Экспериментальные исследования | **The Experimental Researches**

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MOLECULAR AND BIOLOGICAL CHARACTERIZATION OF LOW-PATHOGENIC H9N2 INFLUENZA VIRUSES ISOLATED IN ISRAEL

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All Israeli H9N2-strain viruses used in the present study were subdivided among three groups: groups A and B viruses contained – in all eight segments – 90658/00-like and 1304/03-like sequences, respectively; Group C comprised viruses isolated in 2006-2010, which contained four 1304/03-like segments and four 1525/06-like segments. Molecular analysis revealed that most of the isolates had an RSSR motif at the cleavage site of haemagglutinin. Most of the viruses had an haemagglutinin with L216 typical of humans, and internal proteins associated with the avian host specificity. The studied viruses infected the majority of the directly inoculated birds.

Exposure to the virus with full-length PB1-F2 protein for 24 h caused destruction of mitochondria and cell death in cultures of human macrophages, and this effect was not associated with activation of protein p53. A virus with truncated PB1-F2 protein exhibited no destructive effect on mitochondria, but induced enhanced production of pro-apoptotic P53 protein.

Keywords: *H9N2 virus; phylogenetic analysis; viral genes; virus replication; PB1-F2 protein; apoptosis*

Introduction

Avian influenza A viruses of subtype H9N2 were first isolated and studied in the United States in 1966 [1]. Since then, H9N2 viruses have been isolated from wild birds and poultry in various countries of the Old and New Worlds [2-5]. Outbreaks of H9N2-virus-associated disease have been reported in many European, Asian, African, and American countries [5], but these viruses were found to be endemic in only two regions: China and the Middle East [6-13]. At least two main lineages – A/Duck/Hong Kong/Y280/97-like and A/Quail/Hong Kong/G1/97 – were established amongst H9N2 viruses isolated from domestic poultry birds in Asia [14].

Similarly to the two highly pathogenic viruses, subtypes H5N1 and H7, low-pathogenic viruses can cross the interspecies barrier and be directly transmitted from birds to mammalian species, including pigs and humans [15-16]. In Israel, H9N2 virus was initially isolated in May 2000 and subsequently, after a 1.5-yr break, from December 2001 to mid-2003, this virus became the predominant avian influenza virus in the country [17]. In a previous study ([18] Golender *et al*., 2008) we characterized the virus that circulated in Israel until 2006; the main objective of the present study was to conduct phylogenetic and biological characterization of H9N2 avian influenza viruses isolated in Israel during 2000-2010.

Materials and methods

Viruses

The isolation and identification methods used were based on classical laboratory methodology ([18] Golender *et al*., 2008). Generally, samples were collected from suspect birds by taking

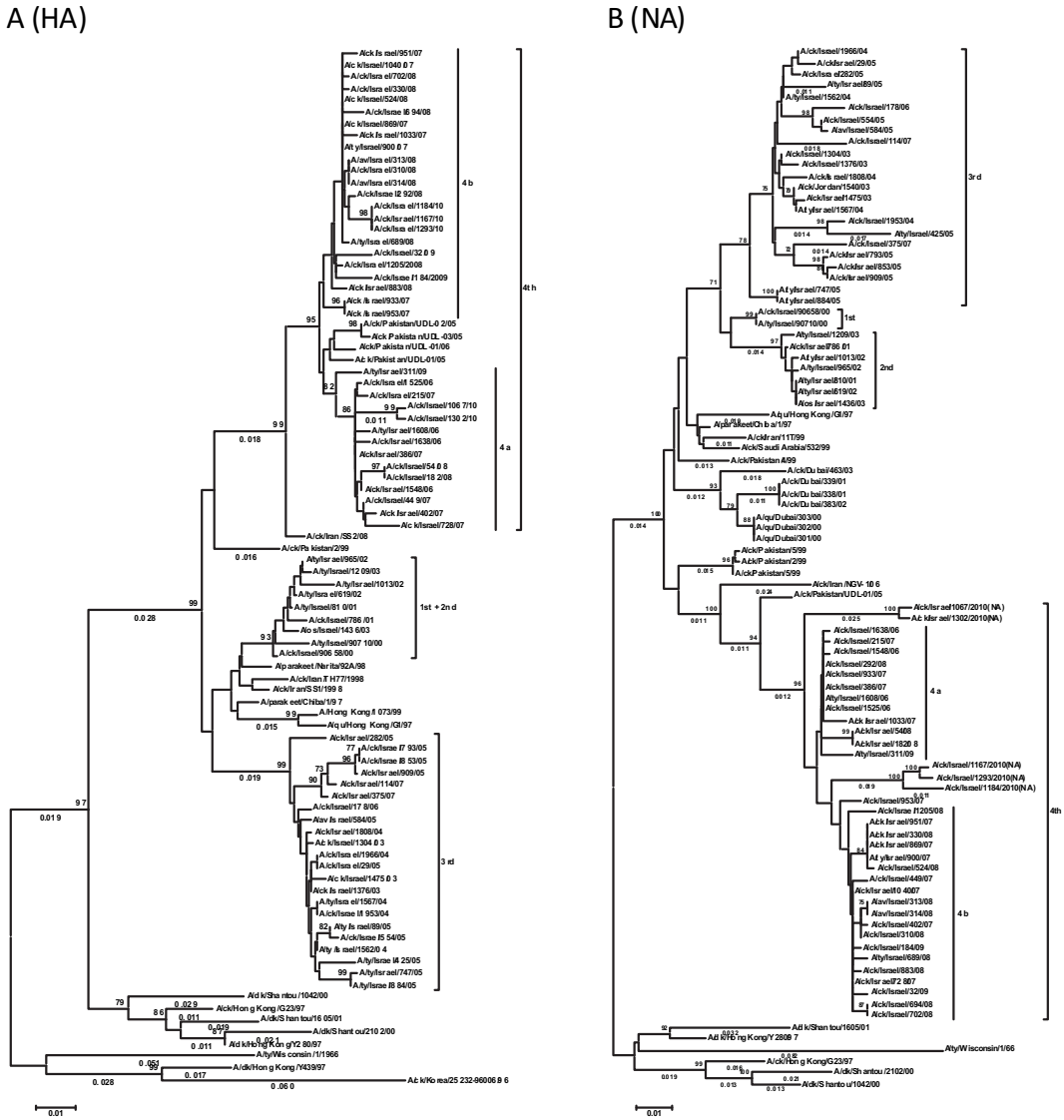

Fig. 1. Phylogenetic relationships of HA (A) and NA (B) genes from H9N2 influenza viruses isolated in Israel and from those from other countries. The phylogenetic trees based on nucleotide sequences were generated by neighbour-joining analysis with the Maximum Composite Likelihood model, using MEGA 5.0 software. Numbers below branches indicate neighbour-joining bootstrap values; numbers above branches indicate branch lengths.

smears from the trachea. The isolates were propagated in specific-pathogen-free embryonated chicken eggs for 10 to 11 days by the allantoic route. A haemagglutination inhibition test with monospecific polyclonal antisera obtained from Dr. Capua of the Office International des Epizooties [OIE] (Food and Agriculture Organization of the United Nations), and the National Reference Laboratory for Newcastle Disease and Avian Influenza, Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy, was used for serological identification of the avian influenza virus (AIV) isolates. Identification was confirmed by reverse transcription-polymerase chain reaction (RT PCR) with two sets of primers for HA and NA subtypes, respectively [18]. The studies used sixty-eight H9N2 viruses selected from the Kimron Veterinary Institute's collection.

Genetic and sequence analysis

The viral RNA was extracted directly from the allantoic fluid with the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA). Purified genomic RNA was used to generate cDNA clones by RT-PCR, according to the standard procedure. The

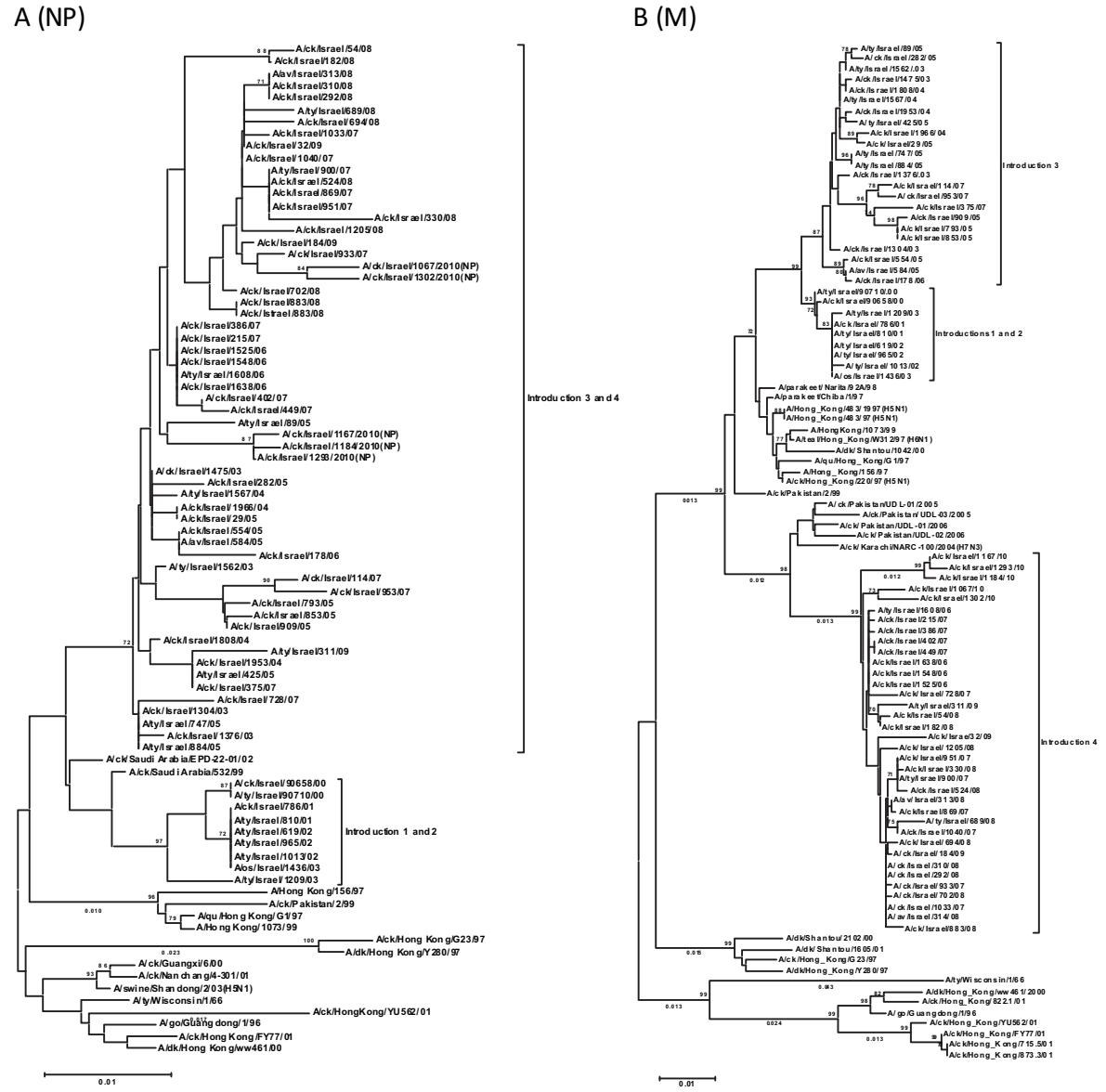

Fig. 2. Phylogenetic relationships between NP (A) and M (B) genes from H9N2 influenza viruses isolated in Israel and from those from other countries. The phylogenetic trees based on nucleotide sequences were generated by neighbour-joining analysis with the Maximum Composite Likelihood model, using MEGA 5.0 software. Numbers below branches indicate neighbour-joining bootstrap values; numbers above branches indicate branch lengths.

sets of primers that were used for the RT-PCR reaction and for the subsequent sequence analysis are available from the Avian Diseases Division of the Kimron Veterinary Institute. The PCR products were subjected to electrophoresis in agarose gel, and specific DNA was excised, purified with the MEGAquick-spin PCR and Agarose Gel Extraction System (iNtRON Biotechnology, Gyeonggi-do, Korea), and then sequenced at the Weizmann Institute of Science, Rehovot, Israel, by means of capillary electrophoresis with a Model 3700 DNA Analyzer (Perkin Elmer, Foster City, CA, USA).

