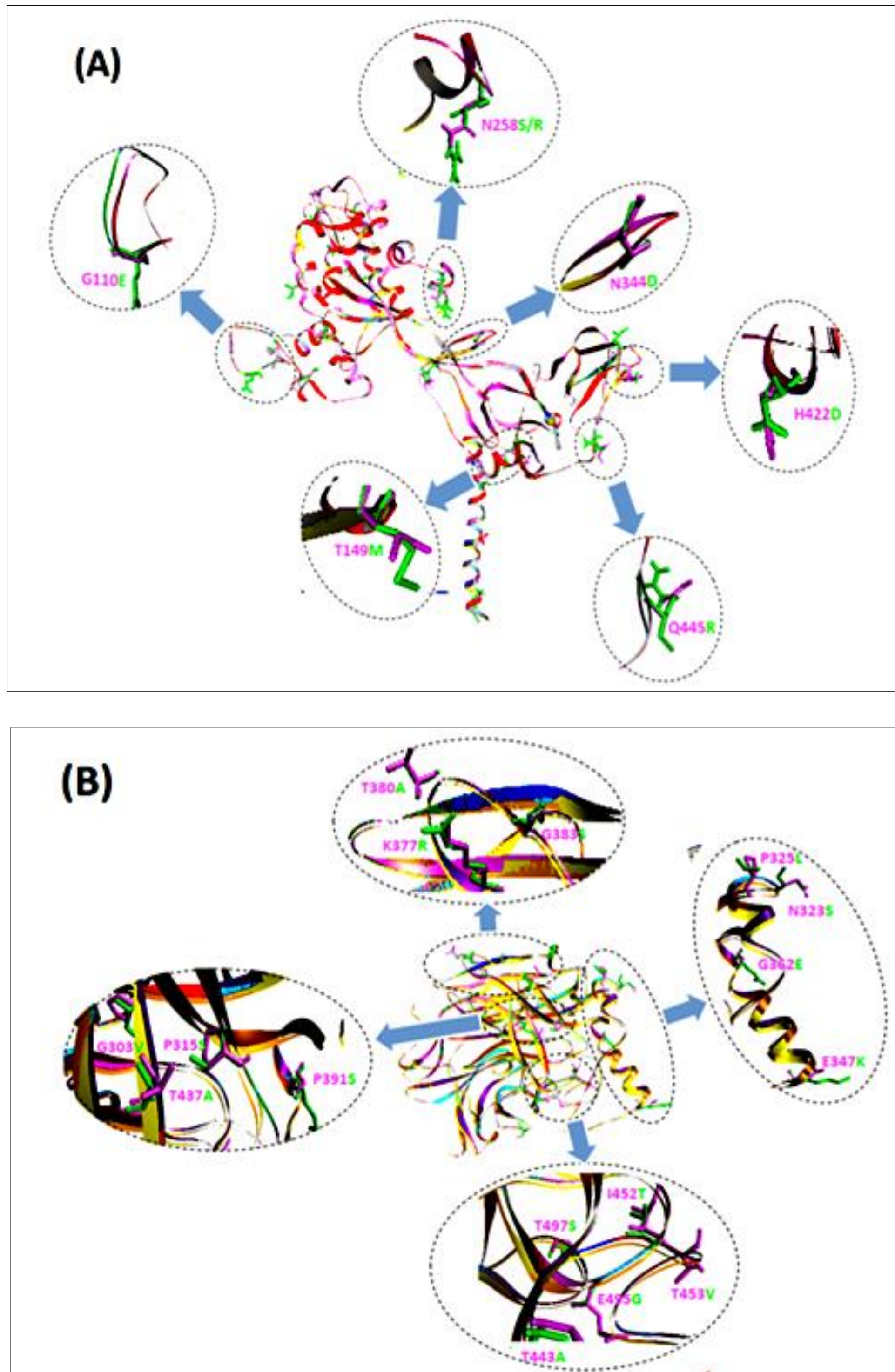

Figure 4 Visualization of superimposed 3D structures of F and HN proteins in D.S v2017. A single monomer was presented as a ribbon with different colors: MG-1992 (purple), MG-MEOLA (yellow), MGMNJ (blue), MG-725 (red), MGF082T (light green), MGF015T (beige), MGF092C (light blue), MGF120T (greenish yellow), MGS1130T (gray), MGF166 (brown), MGF003C (purple). MG-39 is excluded from the superposition because its structure is not complete. (A) Superposition of 3D structures of the F protein. (B) Superposition of 3D structures of the HN protein. Mutated residues of amino acids resulting in a change in the structure are represented by green sticks and the amino acids of the reference structure (MG-1992) are represented in purple sticks

3.4. Effects of mutations on protein stability, structures and functions

The effects of mutation on protein stability are analyzed using MUpro server. As results, the mutations K73R in F and G303V in HN obtained a MUpro score greater than 0, indicating that they caused an increase in the stability of the F and HN protein (Table 4). In contrast, the other mutations scored MUpro less than 0, indicating that they decreased the stability of these two proteins (Supplementary Table 1). These results indicate that all mutations have effects on the stability of the F and HN proteins.

The impacts of the mutation in the 3D structure of the proteins are evaluated with the Mutpred 2.0 server. The scores obtained indicated that the D344N mutation was "deleterious" in the structure of the F protein, whereas the N19Y, N120T, G303V, P315S, E347K, P391S, and E495G mutations are predicted to affect the structure of the HN protein (Table 4). The remaining mutations are classified as "neutral" and are not predicted to affect protein structures (Supplementary Table 1).

The PROVEAN server is used to predict the impacts of mutations on the biological function of proteins. Most of the mutations found in the F and HN proteins of MG isolates have a PROVEAN score greater than -2.5 (Supplementary Table 1). Therefore, they are considered "neutral" and thus would not influence the functions of these two proteins. However, the mutations A477T in the F protein and G303V, P315S in the HN protein have PROVEAN scores below -2.5 and are considered "deleterious" on the functions of these proteins (Table 4).