Phylogenetic analysis

All sequence data were compiled and edited by using the SeqManII from the DNASTAR System that used the Illumina, ABI SOLiD, and Helicos Data subsystems (454 Life Sciences, Banford, CT, USA). Nucleotide and deduced amino acid sequences were aligned by using the BioEdit Package, version 7 [19] with ClustalW [20]. Phylogenetic trees were

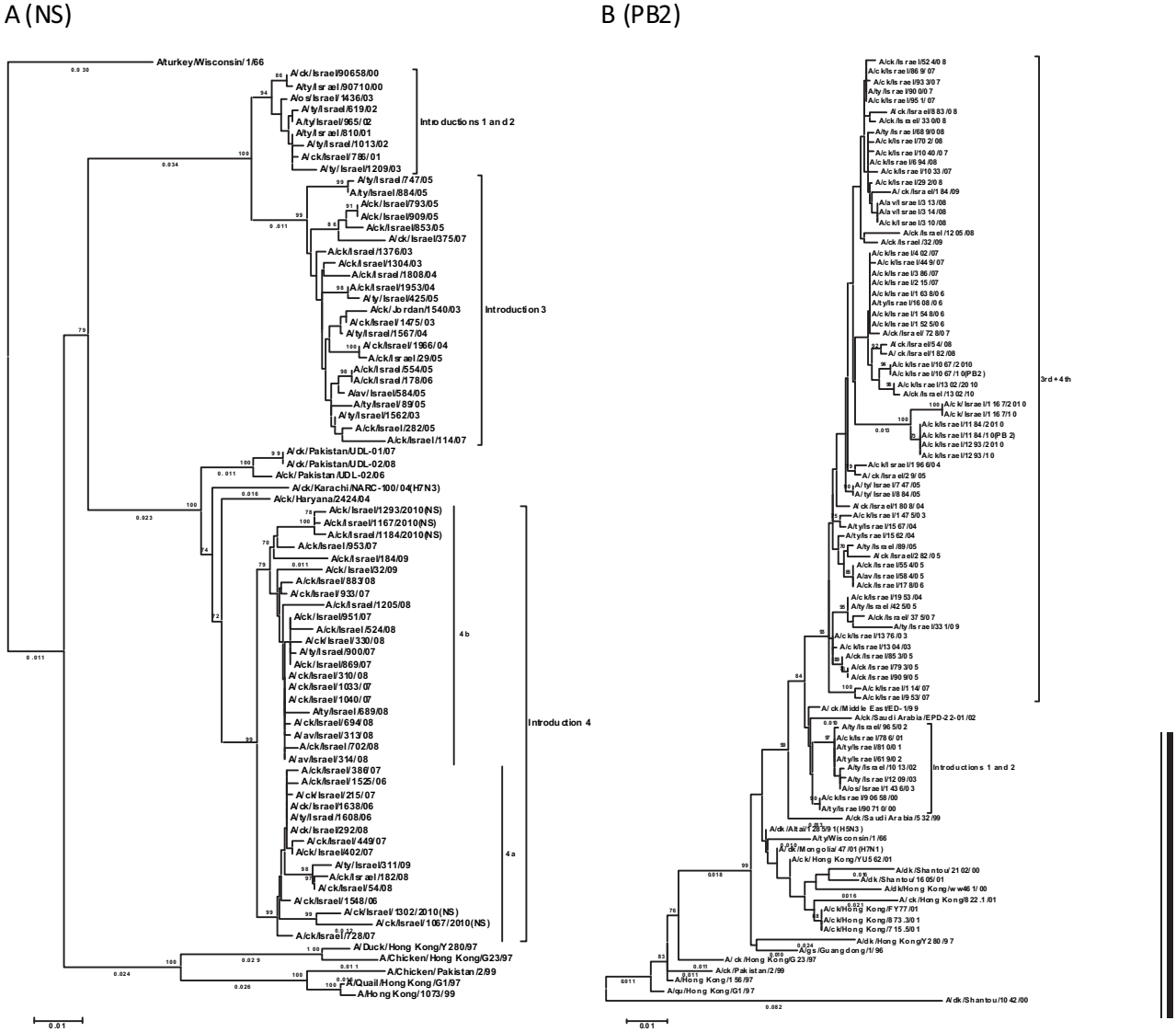

Fig. 3. Phylogenetic relationships between NS (A) and PB2 (B) genes from H9N2 influenza viruses isolated in Israel and from those from other countries. The phylogenetic trees based on nucleotide sequences were generated by neighbour-joining analysis with the Maximum Composite Likelihood model, using MEGA 5.0 software. Numbers below branches indicate neighbour-joining bootstrap values; numbers above branches indicate branch lengths.

generated by using the MEGA Software, version 5 [21]. The nucleotide regions used in phylogenetic analysis were of the following lengths: for the PB2 gene, 2277 nucleotides; for the PB1 gene, 2274 nucleotides; for the PA gene, 2151 nucleotides; for the HA gene, 1680 nucleotides; for the NP gene, 1494 nucleotides; for the NA gene, 1406 nucleotides; for the M gene, 977 nucleotides; and for the NS gene, 834 nucleotides. The nucleotide sequences used in this study are available from GenBank under accession numbers AY738451-AY738456, DQ683025-DQ683047, EF492221-EF492428, FJ464596-FJ464730, GQ120534-GQ140290, GQ148820-GQ148875, EF501983, JQ254936-JQ254991, JQ973661, and EU574919

Human macrophage culture

We studied THP-1 macrophages generated from the THP-1 human monocytic leukaemia cell line. To induce differentiation THP-1 was cultured for 48 h in the presence of phorbol 12-myristate 13-acetate (PMA) at 100 ng/ml.

Fig. 4. Phylogenetic relationships between PB1 (A) and PA (B) genes from H9N2 influenza viruses isolated in Israel and from those from other countries. The phylogenetic trees based on nucleotide sequences were generated by neighbour-joining analysis with the Maximum Composite Likelihood model, using MEGA 5.0 software. Numbers below branches indicate neighbour-joining bootstrap values; numbers above branches indicate branch lengths.

Human macrophage culture virus infection

The 24-h cultures of human THP-1 macrophage cells were infected with 10^4 EID_{50} (50 % egg-infecting dose) of selected H9N2 isolates and, 24 h post-infection, the cells were examined by fluorescent staining of the macrophage culture and apoptosis determination.

Fluorescent staining of macrophages culture

Forty-eight hours after plating, the cells in the culture medium were incubated for 40 min with MitoTracker Red 580

(Molecular Probes, Eugene, OR, USA) at 200 nmol/L, for mitochondrion staining. The dye was then removed. After rinsing with cold PBS (pH 7.4) the cells were fixed in absolute methanol for 10 min. After rinsing with cold PBS the cells were permeabilized with 0.5 % Triton X-100 for 10 min at room temperature. After blocking, Mab-421 (a kind gift from Prof. V. Rotter, of the Weizman Institute of Science, Rehovot, Israel), which recognizes a C-terminal epitope of the p53 protein [22] was added without dilution, and the mixture was incubated at room temperature for 2 h, and then with anti-mouse IgG-FITC (Sigma, St. Louis, MO, USA) at 1:128 dilution for 1 h. After removal of antibodies, the cells were rinsed with PBS and mounted with an aqueous mounting medium Fluorescence was immediately observed by means of an Axioskop 2 (Karl Zeiss, Germany]) or a Leica laser scanning confocal microscope (Leitz, Bensheim, Germany).

All control sections were processed in the absence of primary antibody. The slides were washed, mounted with an aqueous mounting medium, and photographed within a few hours under a digital microscope camera with image processing by the Image Pro Plus version 6 software. Light intensity and contrast were standardized for each culture with an appropriate control.

Apoptosis determination

The apoptotic index (AI) was determined by using the ApopTag Marker (Oncor, Inc., Gaithersburg, MD) and was calculated as the percentage of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) - positive cells per 1,000 cells, according to Gavrieli *et al*. [23].

Animal tests

Eight Israeli H9N2 viruses belonging to the 1st (one isolate), 2nd (one isolate), 3rd (three isolates), and 4th (three isolates) introductions were selected for the animal tests. One millilitre of allantoic fluid containing 10^6 Ч EOD_{50} of one isolate was inoculated into each of six 3-week-old White Leghorn chickens. The dose was divided between the intranasal (0.3 ml), intraocular (0.1 ml), and intratracheal (0.6 ml) routes. Four contact birds were introduced into the inoculated birds' cage 4 h post-inoculation (p.i.) to check transmission properties of the viruses. All the birds were observed daily for signs of disease or mortality. Tracheal and cloacal swabs were collected on days 3, 5, 7, and 10 p.i., and virus was titrated in 10-day-old embryonated chicken eggs.

Results

All the viruses examined in this study were isolated in Israel during 2000-2010. In the light of epidemiological and genetic analyses, the viruses isolated prior to mid-2006 were divided among three introductions. In brief: the first two H9N2 viruses were isolated in May 2000 and the appearance of these viruses in Israel was designated as the 1st introduction. Then, after devastation of affected flocks, no H9N2 viruses were found for 1.5 years. The 2nd introduction was recognized in December 2001, when the virus reappeared in Israel and affected both chicken and turkey flocks. Viruses belonging to the 3rd introduction were first isolated in March 2003 and until April 2006 this virus was totally predominant in Israel

Table 1.

General phylogenetic characterization of H9N2 viruses isolated in Israel in 2000-2010

Introduction of H9N2	Virus genes							
	HA	NA	NP	NS	M	PA	PB1	PB2
1st and 2nd introductions	90658/00-like	90658/00-like	90658/00-like	90658/00-like	90658/00-like	90658/00-like	90658/00-like	90658/00-like
3d introduction	1304/03-like	1304/03-like	1304/03-like	1304/03-like	1304/03-like	1304/03-like	1304/03-like	1304/03-like
4th introduction	1525/06-like	1525/06-like	1304/03-like	1525/06-like	1525/06-like	1304/03-like	1304/03-like	1304/03-like