Table 4 Effects of mutations on the stability, structure and functions of F and HN proteins

<table>
<tr><th>Protein</th><th>Mutation</th><th>Mupro score
(threshold = 0)</th><th>Mutpred 2.0 score
(threshold = 0.5)</th><th>PROVEAN score
(threshold = -2.5)</th><th>Effects of mutations</th></tr>
<tr><td rowspan="7">F</td><td>K73R</td><td>0.147</td><td>0.189</td><td>-0.431</td><td>Alteration of stability</td></tr>
<tr><td rowspan="5">N344D</td><td rowspan="5">-0.691</td><td rowspan="5">0.536</td><td rowspan="5">0.112</td><td>Loss of strand and loop</td></tr>
<tr><td>Loss of disulfide bond at C347</td></tr>
<tr><td>Loss of ADPRibosylation in R349</td></tr>
<tr><td>Loss of Oglycosylation in T348</td></tr>
<tr><td></td></tr>
<tr><td>A477T</td><td>-0.732</td><td>0.295</td><td>-2.572</td><td>Alteration of the biological functions of the protein</td></tr>
<tr><td rowspan="11">HN</td><td rowspan="3">N19Y</td><td rowspan="3">-0.304</td><td rowspan="3">0.508</td><td rowspan="3">-2.087</td><td>Loss of the helix</td></tr>
<tr><td>Alteration of the transmembranaire protein</td></tr>
<tr><td>Gain of strand</td></tr>
<tr><td rowspan="2">N120T</td><td rowspan="2">-0.496</td><td rowspan="2">0.555</td><td rowspan="2">-0.785</td><td>Alteration of the transmembranaire protein</td></tr>
<tr><td>Gain of Nglycosilation site</td></tr>
<tr><td rowspan="5">G303V</td><td rowspan="5">0.052</td><td rowspan="5">0.921</td><td rowspan="5">-8.695</td><td>Alteration of the transmembranaire protein</td></tr>
<tr><td>Loss of B factor</td></tr>
<tr><td>Loss of catalytic site in G304</td></tr>
<tr><td>Gain of allosteric site in G303</td></tr>
<tr><td>Alteration of the biological functions of the protein</td></tr>
<tr><td>P315S</td><td>-0.912</td><td>0.654</td><td>-4.749</td><td>Alteration of the transmembranaire protein</td></tr>
</table>

					Loss of allosteric site in Y317
					Gain of catalytic site in Y317
					Gain of solvant accessibility
					Alteration of the biological functions of the protein
	E347K	-1.146	0.573	0.260	Loss of phosphorylation at Y350
					Loss of sulfatation atY350
					Alteration of the transmembranaire protein
					Gain of ubiquitylation at E347
	P391S	-1.063	0.520	0.418	Alteration of the transmembranaire protein
					Alteration of ordered interface
					Gain of strand
	E495G	-1.447	0.702	-1.313	Modification of ordered interface
					Modification of the metallic bond
					Gain of solvant accessibility, gain of loop, gain of catalytic site at R498

4. Discussion

The development of an effective broad-spectrum vaccine appears to be an unavoidable solution to control the continuous circulation of NDV and the annual explosion of ND in endemic areas. Currently, many researchers are working on the development of a "genotype-matched vaccine" using the two new vaccine platforms such as reverse genetics and immuno-informatics [6]. Despite these efforts, there is a gap in the selection of the target, as recurrent mutations in the targeted viral proteins are not considered in the design. However, the substitution of certain residues in a protein could affect its structure, function and also its antigenicity[22,23]. Therefore, the identification and analysis of mutations is essential to increase the accuracy of target protein selection in ND vaccine design. In this study, we examined mutations in the F and HN protein of twelve NDV strains isolated in Madagascar.

In all previous studies, analysis of mutations in NDV F and HN proteins was performed by *in vitro* and/or *in vivo* methods [24-27]. However, these techniques required sophisticated materials and consumed a lot of time to perform. For this reason, we used the bioinformatics or *in silico* method, which is not expensive but allows to obtain faster and exploitable results [28,29]. Some researchers have already used it for the characterization of mutations in structural proteins of the Severe Acute Respiratory Syndrome Coronavirus 2 or SARS Cov2 [30,31].

First, we identified by multiple sequence alignment 67 mutations including 26 mutations in F proteins and 41 mutations in HN proteins (Table 2; Supplementary Figure 1 and 2). None of these mutations affect the specifics residues of Malagasy strains (genotype XI), as reported by Maminiaina in 2011 [32]. Furthermore, none of them correspond to the characteristic mutations of the new genotypes such as XIV, XVII and XVIII [33-36]. Thus, the identification of these mutations raises an alarm about the existence of a progressive evolution in genotype XI originating from Madagascar.

16 of the 67 mutations identified were located in the functional regions (N-T, HR1, HR2, T.M and C-T) of the F and HN proteins, but they did not cause deleterious consequences on the structures and functions of these proteins (Figures 1A and 1B and Table 4). However, many studies have reported that mutations in these regions reduce the fusion or

neuraminidase activity of these proteins and alter virus replication and pathogenicity [24-27]. Therefore, additional experimental analysis is needed to confirm the accuracy of our predictive results.

The remaining 51 mutations are dispersed along the linear sequence of F and HN protein (Supplementary Figure 1 and 2) or are located in the head and stalk domains of the 3D structures of these proteins (Figures 2C and 3C). All of these mutations affect the stability of these two proteins (Supplementary Table 1). However, only 6 mutations (N344D G303V, P315S, E347K, P391S and E495S) altered their tertiary structures. In addition, only three mutations (A477T, G303V and P315S) affected their functions (Table 4). Through experimental studies, Cho and colleagues have previously reported that the mutation E347K is one of the potential markers to understand the antigenic variation of NDV [37]. In addition, Hu and colleagues also demonstrated that this mutation could affect the characteristics of the HN protein and would allow it to escape the recognition of epitopes by monoclonal antibodies [38]. On the other hand, Ruyan and colleagues also confirmed that the P315S mutation in the HN protein increases the thermostability of NDV [39]. So, both E347K and P315S mutations have already been characterized by other researchers. On the contrary, no studies have been performed on the other mutations such as N344D, G303V, P391S and E495S. Thus, our *in silico* results provided important information suggesting that they may alter structures, affect functions, and modify the stability of F and HN proteins in NDV. Nevertheless, further experimental studies are still needed to valorize these results.

Appendix

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                    10         20         30         40         50
          ....|....| ....|....| ....|....| ....|....| ....|....|
MG-92     MGSKSSTWIP ISPMPTILIA LALGCVRLTS SLDGRPLAAA GIVVTGDKAV
MEOLA     ...Q...... .F........ .......S.. .......... ..........
MG-39     ...Q...... .F........ .......S.. .......... ..........
MGFMNJ    ...Q...... .F........ .......S.. .......... ..........
MG-725    .......... .......... .......... .......... ..........
MGF003C   ...Q...... .F....V..V .......... .......... ..........
MGF082T   ...Q...... .F....V..V .......... .......... ..........
MGF015T   ...Q...... .F....V..V .......... .......... ..........
MGF120T   ...Q...... .F....V..V .......... .......... ..........
MGF166    ...Q...... .F....V..V .......... .......... ..........
MGF192C   ...Q...... .F....V..V .......... .......... ..........
MGS1130T  ...Q...... .F....V..V .......... .......... ..........