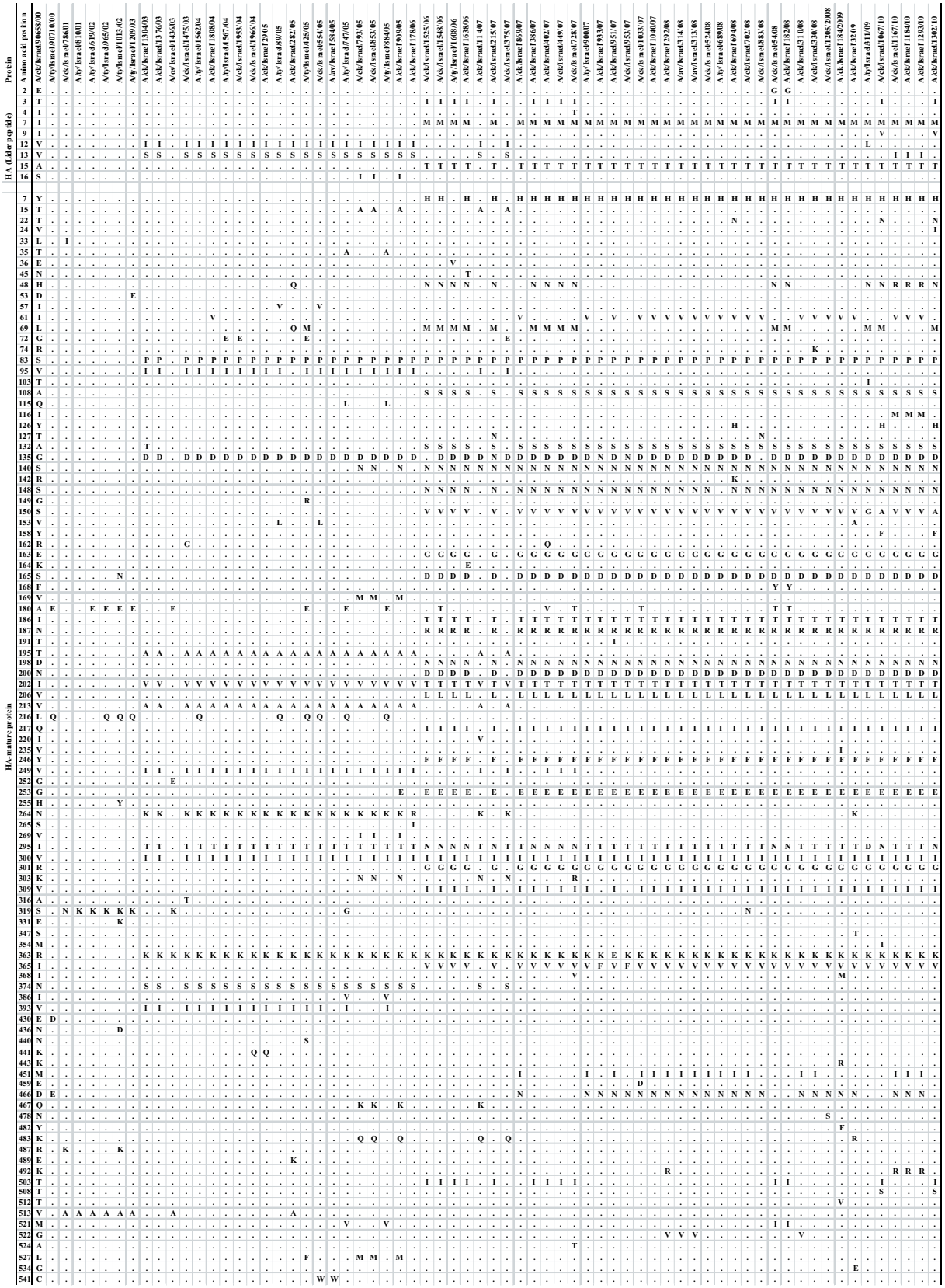

Fig. S1. Amino acid substitutions in HA proteins of H9N2 viruses isolated in Israel during 2000-2010.

Golender *et al*. [18]. The existence of a 4th introduction was suspected in November 2006, when the first virus that differed significantly from the previously isolated strains was found. Despite molecular differences, all the Israeli H9N2 viruses

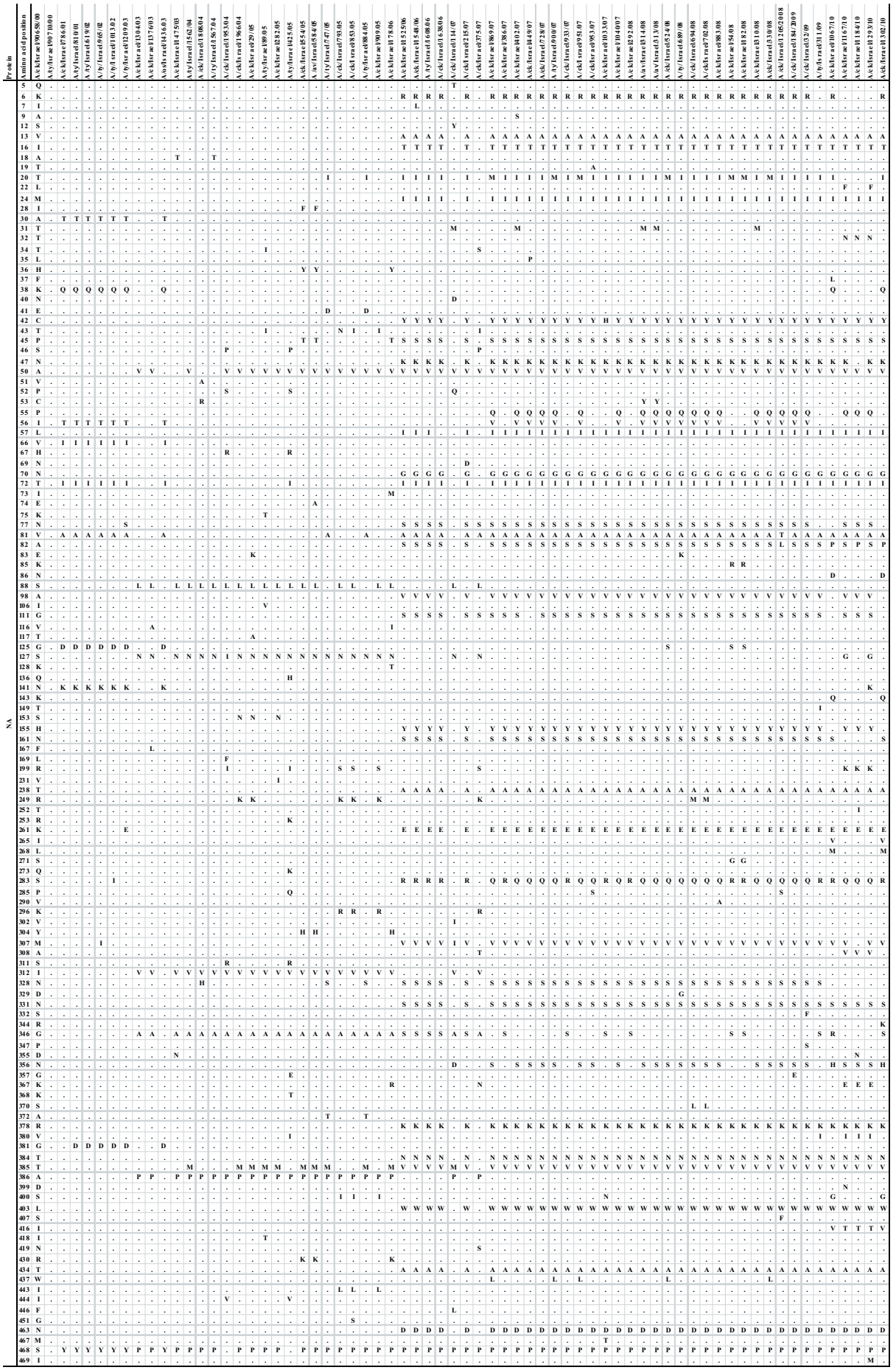

Fig. S2. Amino acid substitutions in NA proteins of H9N2 viruses isolated in Israel during 2000-2010.

caused mild respiratory disease accompanied by a drop in egg production.

Phylogenetic analysis of the envelope glycoprotein genes of H9N2 viruses

According to the phylogenetic analysis of the HA gene, all the Israeli H9N2 isolates fell into a single G1-like lineage (Fig. 1 A). Inside this lineage, the Israeli AIVs fell into two groups: the first contained AIVs belonging to the1st, 2nd and 3rd introductions; the second group comprised strains isolated after the end of 2006, which were designated as the 4th introduction. The Israeli viruses belonging to the first group clustered with AIVs isolated in Asian and Middle-Eastern countries during 1997-1999; local isolates belonging to the second group clustered together with H9N2

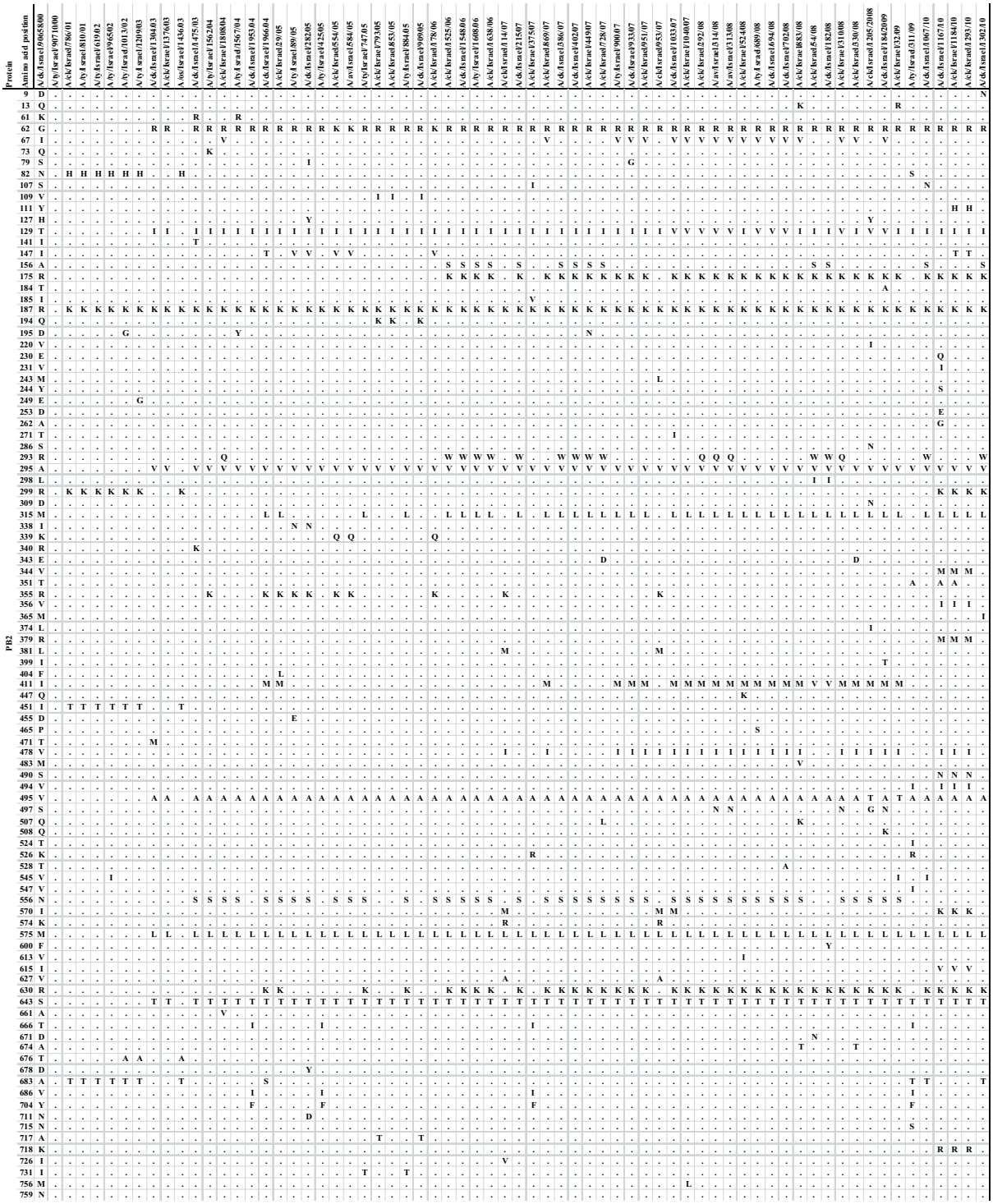

Fig. S3. Amino acid substitutions in PB2 proteins of H9N2 viruses isolated in Israel during 2000-2010.

viruses isolated in Iran and Pakistan during 2005-2008 (Fig. 1A). The latter group was subdivided into two phylogenetically neighbouring subgroups: subgroup A comprised isolates closely related to prototype virus A/ck/Israel/1525/2006; subgroup B comprised viruses related to prototype A/ck/Israel/869/07, and within the latter subgroup might be distinguished a cluster containing three viruses isolated in 2010.