                    60         70         80         90        100
          ....|....| ....|....| ....|....| ....|....| ....|....|
MG-92     NIYTSSQTGS IIVKLLPNMP KDKEACAKAP VEAYNRTLTT LLTPLGDSIR
MEOLA     .......... ..I....... ..R.....S. .......... ..........
MG-39     .......... ..I....... ..R.....S. .......... ..........
MGFMNJ    .......... ..I....... ..R....... .......... ..........
MG-725    .......... .......... .......... .......... ..........
MGF003C   .......... .......... ..R....... .......... ..........
MGF082T   .......... .......... ..R....... .......... ..........
MGF015T   .......... .......... ..R....... .......... ..........
MGF120T   .......... .......... ..R....... .......... ..........
MGF166    .......... .......... ..R....... .......... ..........
MGF192C   .......... .......... ..R....... .......... ..........
MGS1130T  .......... .......... ..R....... .......... ..........
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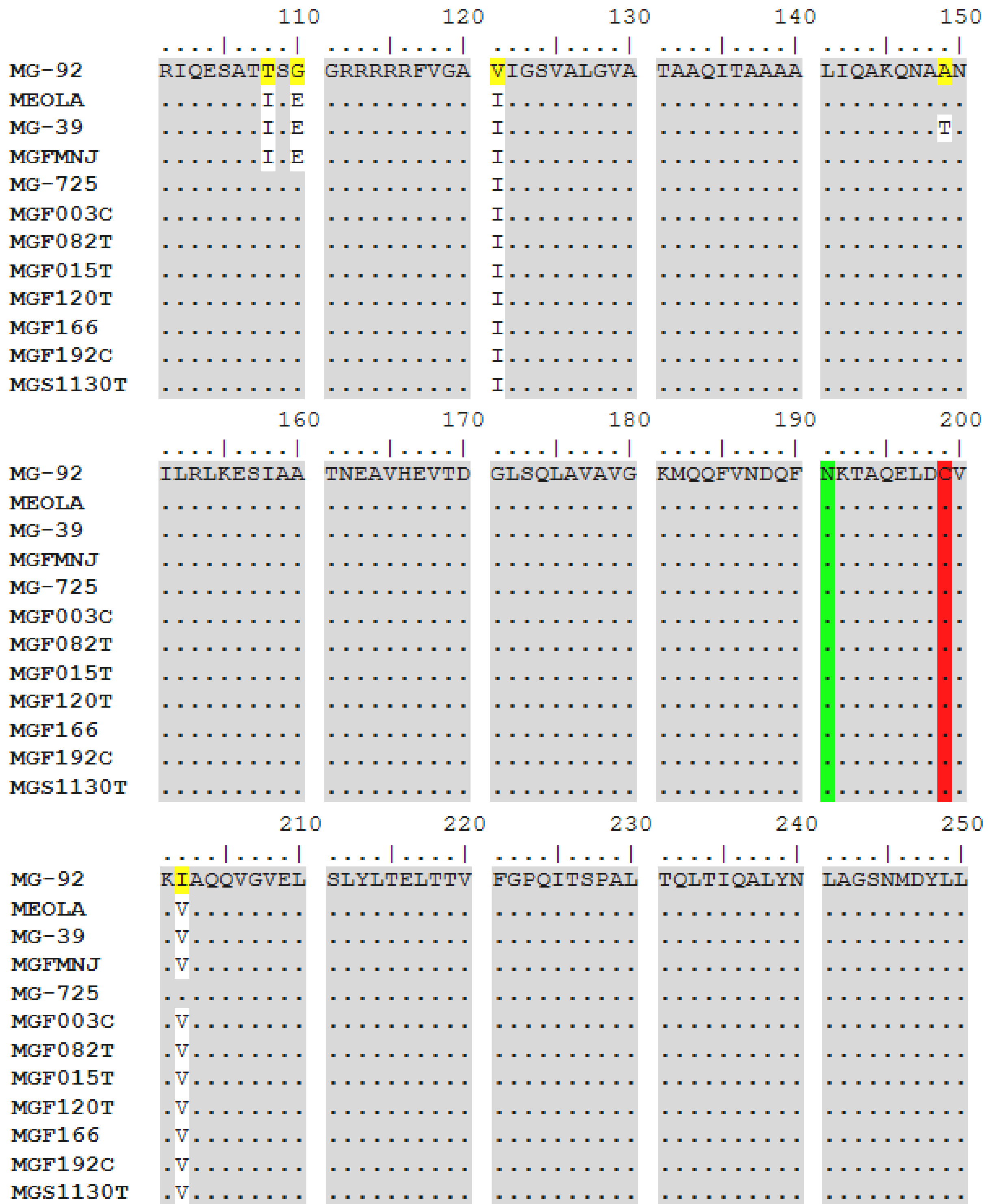