Phylogenetic analysis of the NA gene placed all Israeli isolates into a single G1-like lineage within which they formed two separate groups (Fig. 1B) that were analogous to the H9 groups described above. Local isolates of the first group contained AIVs belonging to the 1st, 2nd and 3rd introductions. The second group contained Israeli isolates of AIVs belonging to the 4th introduction, which clustered with A/ck/Iran/NGV-1/06 and A/ck/Pakistan/

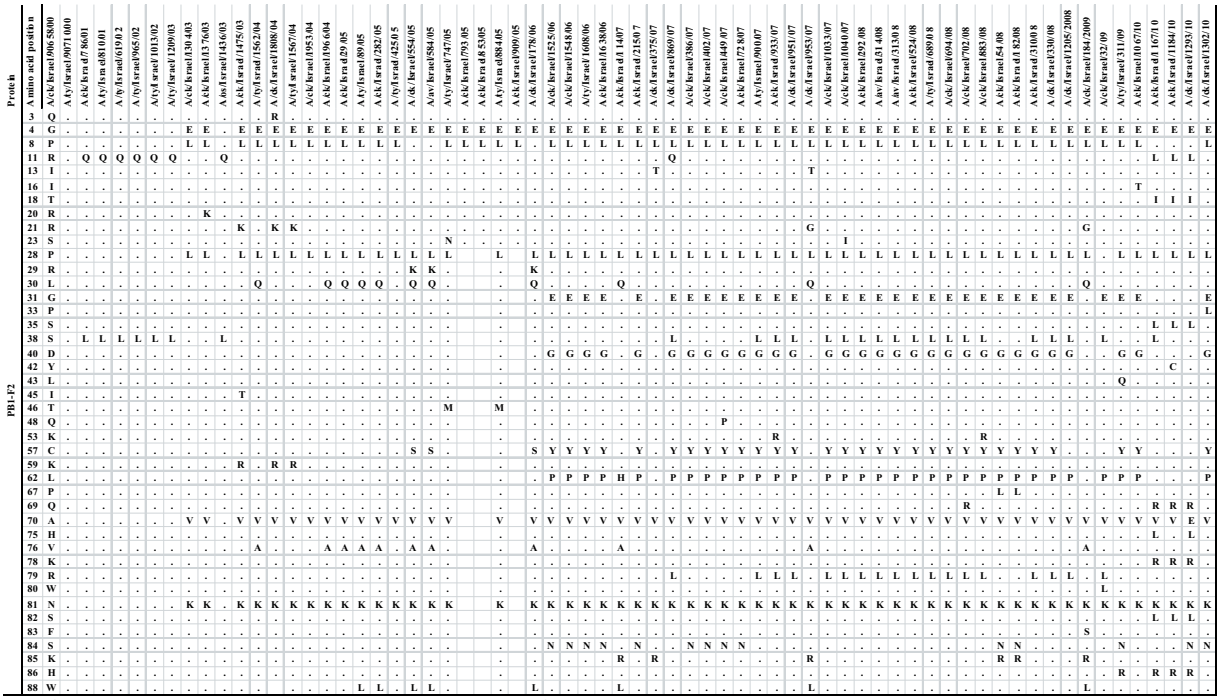

Fig. S4. Amino acid substitutions in PB1-F2 proteins of H9N2 viruses isolated in Israel during 2000-2010.

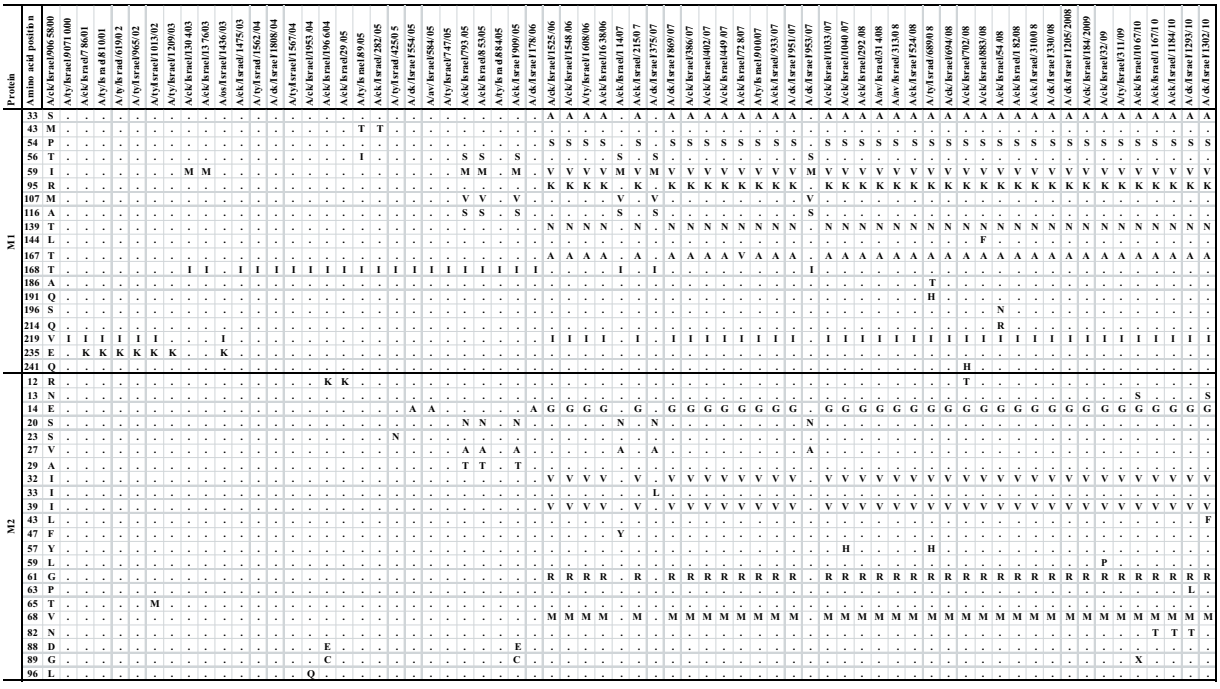

Fig. S5. Amino acid substitutions in M1 and M2 proteins of H9N2 viruses isolated in Israel during 2000-2010.

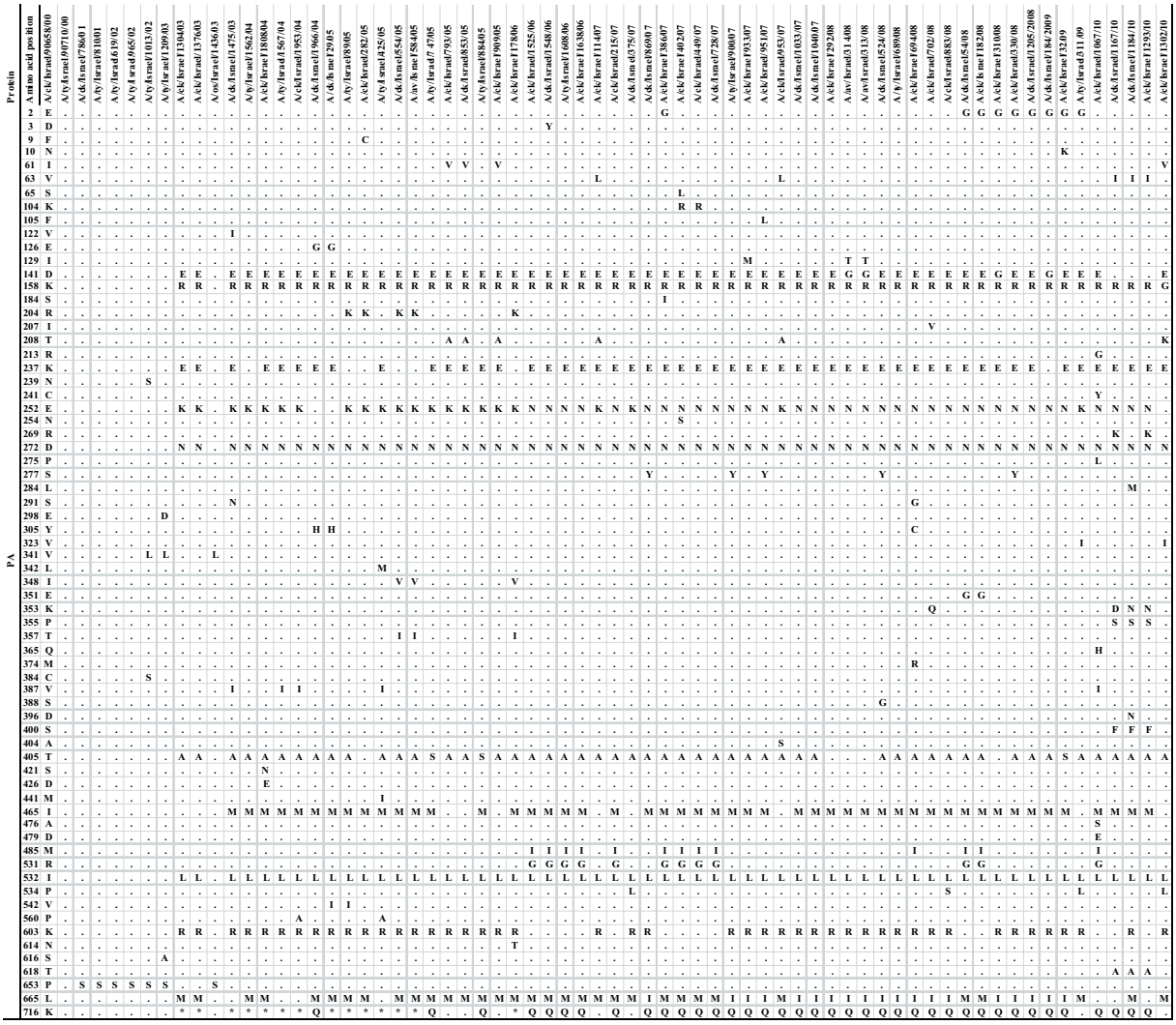

Fig. S6. Amino acid substitutions in PA proteins of H9N2 viruses isolated in Israel during 2000-2010.

UDL-01/05. This second group might be subdivided into two subgroups, but differences between these subgroups are only about 1 %. All viruses isolated in 2010 also belonged to the second group, within which they formed two small clusters, separated by a difference greater than 5 %.

Phylogenetic analysis of the H9N2 internal genes

Phylogenetic analysis of NP gene revealed that all Israeli isolates, together with some other Middle-East viruses, fell into one G1-like Asian lineage (Fig. 2 A), within which the Israeli isolates fell into two groups: the first contained viruses belonging to the 1st and 2nd introductions, and comprised 90658/00-like viruses, isolated during 2000-2003; the second contained viruses belonging to the 3rd and 4th introductions, which were 1304/03-like. The viruses isolated in 2010 formed two clusters.

Phylogenetic analysis of the M gene showed that all Israeli isolates belonged to the G1-like lineage, within which they fell into two main groups. The first group contained 90658/00-like viruses belonging to the 1st, 2nd and 3rd introductions; the second group included 1525/06-like viruses belonging to the 4th introduction. The latter group could be separated into two clusters containing viruses isolated in 2010 (Fig. 2B).

Phylogenetic analysis of the NS gene placed all Israeli isolates in two main phylogenetic groups, both different from the G1-like and Y280-like Asian lineages (Fig. 3 A): the first group comprised 90658/00-like and 1304/03-like clusters; the