110 120 130 140 150
MG-92 RIQESATTSG GRRRRRFVGA VIGSVALGVA TAAQITAAAA LIQAKQNAAN
MEOLA ....I.E .... I......... ....
MG-39 ....I.E .... I......... ....T.
MGFMNJ ....I.E .... I......... ....
MG-725 .... I......... ....
MGF003C .... I......... ....
MGF082T .... I......... ....
MGF015T .... I......... ....
MGF120T .... I......... ....
MGF166 .... I......... ....
MGF192C .... I......... ....
MGS1130T .... I......... ....
160 170 180 190 200
MG-92 ILRLKESIAA TNEAVHEVTD GLSQLAVAVG KMQQFVNDQF NKTAQELDCV
MEOLA ....
MG-39 ....
MGFMNJ ....
MG-725 ....
MGF003C ....
MGF082T ....
MGF015T ....
MGF120T ....
MGF166 ....
MGF192C ....
MGS1130T ....
210 220 230 240 250
MG-92 KIAQQVGVEL SLYLTELTTV FGPQITSPAL TQLTIQALYN LAGSNMDYLL
MEOLA .V........ ....
MG-39 .V........ ....
MGFMNJ .V........ ....
MG-725 ....
MGF003C .V........ ....
MGF082T .V........ ....
MGF015T .V........ ....
MGF120T .V........ ....
MGF166 .V........ ....
MGF192C .V........ ....
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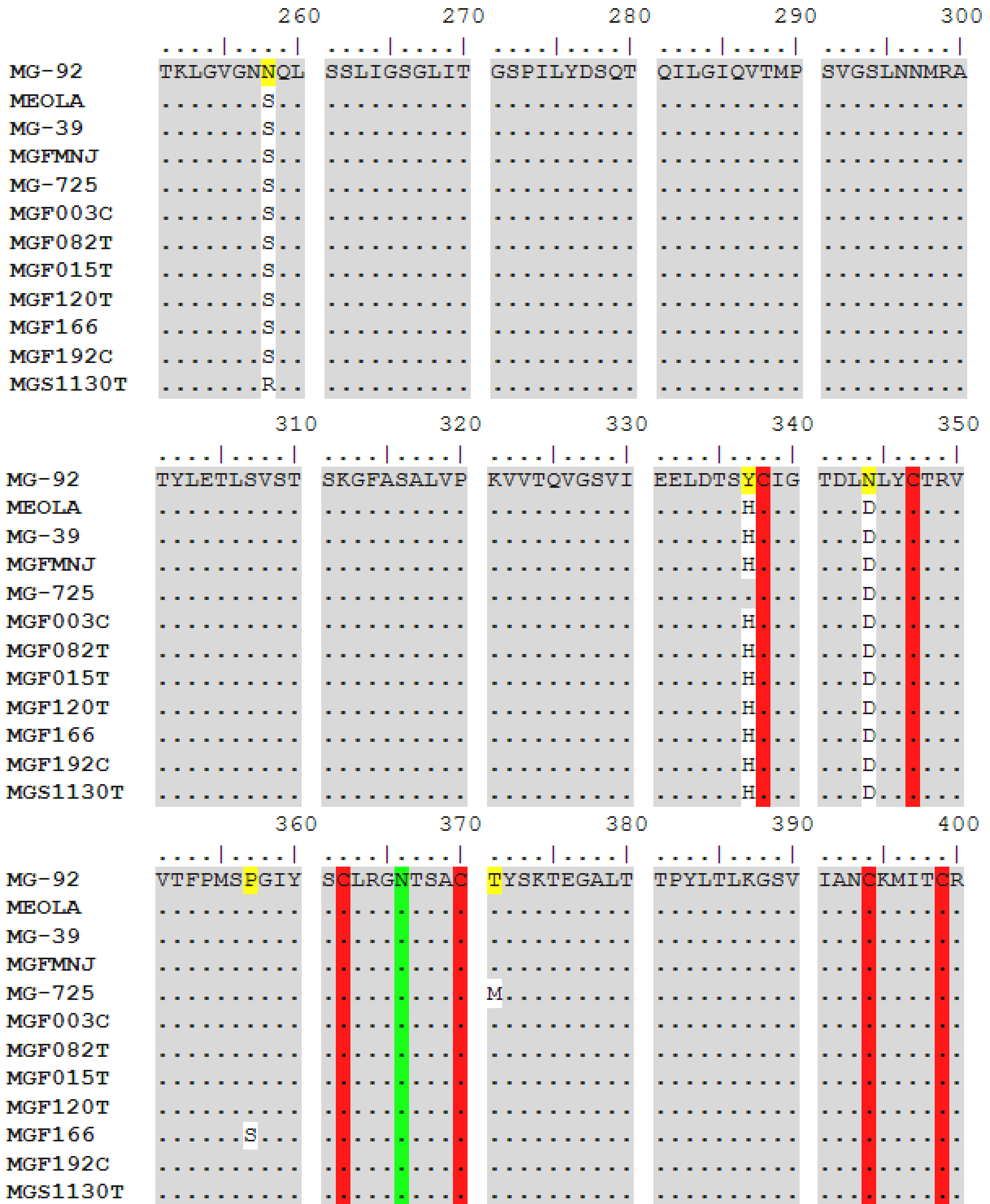