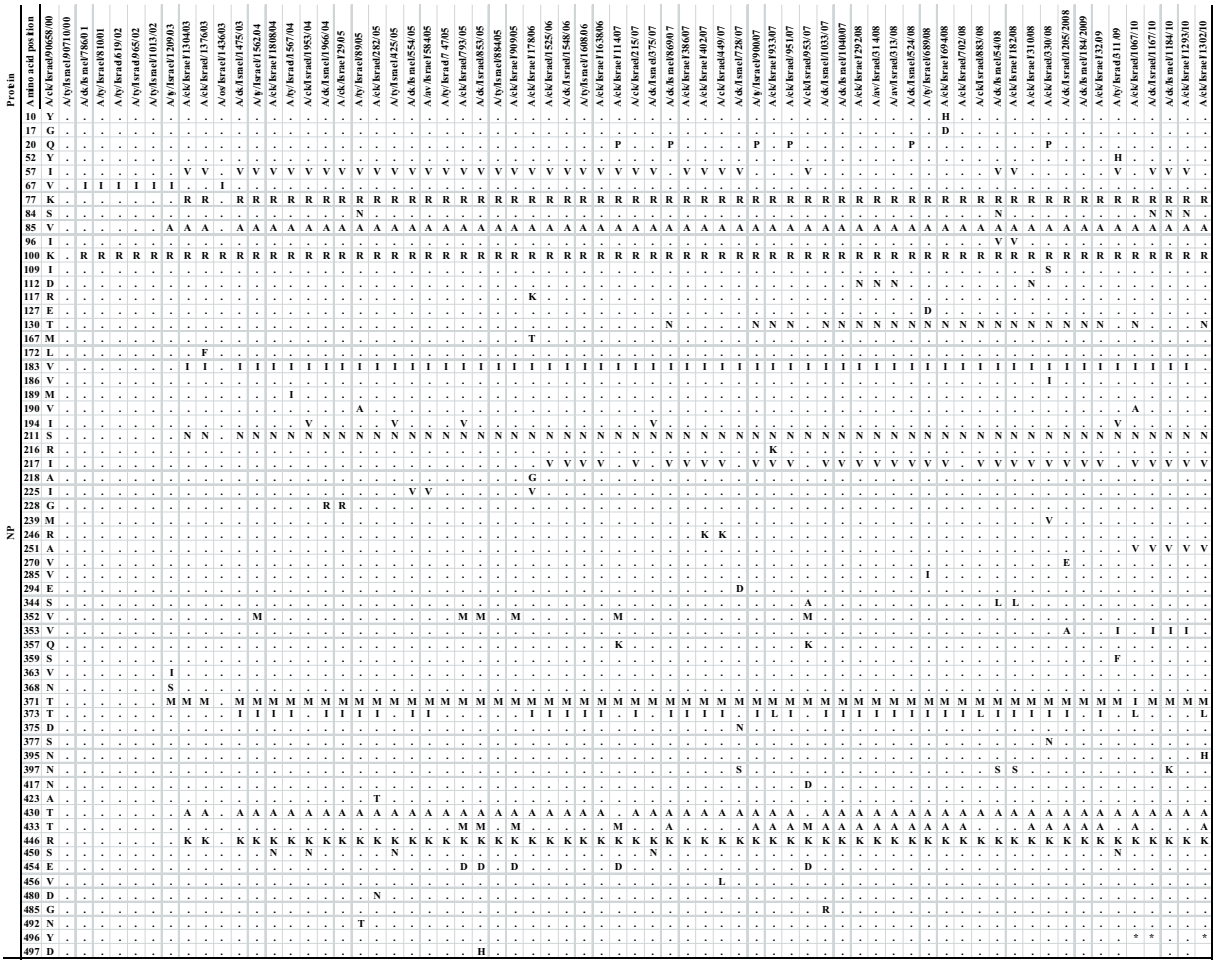

Fig. S7. Amino acid substitutions in NP proteins of H9N2 viruses isolated in Israel during 2000-2010.

second group comprised 1525/06-like and 869/07-like clusters. In turn, the 1525/06-like cluster contained 54/08-like and 1067/10-like sub-clusters, and the 869/07-like cluster contained an 1167/10-like sub-cluster.

Analysis of the three polymerase genes, PB2, PB1 and PA showed that Middle-Eastern H9N2 viruses (all Israeli isolates together with A/ck/Middle East/ED-1/ 99 and A/ck/Saudi Arabia/532/99) fell into one lineage which was different from G1-like and Y280-like Asian lineages.

In the case of PB2 gene (Fig. 3 B), Israeli isolates formed two main groups: the first contained ck/Israel/90658/00-like viruses isolated in Israel during 2000-2003, i.e., the 1st and 2nd introductions; the second group contained viruses isolated during 2003-2010, i.e., the 3rd and 4th introductions. The second group includes the cluster containing viruses isolated in Israel in 2010.

In the case of the PB1 gene, Israeli isolates formed two main phylogenetic groups: the first group contained only 90658/00-like viruses, i.e., the 1st and 2nd introductions; the second group contained AIVs isolated during 2003-2010, i.e., the 3rd and 4th introductions. The second group was found to contain two clusters comprising viruses isolated in 2010 (Fig. 4 A).

Analysis of the PA gene (Fig. 4 B) placed Israeli isolates into two phylogenetic groups, which differed from Asian G1-like and Y280-like lineages. The first group comprised 90658/00-like viruses isolated during 2000-2003, i.e., the 1st and 2nd introductions; the second group included viruses isolated during 2003-2010, i.e., the 3rd and 4th introductions. Three of the viruses isolated in 2010 formed one cluster, whereas other two – A/ck/Israel/1067/10

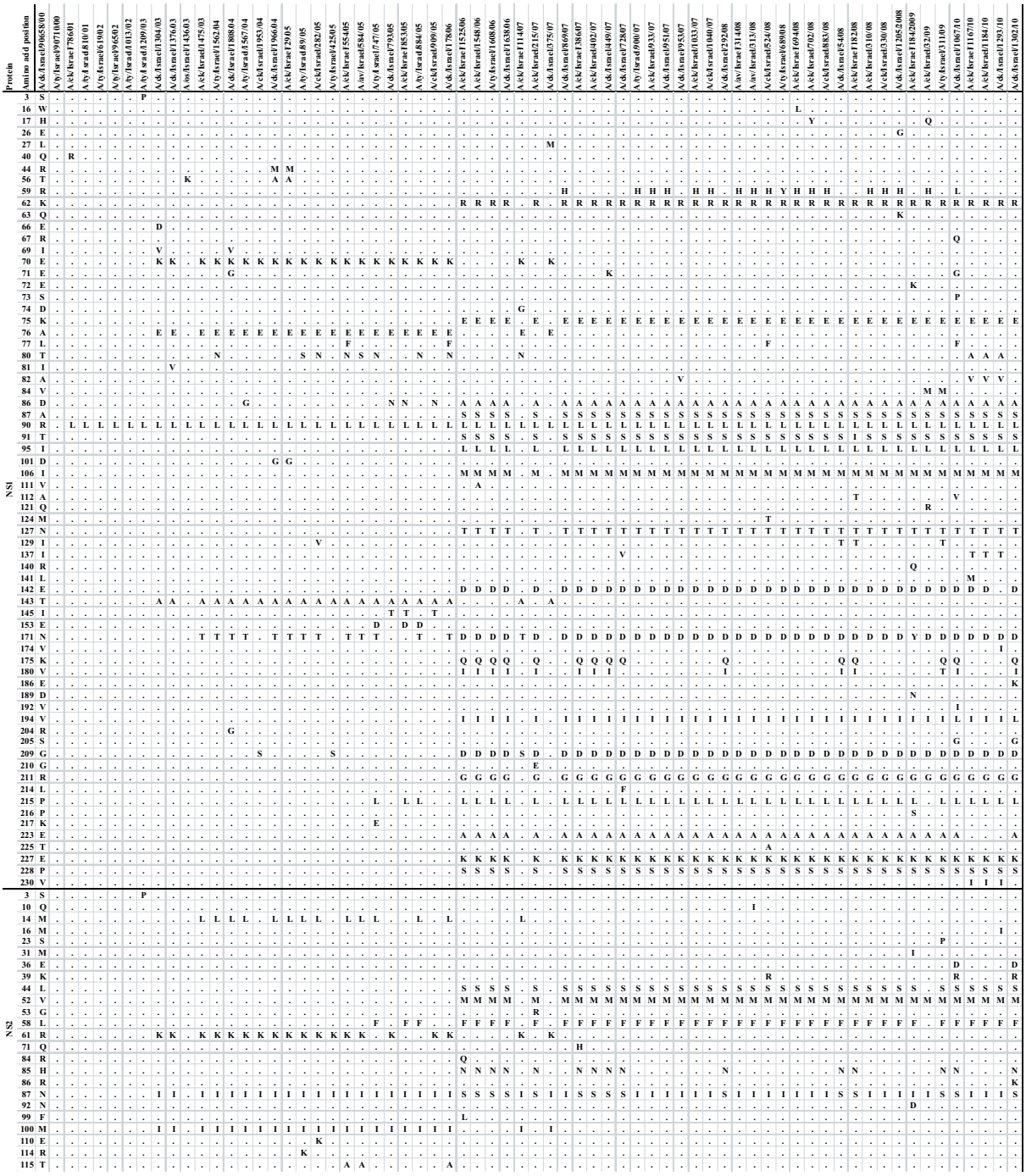

Fig. S8. Amino acid substitutions in NS1 and NS2 proteins of H9N2 viruses isolated in Israel during 2000-2010.

and A/ck/Israel/1302/10 – clustered with different isolates.

In the light of the phylogenetic characteristics of all eight gene segments, all Israeli H9N2 strains examined in the present study fell into three groups: A, containing 90658/00-like sequences in all eight segments – the 1st and 2nd introductions; B, containing 1304/03-like sequences in all segments – the 3rd introduction; and C, containing four 1525/06-like and four 1304/03-like segments – the 4th introduction (Table 1).

Virus proteins

The envelope glycoproteins of the analyzed H9N2 were typical for this subtype and did not contain any deletions or insertions. In HA, four variable positions were found in the receptor binding pocket: in position 216 (H9 numbering) 58 viruses had leucine (216L) and 10 had glutamine (216Q); in position 180, fifty-three isolates

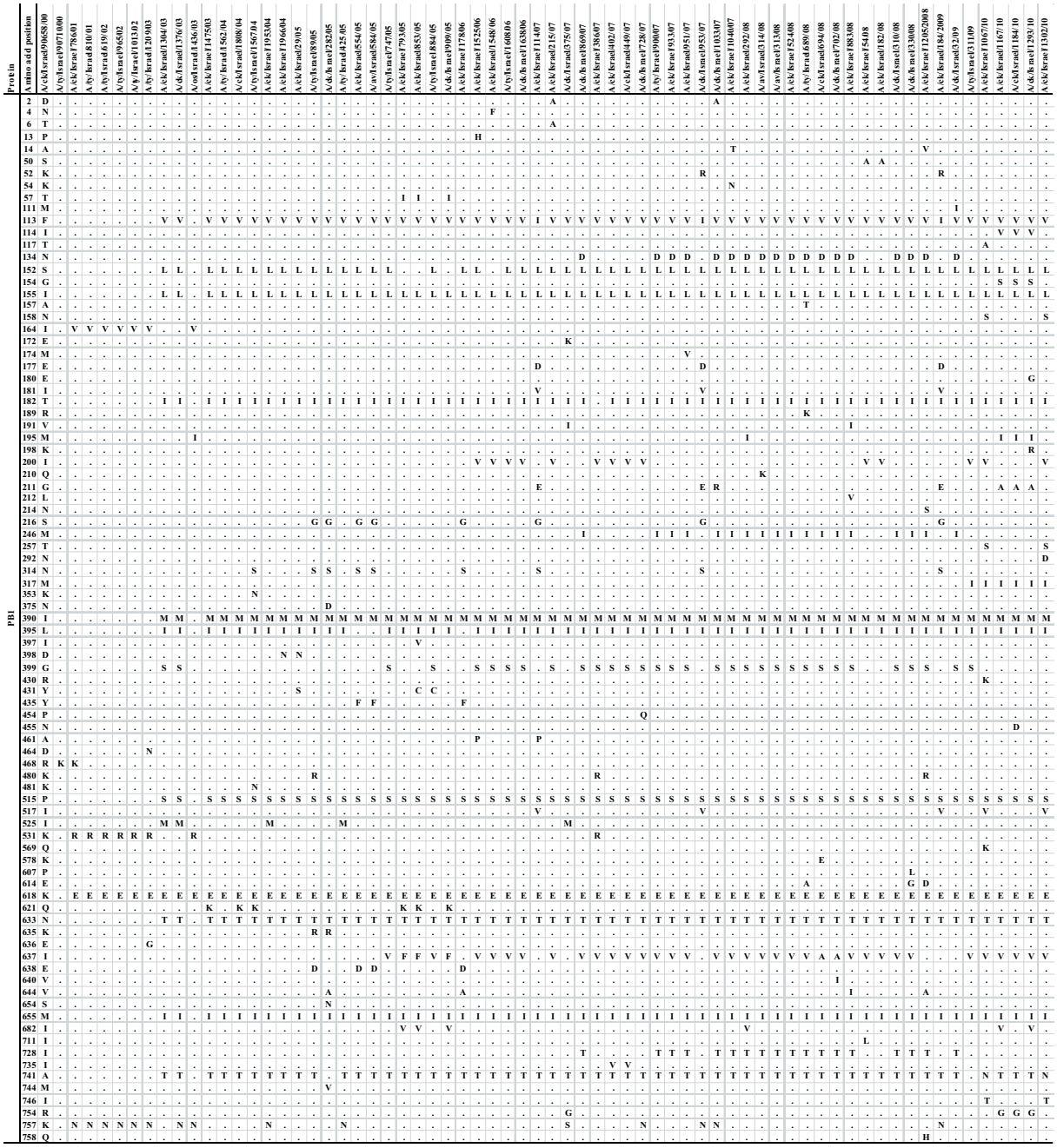

Fig. S9. Amino acid substitutions in NS1 and NS2 proteins of H9N2 viruses isolated in Israel during 2000-2010.

had alanine (180A), nine had glutamate (180E), five had threonine (180T), and one had valine (180V); in position 217, thirty-one isolates had glutamine (217Q) and 37 had isoleucine (217I); in position 132, thirty strains had alanine (132A), 37 had serine (132S) and one had threonine (132T). Most of the studied H9N2 isolates maintained an arginine-serine-serine-arginine (RSSR) motif at the connecting peptide of their HA, but a few substitutions were observed in this peptide: in position 319, six isolates had an RSKR motif, two an RSNR motif and one an RSGR motif (Fig. S1). In the NA protein of the analyzed H9N2 isolates no R292 K substitutions associated with resistance to neuraminidase inhibitor drugs (oseltamivir) were observed (Fig. S2).

All internal proteins of the Israeli isolates were typical of avian H9N2 viruses, but in some positions they had amino acids characteristic of mammalian viruses.

In PB2 proteins, 66 analyzed isolates had 674A typical of avian hosts, and the mammalian-specific variant 674T was found in only two isolates; in position 627, no isolates had 627K (lysine), which is

Table 2.

Tracheal and cloacal shedding of virus after inoculation with three H9N2 viruses

Introduction	virus	infected birds, post infection day			contacted birds, post infection day		
		3	5	7	3	5	7
	Tracheal shedding of virus, number shedding/total (mean tracheal virus titers*)						
1st introduction	A/ty/Israel/90710/00	5/6 (1.5)	4/6 (2.8)	0/6	0/4	0/4	0/4
2nd introduction	A/ck/Israel/786/01	6/6 (2.4)	1/6 (1)	0/6	0/4	0/4	0/4
3rd introduction	A/ck/Israel/1376/03	6/6 (3)	6/6 (2.2)	2/6(1.5)	4/4 (1.5)	4/4 (2.8)	4/4(2)
	A/ty/Israel/1567/04	1/6 (3)	1/6(4)	1/6 (1)	0/3	0/3	0/3
	A/ck/Israel/114/07	6/6 (2)	3/6 (1.2)	0/6	3/4 (1.4)	4/4 (2.2)	4/4 (2.2)
4th introduction	A/ck/Israel/1525/06	6/6 (4.6)	5/6 (1.6)	1/6 (2)	3/3 (3)	3/3 (3)	3/3 (1)
	A/ck/Israel/386/07	6/6 (4.2)	5/6 (1.6)	0/6	4/4 (4)	4/4 (2.3)	0/4
	A/ck/Israel/449/07	6/6 (3.2)	1/6 (1)	1/6 (1)	1/4 (1)	4/4 (3.2)	4/4 (3.2)
	Cloacal shedding of virus, number shedding/total (mean cloacal virus titers*)						
1st introduction	A/ty/Israel/90710/00	3/6 (1)	2/6 (3)	2/6(1.8)	0/4	0/4	0/4
2nd introduction	A/ck/Israel/786/01	3/6 (2.2)	0/6	0/6	0/4	0/4	0/4
3rd introduction	A/ck/Israel/1376/03	6/6 (1.5)	1/6 (2)	1/6(1)	0/4	1/4 (1)	0/4
	A/ty/Israel/1567/04	1/6 (1)	0/6	1/6 (2)	0/3	0/3	0/3
	A/ck/Israel/114/07	5/6 (2)	0/6	1/6 (1)	0/4	0/4	0/4
4th introduction	A/ck/Israel/1525/06	3/6 (2.4)	1/6 (3)	0/6	0/3	0/3	0/3
	A/ck/Israel/386/07	5/6 (2)	1/6 (1)	0/6	0/4	0/4	0/4
	A/ck/Israel/449/07	6/6 (1.5)	0/6	0/6	0/4	0/4	0/4

*In brackets presented titer of the virus expressed as $LgEID_{50}$/ml

associated with the mammalian adaptation, most H9N2 isolates had the avian-virus-specific 627V, and only two had 627A, which had not been observed previously; no D701N or A199S substitutions – also associated with host specificity – were revealed (Fig. S3).

In the PB1-F2 protein, 76A and 82S, which is typical of mammalian hosts, were found in11 and 65 isolates, respectively (Fig. S4). Three H9N2 isolates – A/ck/ Israel/793/05, A/ck/Israel/853/05 and A/ck/Israel/909/05 – contained the PB1-F2 protein truncated to 25 amino acid residues. The last three viruses and three isolates – A/ck/Israel/114/07, A/ck/Israel/375/07 and A/ck/Israel/953/07 – had a V27A

Table 3.

Amino acid substitutions typical for mammalian and human AIVs in genes of Israeli H9N2 isolates

Protein	aa position	Predicted aa		Introduction			
		Avian	Mammalian/ Human	1st	2nd	3rd	4th
PB2	44	A	S	A	A	A	A
	64	M	T	M	M	M	M
	199	A	S	A	A	A	A
	588	A	I	A	A	A	A
	627	V	K	V	V	V/A[13]	V/A[14]
	701	D	N	D	D	D	D
	674	A	T	A	A	A	A/T[1]
PB1-F2	76	V	A	V	V	V/A[7]	V/A[8]
	82	L	S	S	S	S	S/L[9]
	87	E	G	E	E	E	E
PA	57	R	Q	R	R	R	R
	356	K	R	K	K	K	K
	404	A	S	A	A	A	A/S[2]
	409	S	N	S	S	S	S
NP	109	I	V	I	I	I	I/S[3]
	214	R	K	R	R	R	R
	357	Q	K	Q	Q	Q	Q/K[4]
	372	E	D	E	E	E	E
M2	11	T	I	T	T	T	T
	20	S	N	S	S	S/N[10]	S/N[11]
	57	Y	H	Y	Y	Y	Y/H[12]
	86	V	A	V	V	V	V
NS1	215	P	T	P	P/L[5]	P/L[5]	P/L[5]
	227	E	R/K	E	E	E	K[6]
NS2	70	S	G	S	S	S	S

1- A/ck/Israel/883/08, A/ck/Israel/330/08
2- A/ck/Israel/953/07
3- A/ck/Israel/330/08
4- A/ck/Israel/114/07, A/ck/Israel/953/07
5- P – 29 viruses, L – 39 viruses
6- All viruses belonging to 4th introduction
7- V – 10, A – 9
8- A-2 [A/ck/Israel/953/07, A/ck/Israel/184/09]
9- L - A/ck/Israel/1167/2010, A/ck/Israel/1184/2010, A/ck/Israel/1293/2010
10- A/ck/Israel/793/05, A/ck/Israel/853/05, A/ck/Israel/909/05, A/ck/Israel/114/07, A/ck/Israel/375/07
11- A/ck/Israel/953/07
12- A/ty/Israel/689/08, A/ck/Israel/1040/07
13- A/ck/Israel/114/07
14- A/ck/Israel/953/07

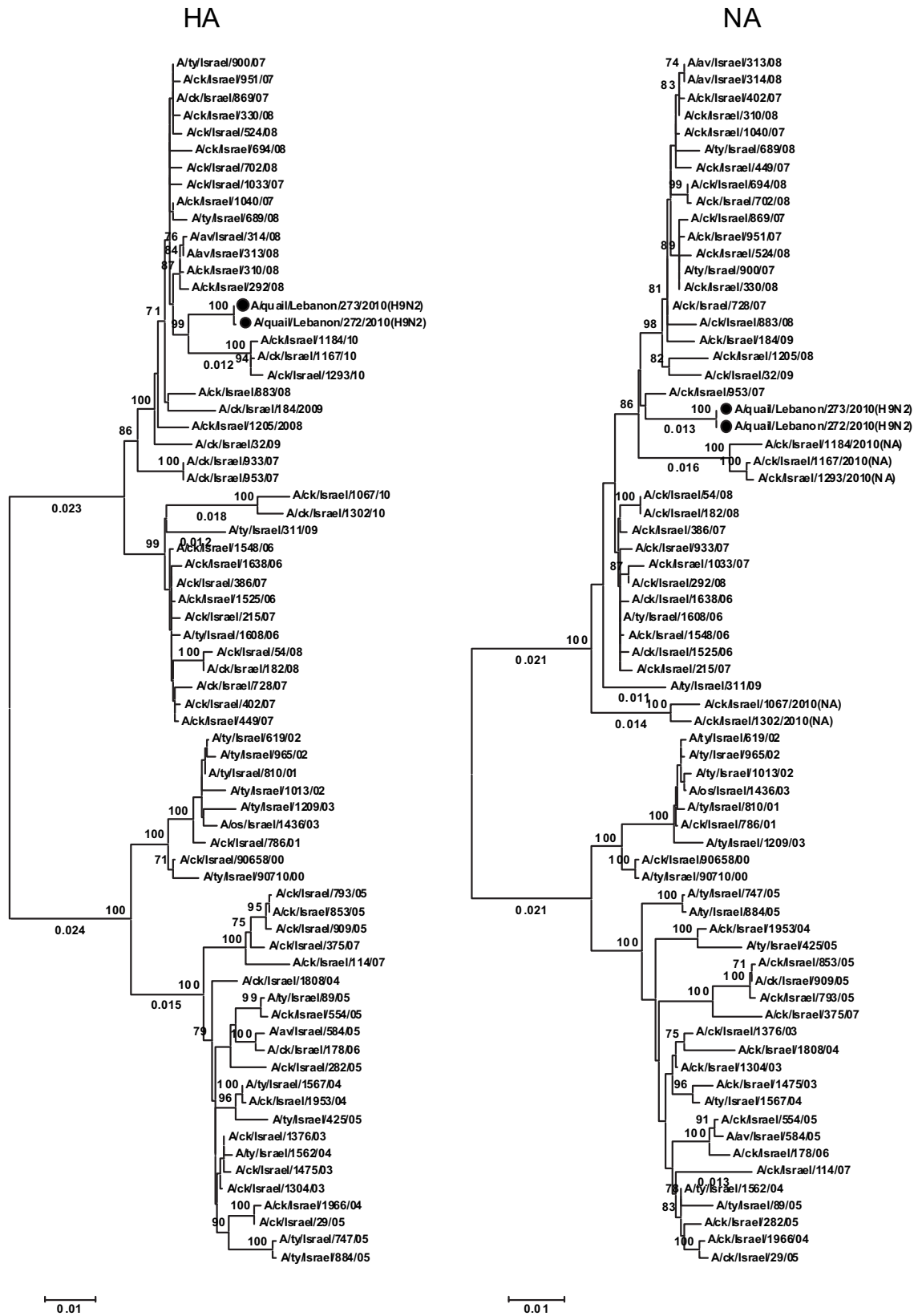

Fig. S10. Phylogenetic relationships of HA and NA genes from H9N2 influenza viruses isolated in Israel and Lebanon. The phylogenetic trees based on nucleotide sequences were generated by neighbour-joining analysis with the Maximum Composite Likelihood model, using MEGA 5.0 software. Numbers below branches indicate neighbour-joining bootstrap values; numbers above branches indicate branch lengths. Viruses isolated in Lebanon are marked with black circles.

Fig. S11. Phylogenetic relationships of NP and NS genes from H9N2 influenza viruses isolated in Israel and Lebanon. The phylogenetic trees based on nucleotide sequences were generated by neighbour-joining analysis with the Maximum Composite Likelihood model, using MEGA 5.0 software. Numbers below branches indicate neighbour-joining bootstrap values; numbers above branches indicate branch lengths. Viruses isolated in Lebanon are marked with black circles.