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MEOLA ....S.. ....
MG-39 ....S.. ....
MGFMNJ ....S.. ....
MG-725 ....S.. ....
MGF003C ....S.. ....
MGF082T ....S.. ....
MGF015T ....S.. ....
MGF120T ....S.. ....
MGF166 ....S.. ....
MGF192C ....S.. ....
MGS1130T ....R.. ....
310 320 330 340 350
MG-92 TYLETLSVST SKGFASALVP KVVTQVGSVI EELDTSYCIG TDLNLYCTRV
MEOLA ....H... ...D......
MG-39 ....H... ...D......
MGFMNJ ....H... ...D......
MG-725 ....D......
MGF003C ....H... ...D......
MGF082T ....H... ...D......
MGF015T ....H... ...D......
MGF120T ....H... ...D......
MGF166 ....H... ...D......
MGF192C ....H... ...D......
MGS1130T ....H... ...D......
360 370 380 390 400
MG-92 VTFPMSPGIY SCLRGNTSAC TYSKTEGALT TPYLTLKGSV IANCKMITCR
MEOLA ....
MG-39 ....
MGFMNJ ....
MG-725 .... M......... ....
MGF003C ....
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MGF192C ....
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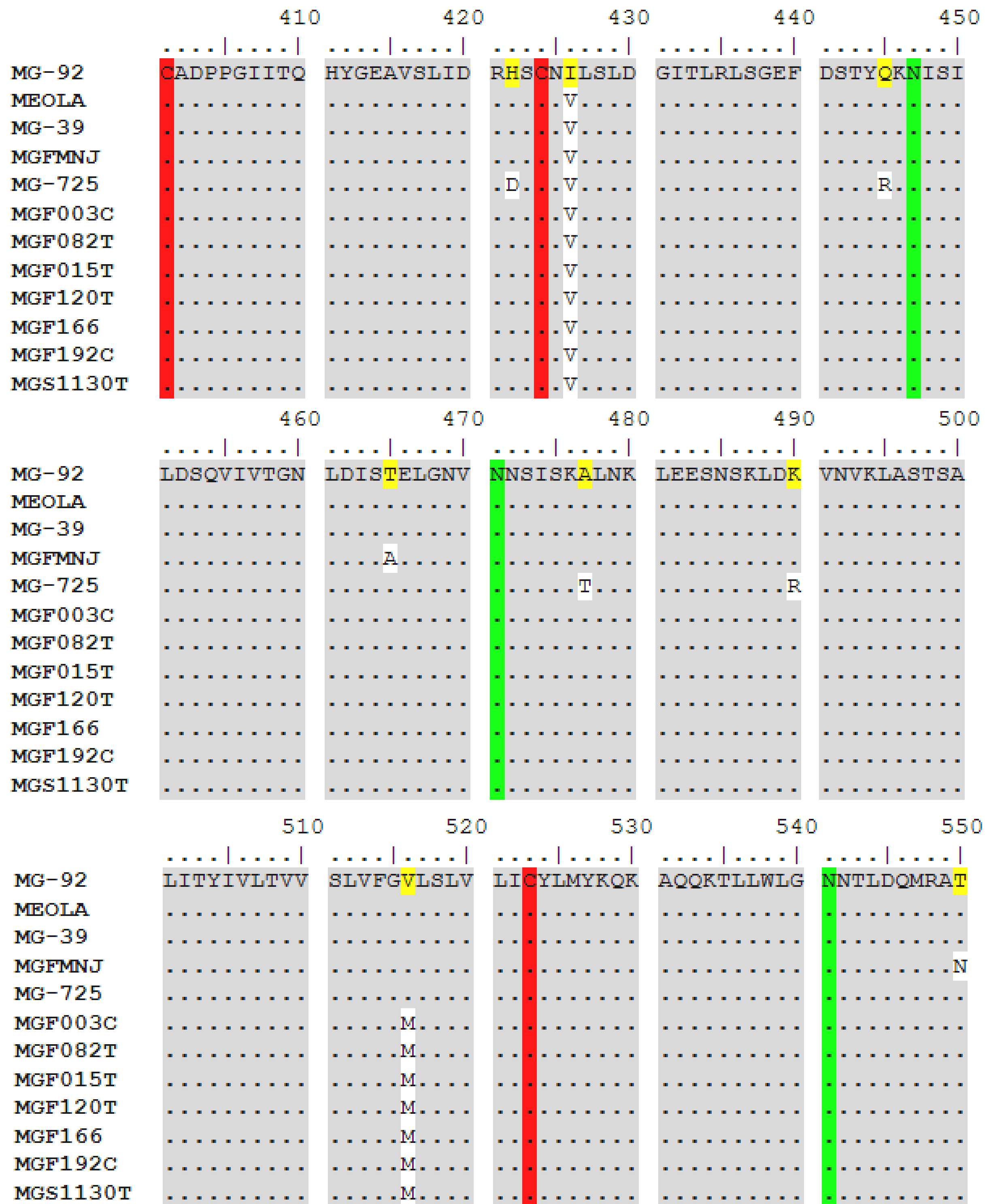
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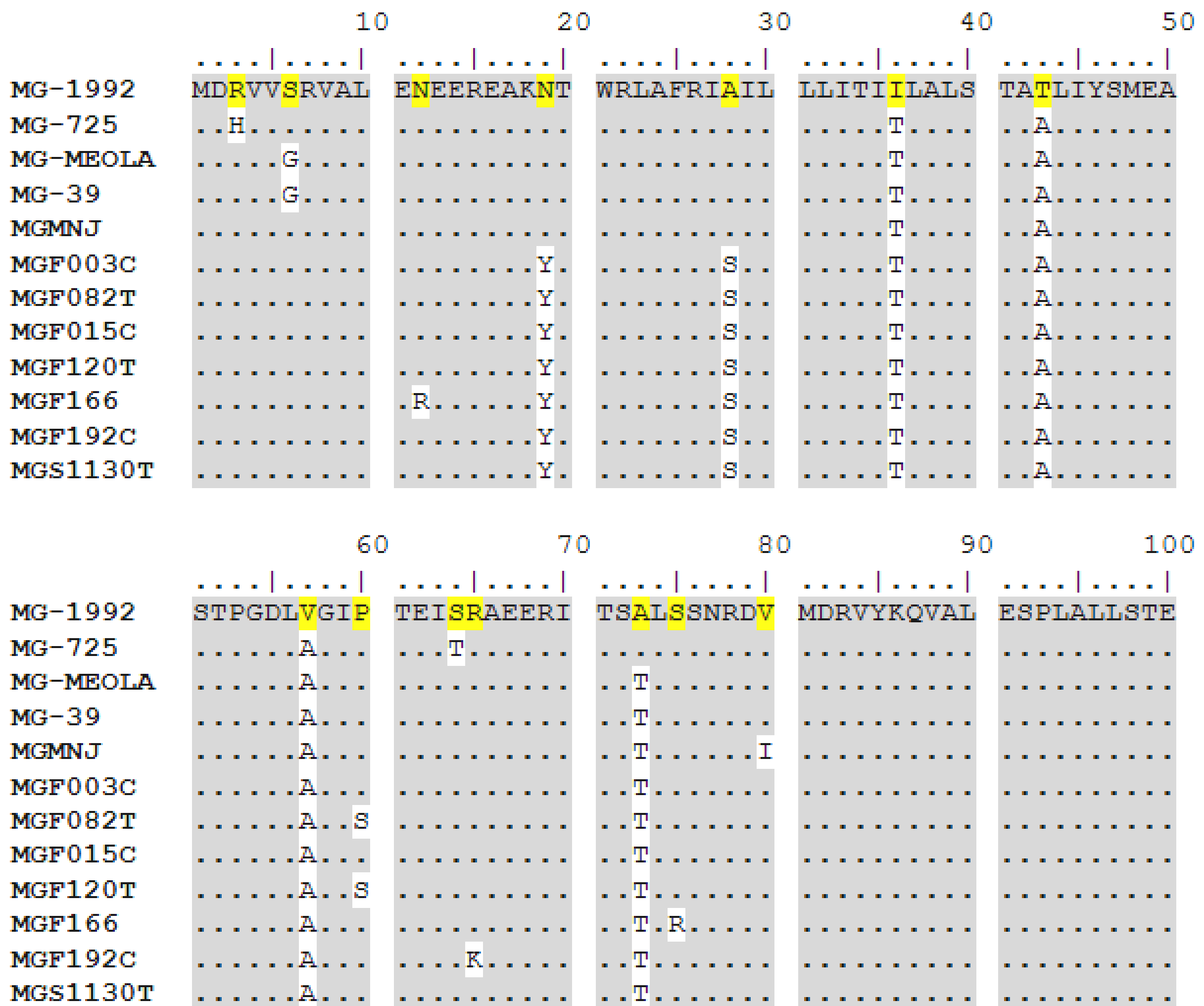