Fig. S12. Phylogenetic relationships of M and PB1genes from H9N2 influenza viruses isolated in Israel and Lebanon. The phylogenetic trees based on nucleotide sequences were generated by neighbour-joining analysis with the Maximum Composite Likelihood model, using MEGA 5.0 software. Numbers below branches indicate neighbour-joining bootstrap values; numbers above branches indicate branch lengths. Viruses isolated in Lebanon are marked with black circles.

substitution at the M2 protein, which is associated with amantadine resistance. Also in this protein, some viruses had 20N and 57H amino acids, which are associated with the specificity of the mammalian host (Fig. S5). The PA protein in only one virus – A/ck/Israel/953/07 – had the mammalian-typical 404S (Fig S6). The NP protein of two viruses – A/ck/Israel/953/07 and A/ck/Israel/114/07 – had the

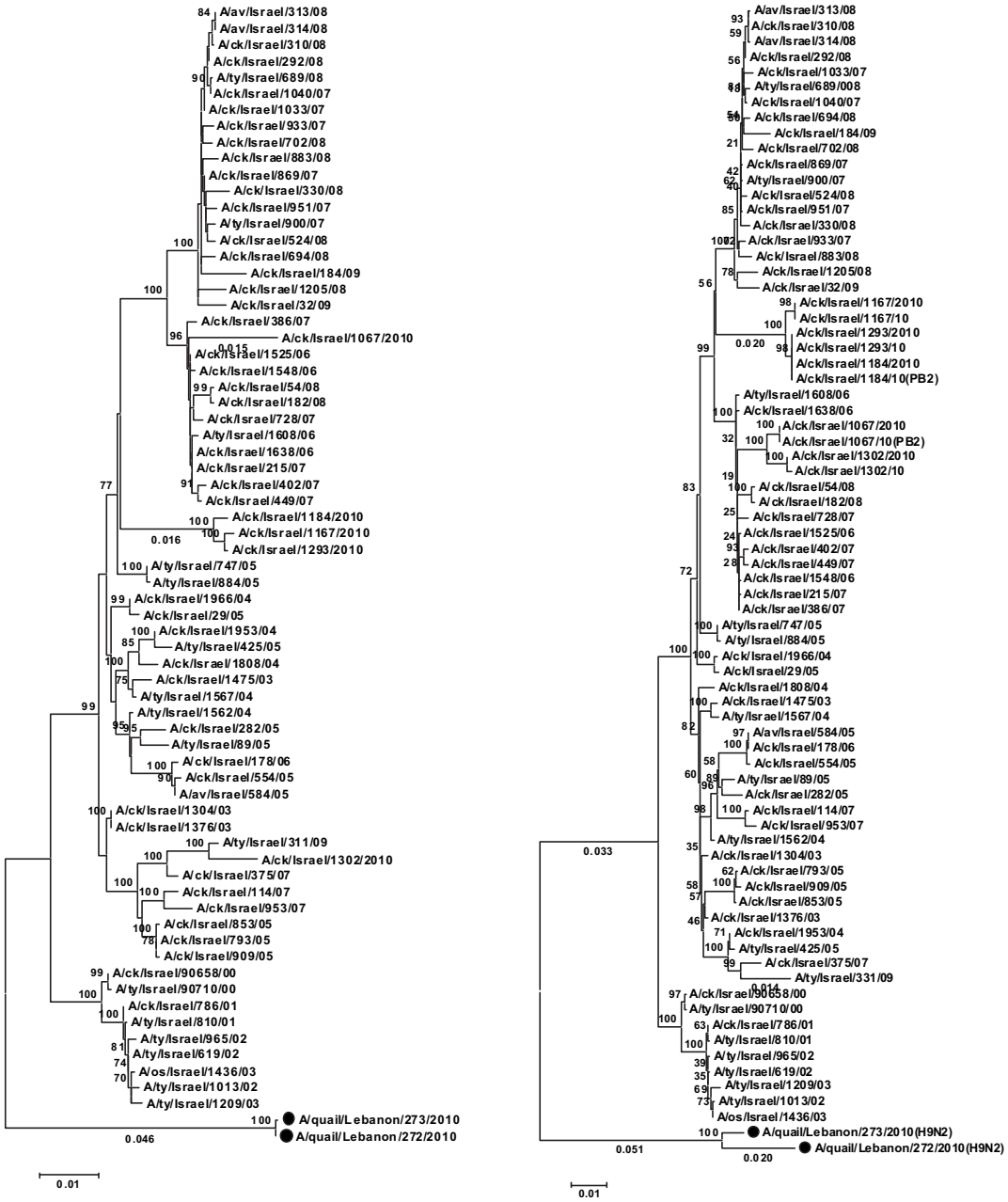

Fig. S13. Phylogenetic relationships of PA and PB2 genes from H9N2 influenza viruses isolated in Israel and Lebanon. The phylogenetic trees based on nucleotide sequences were generated by neighbour-joining analysis with the Maximum Composite Likelihood model, using MEGA 5.0 software. Numbers below branches indicate neighbour-joining bootstrap values; numbers above branches indicate branch lengths. Viruses isolated in Lebanon are marked with black circles.

mammalian-specificity-associated 375K (Fig. S7).

The NS1 protein of all viruses belonging to the 4th introduction had the mammalian-specific 227K. The NS1 of the analyzed H9N2 possessed the EPEV, KSEV and KSEI PDZ ligand motif (Fig. S8). The F103L and M106I NS1 mutations were adaptive genetic determinants of growth and virulence in both human and avian NS1 genes in the mouse model. All Israeli viruses had 103F; viruses belonging to the

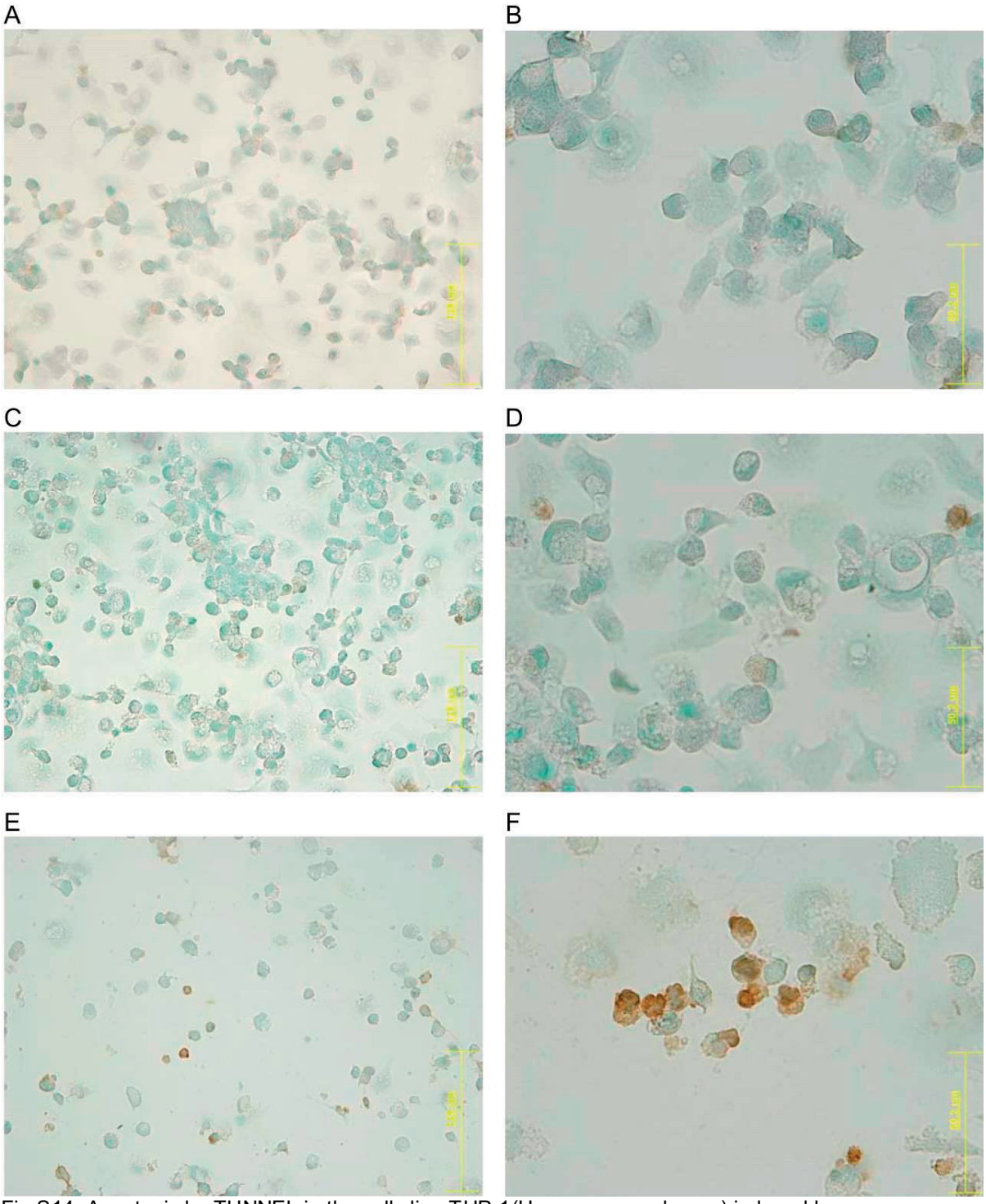

Fig S14. Apoptosis by TUNNEL in the cells line THP-1(Human macrophages) induced by A/ck/Israel/853/2005 and A/ty/Israel/884/2005 H9N2 viruses.
A – control, Ч 400 ; B – control, Ч 1000; C – 24 h after infection with virus A/ck/Israel/853/2005, Ч 400;
D – 24 h after infection with virus A/ck/Israel/853/2005, Ч 1000. This virus stimulated apoptosis only slightly; it induced minimal difference from the virally uninfected macrophage control culture.
E – 24 h after infection with virus A/ty/Israel/884/2005, Ч 400.
F – 24 h after infection with virus A/ty/Israel/884/2005, Ч 1000. This virus strongly induced pronounced apoptosis (brown-stained nucleus)

1st, 2nd and 3rd introductions had 103I, whereas, viruses belonging to the 4th introduction had 103M. No amino acid substitutions associated with mamalian specificity were found in the PB1 protein (Fig. S9)

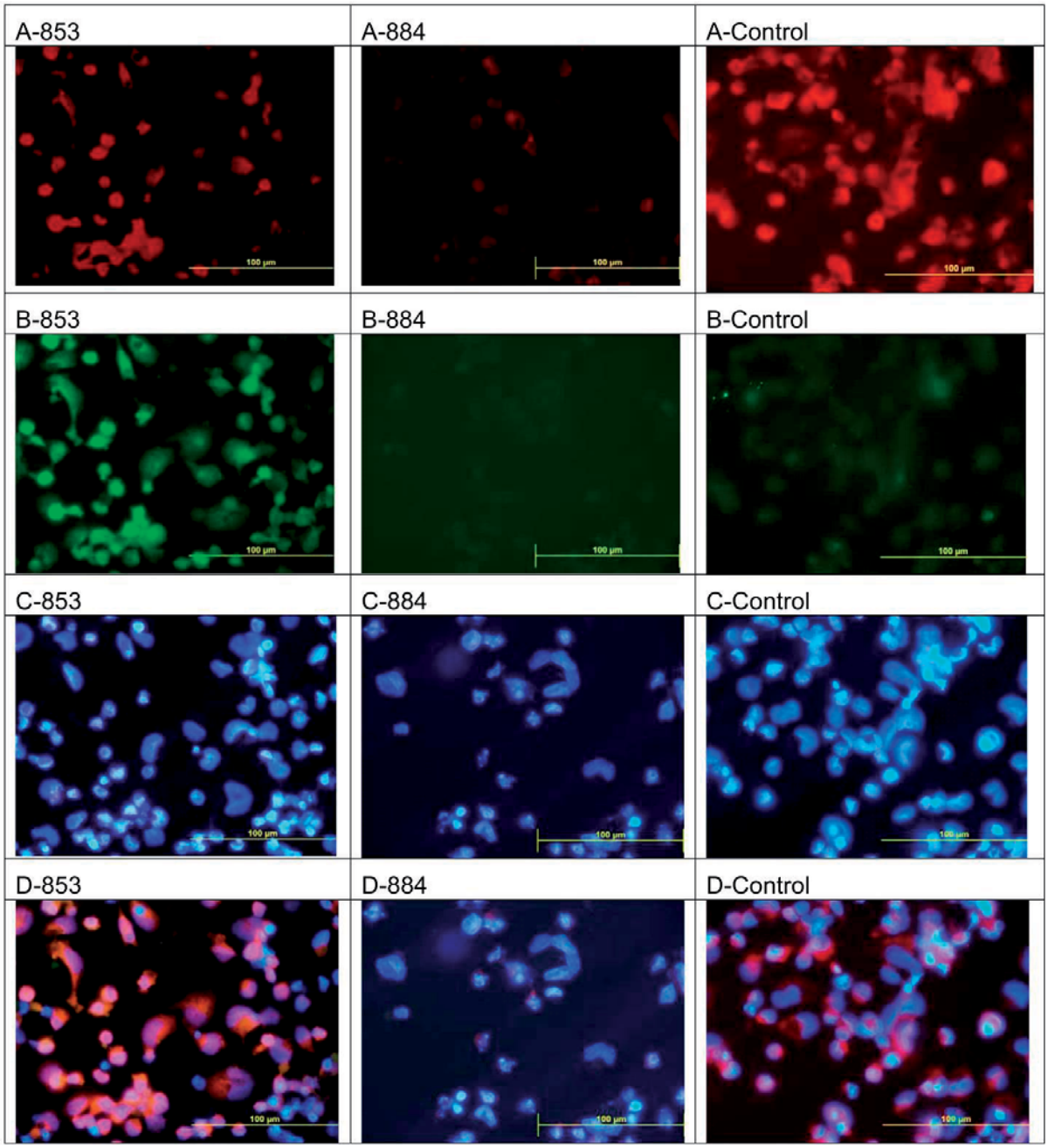

Fig. S15. Influence of viruses A/ck/Israel/853/2005 and A/ty/Israel/884/2005 on mitochondrion and p53 staining.
853 – A/ck/Israel/853/2005 (truncated PB1-F2, length of 25 aa);
884 – A/ty/Israel/884/2005 (PB1-F2, length of 90 aa).
A-853, A-884 and A-control. Mitochondrion staining of infected macrophages with MitoTracker Red 580. 24 h post-infection.
A-853: virus A/ck/Israel/853/2005-infected macrophages had intense staining of mitochondrion with MitoTracker.
A-884 : virus A/ty/Israel/884/2005-infected macrophages completely free of mitochondrion staining. A-control: MitoTracker Red 580 mitochondrion staining of uninfected macrophages.
B-853, B-884 and B-control. Pab 421 staining against p53 protein.
B-853: Intense staining was found in the cytoplasm and nucleus of the infected macrophages.
B-884 and B-control: No P53 staining of the cytoplasm or nucleus was found.
C-853, C-884 and C-control. DAPY nucleus staining.
D-853, D-884 and D-control. Merged images

Replication and transmission of H9N2 viruses in chickens

The animal test data are presented in Table 2. It was shown that viruses belonging to the 1st and 2nd introductions replicated to a limited extent in the tracheas and cloacae of directly infected birds, but were not transmitted to contact chickens

introduced into the same cage. Two out of three studied isolates belonging to the 3rd introduction – A/ck/Israel/1376/03 and A/ck/Israel/114/07 – replicated in the tracheas and cloacae of inoculated chickens and were transmitted and replicated in tracheas of contact chickens (Table 2), whereas A/ty/Israel/1567/04 directly infected only one bird; though this virus replicated successfully, it was not transmitted into contact birds. All viruses belonging to the 4th introduction replicated into tracheas and cloacae of inoculated birds and transmitted to contacted chickens. None of the inoculated or contacted birds showed visible signs of disease, and all groups continued to gain weight.

Influence of H9N2 viruses with complete and truncated PB1-F2 proteins on human macrophages *in vitro*

To study the influence of the PB1-F2 gene on human macrophages in culture we selected two H9N2 viruses belonging to the same introduction but having considerable differences in their PB1-F2 gene sequences: A/ty/Israel/884/2005, with PB1-F2 protein, 90 aa in length; and A/ck/Israel/853/2005, with truncated PB1-F2 protein, 25 aa in length. At 24 h post-infection, virus A/ty/Israel/884/2005 strongly induced pronounced apoptosis of the human macrophage cells and complete loss of the mitochondrion MitoTracker staining, but no P53 staining of the cytoplasm or nucleus was found. In contrast, virus A/ck/Israel/853/2005 stimulated apoptosis only slightly, and elicited only minimal differences as compared with control uninfected macrophage culture. Similarly to the control macrophage culture, macrophages infected with A/ck/Israel/853/2005 exhibited intense MitoTracker staining of the mitochondrion. In this case, the cytoplasm and nucleus of the infected macrophages exhibited intense staining with antibody Pab 421 against p53 protein (Fig. S14-S15).

Discussion

During 2000-2010 the Israeli poultry industry was exposed to four introductions of LPAI–H9N2 Panshin *et al*. [24]. As noted above, on the basis of phylogenetic characteristics of complete AIV genomes, all Israeli H9N2 strains examined in the present study fell into three groups. Viruses belonging to group A – the 1st and 2nd introductions – contained 90658/00-like sequences that were closely related to those in Asian H9N2 strains. Local AIVs from Group B – 3rd introduction – contained 1304/03-like sequences that differed significantly from 90658/00-like sequences, in all segments. Group C comprised viruses belonging to the 4th introduction; these viruses contained four 1304/03-like segments and four 1525/06-like segments (HA, NA, NS, and M) which differed significantly from 90658/00-like and 1304/03-like sequences (Table 1). Thus, these viruses occurred as a result of reassortment between the new variant of H9N2 and the viruses circulating previously in the local region. Unfortunately, the Gene Bank contains only a limited set of viruses isolated in countries that share borders with Israel during 2000-2010. Among them were found four viruses, namely: A/chicken/Jordan/1540/2003(H9N2) – closely related to the Israeli H9N2 of the 3rd introduction; A/avian/Egypt/920431/2006(H9N2) – significantly different from all the Israeli isolates; and two Lebanese viruses – A/quail/Lebanon/272/2010(H9N2) and A/quail/Lebanon/273/2010(H9N2) – which had envelope genes closely related to those of Israeli isolates of group C, and internal genes significantly different from those of all the Israeli strains (Figs S10-S13). Thus, the H9N2 strains circulating in this region have internal genes from several different sources: in the case of the Israeli isolates – from local H9N2 AIVs circulating in Israel; in the case of the Lebanese viruses – from the Eurasian viruses belonging to various subtypes other than H9N2. Experimental studies of H9N2 viruses in infected and contacted chickens showed no differences among local H9N2

isolates belonging to different introductions.

Some molecular changes in the envelope and internal proteins have been associated with the host specificity of Israeli H9N2 viruses for mammals, including humans. In general, differences in host specificity and pathogenicity correspond to changes in the envelope glycoproteins, in addition these properties may be associated with substitutions in internal genes Fusaro *et al*. [25]. Chen *et al*. [7] validated 50 amino acid signatures that distinguish AIV from human influenza viruses. In most positions of all internal proteins our local isolates contained amino acids typical of avian viruses; amino acids typical of human influenza viruses were found in only a few cases (see Table 3).

The results of experiments *in vivo* imply that all the studied viruses infected the majority of the directly inoculated birds, and the viruses belonging to the 3rd and 4th introductions were able to infect contacted birds (Table 2). Although it seems that viruses related to the more recent introductions multiply efficiently in the trachea, no significant differences were found among the studied viruses, in their virulence and invasiveness.

Protein PB1-F2 was encoded in an alternative reading frame of the PB1 gene. This protein was found in most PB1-coding sequences of influenza viruses isolated from birds [26]; it increases virulence and the risk of secondary infections [27-28] by inducing apoptosis in macrophages [29]. This protein is functionally active if it has a length of 79 or more amino acids. Our present data show that the complete PB1-F2 gene must be present for induction of mitochondrion death and apoptosis. Most local AIV isolates had the full-length PB1-F2 protein, except for three H9N2 isolates that were truncated. It is not clear why the percentage of truncated PB1-F2 protein is much smaller in avian influenza A viruses than in swine and human influenza A isolates. The above findings show that exposure to the virus with full-length protein for 24 h caused destruction of mitochondria and cell death in cultures of human macrophages (Figs. S14 and S15), and this effect was not associated with activation of protein p53 (Fig. S15). In contrast, a virus with truncated PB1-F2 protein did not have a destructive effect on mitochondria, but induced enhanced production of pro-apoptotic P53 proteins (Figs. S14 and S15).

It can be assumed that the virus with an inactive PB1-F2, in contrast to viruses with the full-length protein, can multiply in macrophages to some extent, and, consequently, reassortment of genes between avian and human AIVs with truncated PB1-F2 can occur directly in human macrophages.

Our present study demonstrated that low-pathogenic H9N2 influenza viruses have been circulating continuously in the Middle-East region, including Israel, during the last decade. Periodical introductions of highly pathogenic H5N1 and pandemic H1N1viruses have aggravated the epidemiological situation in this region. Although we have found only a few substitutions in AIV internal protein genes that might be associated with a high risk of interspecies transmission, including avian-to-human and human-to-human, the possibility of the occurrence of such mutations remains sufficiently high to cause concern.

Acknowledgment

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Резюме

МОЛЕКУЛЯРНАЯ И БИОЛОГИЧЕСКАЯ ХАРАКТЕРИСТИКА НИЗКОПАТОГЕННЫХ ВИРУСОВ ГРИППА H9N2, ВЫДЕЛЕННЫХ В ИЗРАИЛЕ

Тендлер Е., Голендер Н., Шкода И., Драбкин М., Лапин К., Паншин А.

Все израильские вирусы штамма H9N2, используемые в настоящем исследовании, были разделены на группы: вирусы групп А и В содержали соответственно - во всех восьми сегментах -

90658/00-подобные и 1304/03-подобные последовательности; группа С состояла из вирусов, выделенных в 2006-2010 годах, которые содержали четыре 1304/03-подобных сегмента и четыре 1525/06-подобных сегмента. Молекулярный анализ показал, что большинство штаммов имели RSSR-мотив в сайте расщепления гемагглютинина. Большинство вирусов содержали гемагглютинин с типичным для людей L216, и, кроме того, внутренние белки, характерные для птиц. Изученные вирусы заразили большинство привитых птиц непосредственно.

Воздействие вируса с полноразмерной PB1-F2 белка в течение 24 ч вызвал разрушение митохондрий и гибель клеток в культурах человеческих макрофагов, и этот эффект не был связан с активацией белка p53. Вирус с усеченной цепью белка PB1-F2 не оказывал разрушительного воздействия на митохондрии, но индуцировал повышенное производство проапоптотического белка P53.

***Ключевые слова**: вирус H9N2, филогенетический анализ, вирусные гены, репликация вируса, PB1-F2 белок, апоптоз.*

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Резюме

МОЛЕКУЛЯРНА І БІОЛОГІЧНА ХАРАКТЕРИСТИКА НИЗЬКОПАТОГЕННИХ ВІРУСІВ ГРИПУ H9N2, ВИДІЛЕНИХ В ІЗРАЇЛІ

Тендлер Є., Голендер Н., Шкода І., Драбкін М., Лапін К., Паншин О.

Всі ізраїльські віруси штаму H9N2, що використовуються в цьому дослідженні, були розділені на групи: віруси груп А і В містили відповідно - у всіх восьми сегментах - 90658/00-подібні і 1304/03-подібні послідовності; група С складалася з вірусів, виділених у 2006-2010 роках, які містили чотири 1304/03-подібних сегмента і чотири 1525/06-подібних сегмента. Молекулярний аналіз показав, що більшість штамів мали RSSR-мотив в сайті розщеплення гемаглютиніну. Більшість вірусів містили гемаглютинін з типовим для людей L216, і, крім того, внутрішні білки, характерні для птахів. Вивчені віруси заразили більшість щеплених птахів безпосередньо.

Вплив вірусу з повнорозмірною PB1-F2 білка протягом 24 год викликав руйнування мітохондрій і загибель клітин в культурах людських макрофагів, і цей ефект не був пов'язаний з активацією білка p53. Вірус з усіченою ланцюгом білка PB1-F2 не чинив руйнівного впливу на мітохондрії, але індукував підвищений сінтез проапоптотического білка P53.

***Ключові слова**: вірус H9N2, філогенетичний аналіз, вірусні гени, реплікація вірусу, PB1-F2 білок, апоптоз.*