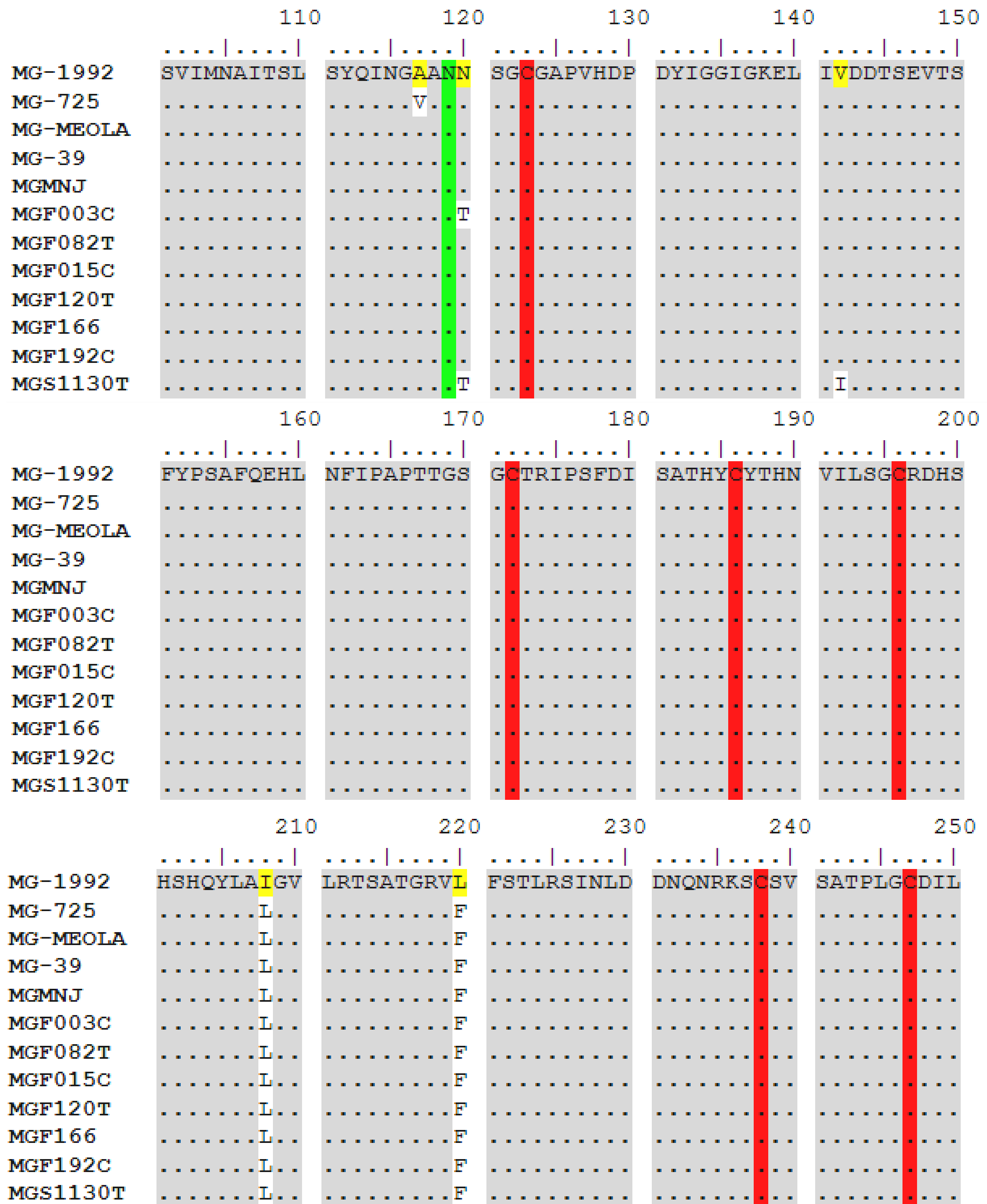
MEOLA ....V.... ....
MG-39 ....V.... ....
MGFMNJ ....V.... ....
MG-725 .... .D...V.... ....R.....
MGF003C ....V.... ....
MGF082T ....V.... ....
MGF015T ....V.... ....
MGF120T ....V.... ....
MGF166 ....V.... ....
MGF192C ....V.... ....
MGS1130T ....V.... ....
460 470 480 490 500
MG-92 LDSQVIVTGN LDISTELGNV NNSISKALNK LEESNSKLDK VNVKLASTSA
MEOLA ....
MG-39 ....
MGFMNJ ....A..... ....
MG-725 ....T... ....R ....
MGF003C ....
MGF082T ....
MGF015T ....
MGF120T ....
MGF166 ....
MGF192C ....
MGS1130T ....
510 520 530 540 550
MG-92 LITYIVLTVV SLVFGVLSLV LICYLMYKQK AQQKTLLWLG NNTLDQMRAT
MEOLA ....
MG-39 ....
MGFMNJ ....N
MG-725 ....
MGF003C ....M.... ....
MGF082T ....M.... ....
MGF015T ....M.... ....
MGF120T ....M.... ....
MGF166 ....M.... ....
MGF192C ....M.... ....
MGS1130T ....M.... ....

```
           ...
MG-92      TRI
MEOLA      ...
MG-39      ...
MGFMNJ     ...
MG-725     ...
MGF003C    ...
MGF082T    ...
MGF015T    ...
MGF120T    ...
MGF166     ...
MGF192C    ...
MGS1130T   ...
```

Supplementary Figure 1 Alignment of F protein in MG isolate: The conserved amino acid residues are presented as dots highlighted in gray. The mutated residues are presented as a single character and highlighted in yellow (in the reference sequence) as well as in white (in the other sequences). The conserved cysteine (C) and asparagine (N) residues are highlighted in red and green, respectively





110 120 130 140 150
MG-1992 SVIMNAITSL SYQINGAANN SGCGAPVHDP DYIGGIGKEL IVDDTSEVTS
MG-725
MG-MEOLA
MG-39
MGMNJ
MGF003C
MGF082T
MGF015C
MGF120T
MGF166
MGF192C
MGS1130T
V
T
T
I
160 170 180 190 200
MG-1992 FYPSAFQEHL NFIPAPTTGS GCTRIPSFDI SATHYCYTHN VILSGCRDHS
210 220 230 240 250
MG-1992 HSHQYLAIGV LRTSATGRVL FSTLRSINLD DNQNRKSCSV SATPLGCDIL
L
F

```
                  260        270        280        290        300
           ....|....| ....|....| ....|....| ....|....| ....|....|
MG-1992    CSKITETEEE DYRSVTPTSM VHGRLGFDGQ YHEKDLDVIT LFKDWVANYP
MG-725     ...V...... .......... .......... .......... ..........
MG-MEOLA   ...V...... .......... .......... .......... ..........
MG-39      ...V...... .......... .......... .......... ..........
MGMNJ      ...V...... .......... .......... .......... ..........
MGF003C    ...V...... .......... .......... .......... ..........
MGF082T    ...V...... .......... .......... .......... ..........
MGF015C    ...V...... .......... .......... .......... ..........
MGF120T    ...V...... .......... .......... .......... ..........
MGF166     ...V...... .......... .......... .......... ..........
MGF192C    ...V...... .......... .......... .......... ..........
MGS1130T   ...V...... .......... .......... .......... ..........

                  310        320        330        340        350
           ....|....| ....|....| ....|....| ....|....| ....|....|
MG-1992    GVGGGSFIDG RVWFPVYGGL KPNSPSDIAQ EGRYVIYKRY NDTCPDEQDY
MG-725     .......... .......... .......... .......... ..........
MG-MEOLA   .......... ....S..... .......... .......... ..........
MG-39      ..V.------ ---------- ---------- ---------- ----------
MGMNJ      .......... ....S..... .......... .......... ..........
MGF003C    .......... ....S..... .......... .......... ..........
MGF082T    .......... ....S..... .......... .......... ..........
MGF015C    .......... ....S..... .......... .......... ..........
MGF120T    .......... ....S..... .......... .......... ......K...
MGF166     .......... ....S..... ..S....... .......... ..........
MGF192C    .......... ....S..... .......... .......... ..........
MGS1130T   .......... ....S..... ....L..... .......... ..........

                  360        370        380        390        400
           ....|....| ....|....| ....|....| ....|....| ....|....|
MG-1992    QIRMAKSSYK PGRFGGKRVQ QAILSIKVST SLGEDPMLTV PPNTVTLMGA
MG-725     .......... .......... ......R..A ......V... ..........
MG-MEOLA   .......... .E........ .......... ......V... ..........
MG-39      ---------- ---------- ---------- ---------- ----------
MGMNJ      .......... .E........ .......... ......V... ..........
MGF003C    .......... .E........ .......... ..S...V... ..........
MGF082T    .......... .E........ .......... ..S...V... ..........
MGF015C    .......... .E........ .......... ..S...V... ..........
MGF120T    .......... .E........ .......... ..S...V... ..........
MGF166     .......... .E........ .......... ..S...V... ..........
MGF192C    .......... .E........ .......... ..S...V... S.........
MGS1130T   .......... .E........ .......... ..S...V... ..........
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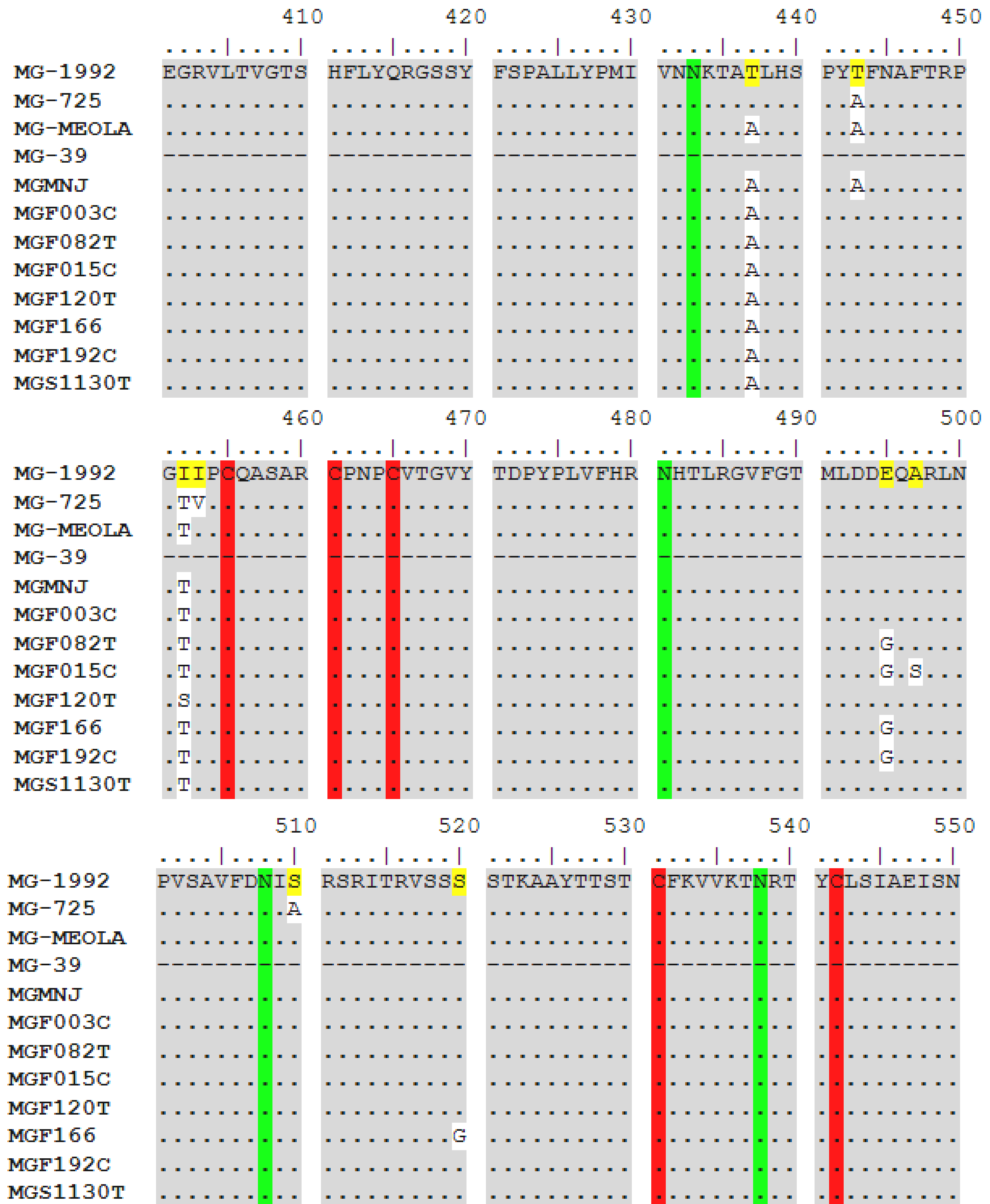
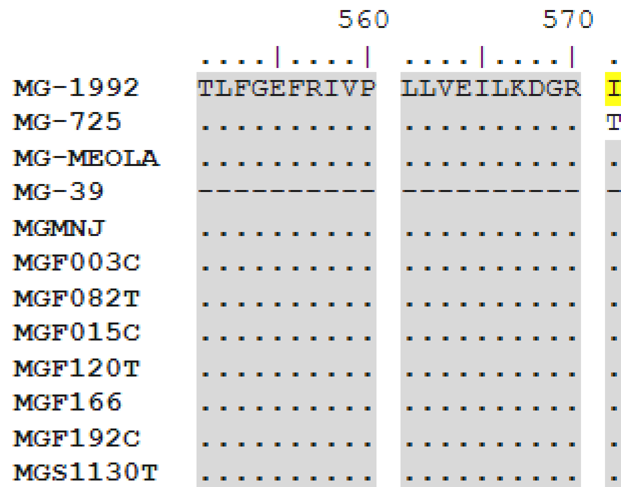

410 420 430 440 450
MG-1992 EGRVLTVGTS HFLYQRGSSY FSPALLYPMI VNNKTATLHS PYTFNAFTRP
MG-725
MG-MEOLA
MG-39
MGMNJ
MGF003C
MGF082T
MGF015C
MGF120T
MGF166
MGF192C
MGS1130T
460 470 480 490 500
MG-1992 GIIPCQASAR CPNPCVTGVY TDPYPLVFHR NHTLRGVFGT MLDDEQARLN
510 520 530 540 550
MG-1992 PVSAVFDNIS RSRITRVSSS STKAAYTTST CFKVVKTNRT YCLSIAEISN



Supplementary figure 2 Alignment of HN protein in MG isolate: The conserved amino acid residues are presented as dots highlighted in gray. The mutated residues are presented as a single character and highlighted in yellow (in the reference sequence) as well as in white (in the other sequences). The conserved cysteine (C) and asparagine (N) residues are highlighted in red and green, respectively.

Supplementary Table 5 Effects of mutations on the stability, structure and functions of F and HN proteins

Protein	Mutation	Mupro score (threshold = 0)	Interpretation	PROVEAN score (threshold = -2.5	Interpretation	Mutpred 2.0 score (threshold = 0.5)	Interpretation
F	K4R	-0.065	Decrease	-0.004	Neutral	0.079	Neutral
	K4Q	-0.064	Decrease	-0.272	Neutral	0.139	Neutral
	S12F	-0.510	Decrease	-0.696	Neutral	0.089	Neutral
	I17V	-0.798	Decrease	-0.125	Neutral	0.059	Neutral
	A20V	-0.535	Decrease	1.187	Neutral	0.131	Neutral
	L28S	-2.316	Decrease	0.497	Neutral	0.253	Neutral
	V63I	-0.574	Decrease	-0.047	Neutral	0.219	Neutral
	K73R	0.147	Increase	-0.431	Neutral	0.189	Neutral
	A79S	-1.005	Decrease	0.553	Neutral	0.203	Neutral
	T108I	-0.121	Decrease	-1.103	Neutral	0.286	Neutral
	G110E	-0.365	Decrease	0.713	Neutral	0.311	Neutral
	V121I	-0.819	Decrease	0.301	Neutral	0.177	Neutral
	A149T	-1.532	Decrease	-0.911	Neutral	0.309	Neutral
	I202V	-0.504	Decrease	-0.129	Neutral	0.125	Neutral
	N258S	-0.841	Decrease	0.185	Neutral	0.126	Neutral
	N258R	-0.838	Decrease	-0.991	Neutral	0.320	Neutral
	Y337H	-1.214	Decrease	1.326	Neutral	0.440	Neutral
	N344D	-0.691	Decrease	0.112	Neutral	0.536	Deleterious
	P357S	-0.741	Decrease	-1.503	Neutral	0.360	Neutral

	T371M	-0.629	Decrease	0.245	Neutral	0.369	Neutral
	H422D	-1.057	Decrease	1.794	Neutral	0.515	Deleterious
	I426V	-0.815	Decrease	-0.437	Neutral	0.071	Neutral
	Q445R	-1.043	Decrease	-0.280	Neutral	0.377	Neutral
	T465A	-0.856	Decrease	-1.048	Neutral	0.246	Neutral
	A477T	-0.732	Decrease	-2.572	Deleterious	0.295	Neutral
	K490R	-0.277	Decrease	-0.339	Neutral	0.142	Neutral
	V516M	-0.770	Decrease	-0.918	Neutral	0.158	Neutral
	T550N	-1.081	Decrease	-0.625	Neutral	0.359	Neutral
HN	R3H	-1.362	Decrease	0.002	Neutral	0.114	Neutral
	S6G	-1.402	Decrease	-1.410	Neutral	0.335	Neutral
	N12R	-0.270	Decrease	-0.708	Neutral	0.409	Neutral
	N19Y	-0.304	Decrease	-2.087	Neutral	0.508	Deleterious
	A28S	-0.807	Decrease	-0.649	Neutral	0.213	Neutral
	I36T	-1.693	Decrease	-1.256	Neutral	0.263	Neutral
	T43A	-1.016	Decrease	0.459	Neutral	0.219	Neutral
	V57A	-1.490	Decrease	0.548	Neutral	0.154	Neutral
	P60S	-0.678	Decrease	0.832	Neutral	0.153	Neutral
	S64T	-0.929	Decrease	-0.216	Neutral	0.159	Neutral
	R65K	-1.004	Decrease	0.371	Neutral	0.126	Neutral
	A73T	-0.899	Decrease	0.691	Neutral	0.135	Neutral
	S75R	-0.614	Decrease	-0.206	Neutral	0.350	Neutral
	V80I	-0.413	Decrease	-0.166	Neutral	0.169	Neutral
	Y112V	-0.722	Decrease	-0.419	Neutral	0.457	Neutral
	A117V	-0.760	Decrease	0.670	Neutral	0.194	Neutral
	N120T	-0.496	Decrease	-0.785	Neutral	0.555	Deleterious
	V142I	-0.856	Decrease	-0.236	Neutral	0.133	Neutral
	I208L	-0.885	Decrease	0.386	Neutral	0.255	Neutral
	L220F	-1.000	Decrease	0.700	Neutral	0.161	Neutral
	I254V	-0.851	Decrease	0.722	Neutral	0.090	Neutral
	G303V	0.052	Increase	-8.695	Deleterious	0.921	Deleterious
	P315S	-0.912	Decrease	-4.749	Deleterious	0.654	Deleterious
	N323S	-0.988	Decrease	-1.218	Neutral	0.453	Neutral
	P325L	-0.692	Decrease	-1.248	Neutral	0.453	Neutral
	E347K	-1.146	Decrease	0.260	Neutral	0.573	Deleterious
	G362E	-0.369	Decrease	-0.386	Neutral	0.325	Neutral
	K377R	-0.808	Decrease	-0.468	Neutral	0.227	Neutral
	T380A	-0.829	Decrease	-1.065	Neutral	0.232	Neutral

	G383S	-0.939	Decrease	0.032	Neutral	0.232	Neutral
	M387V	-0.952	Decrease	0.322	Neutral	0.083	Neutral
	P391S	-1.063	Decrease	0.418	Neutral	0.520	Deleterious
	T437A	-0.893	Decrease	-1.119	Neutral	0.341	Neutral
	T443A	-1.213	Decrease	-1.049	Neutral	0.228	Neutral
	I452T	-1.684	Decrease	1.796	Neutral	0.192	Neutral
	I453V	-0.420	Decrease	0.569	Neutral	0.080	Neutral
	E495G	-1.447	Decrease	-1.313	Neutral	0.702	Deleterious
	A497S	-0.628	Decrease	0.588	Neutral	0.301	Neutral
	S510A	-1.348	Decrease	-1.340	Neutral	0.231	Neutral
	S520G	-1.431	Decrease	-2.189	Neutral	0.393	Neutral
	I571T	-2.320	Decrease	0.007	Neutral	0.419	Neutral

5. Conclusion

In conclusion, the application of bioinformatics methods permitted to identify 26 and 41 mutations in the F and HN proteins of MG isolates. All of these mutations affect the stability of these proteins. However, only six mutations (D344N, G303V, P315S, E347K, P391S and E495S) affect the protein 3D structure and only three mutations (A477T, G303V and P315S) have an impact on their functions. These results provide evidence that mutations can impact the structures, stability and functions of F and HN proteins in NDV. However, it is not yet known whether these mutations will also impact the antigenicity and immunogenicity of these two viral proteins.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that they have no competing interests.

Authors' contributions

T. R. performed all molecular work and wrote the manuscript. O.F.M. and A.A. supervised the realization of this work. All authors have read and approved the final manuscript.

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