Molecular characterization of the glycoprotein genes of H5N1 influenza A viruses isolated in Israel and the Gaza Strip during 2006 outbreaks

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Abstract Highly pathogenic H5N1 avian influenza A viruses (AIV) have caused outbreaks among domestic poultry and wild aquatic birds in many Asian, European, and African countries since 1997. In March 2006 an avian H5N1 influenza A virus was isolated from poultry in Israel. In the present study we molecularly characterized the hemagglutinin (HA) and neuraminidase (NA) genes of eleven H5N1 viruses isolated from domestic poultry in Israel and Gaza in March–April 2006. Phylogenetic analysis of the HA and NA genes showed that the Israeli and Gazian viruses were closely related to viruses isolated in Egypt in 2006.

Keywords H5N1 avian influenza virus · Hemagglutinin · Neuraminidase · Phylogenetic analysis

Introduction

Highly pathogenic H5N1 subtypes of the avian influenza (HPAI) virus are considered the most probable precursors of a pandemic influenza virus [1]. An outbreak of H5N1 HPAI in the live birds and human was registered in Hong Kong in 1996–1997. Since 2001, similar HPAI viruses were found in some animal species, such as poultry, wild birds, tigers, and leopards. During recent years the H5N1 influenza virus has been responsible for killing more than 200 million poultry and more than 150 humans in Asia, Europe, and Africa [1, 2]. The first outbreak of H5N1 in Israel was recorded in March 2006, and more than 1 million poultry were killed to stem its spread.

The avian influenza virus (AIV) genome comprises eight separate segments of single-stranded, negative-sense RNA, which code for 10 viral proteins. Avian influenza viruses are covered by a lipid envelope with two surface glycoproteins—hemagglutinin (HA) and neuraminidase (NA)—which perform at least two important functions of the virus life cycle: the receptor binding activity [3] and the sialidase activity that is required for the release of progeny virus from the cell surface.

Glycoprotein HA is coded by the fourth segment of the viral genome; it is synthesized as a polyprotein precursor that is post-translationally cleaved into two subunits HA1 and HA2, conjugated by a disulphide bond. The cleavage step which is realized by intra- or extra-host-cellular protease [4], is necessary for virus propagation [5, 6]. Virus pathogenicity is correlated with addition of polybasic amino acids at the HA cleavage site [7–9] and glycosylation patterns of HA protein [10]. The presence four or more basic amino acids in the HA cleavage site is one of the main characteristic of the highly pathogenic (HP) AIVs.

The NA protein is coded by the sixth segment of the viral genome; through its neuraminidase activity it removes sialic acid residues from the viral HA and facilitates virus release from cells [11–13]. Colman [14] showed that all NA subtypes of the influenza virus had two-highly

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conservative sites: an NA catalytic site that directly interacts with the substrate (R118, D151, R152, R224, E276, R292, R371, and Y406 in N2 numbering) and framework site (E119, R156, W178, S179, D/N198, I222, E227, H274, E277, N294, and E425) that support the catalytic residues [15–18]. The role of NA in AIV pathogenicity was demonstrated in several studies [19–22]. Amino acid substitutions in catalytic or framework sites of NA may interfere with the initiation of infection, by reducing the enzymatic activity and thereby limiting the release of virus from infected cells [14, 23, 24].

This is the first report concerning the HP H5N1 viruses that caused outbreaks of HP influenza in Israel in March 2006. We describe the genetic and phylogenetic properties of these viruses, which provide important insights towards understanding the origins of the HP H5N1 influenza viruses in Israel.

Materials and methods

Viruses

The H5N1 avian influenza viruses were isolated from poultry in Israel and Gaza in March–April 2006. Eluates of the swab samples were inoculated into 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs through the allantoic route. These eggs were incubated at 37°C for up to 7 days, embryonic death was monitored, and then allantoic fluid was collected under routine conditions. The virus subtypes were identified by RT-PCR with a set of subtype-specific primers [25] and confirmed by hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests with monospecific polyclonal goat antisera obtained from Dr. R.G. Webster (St. Jude Children's Research Hospital, Memphis, Tennessee). The pathogenicity of the isolates was determined by means of the intravenous pathogenicity index (IVPI) test. The HI, NI, and IVPI assays were performed in accordance with the WHO Manual [26]. Allantoic fluids containing AIV were used for analysis as described below.

RNA extraction and RT-PCR

The viral RNA was extracted directly from the HI-positive allantoic fluid with the QIAamp Viral RNA mini Kit (Qiagen Ltd, Valencia, CA). RT-PCR was performed as a one-step reaction with the Qiagen OneStep RT-PCR Kit, according to manufacturer's protocol, without Q solution. The sets of H5 HA and N1 NA primers were used for RT-PCR: HA gene-specific primers (forward: 5′-AGCAAAAGCAGGGG-3′ and reverse: 5′-ACCTGCTATAGCTCCAAATAGT-3′) and two sets NA gene-specific primers (forward:

5′-AGCAAAAGCAGGAGT-3′ and reverse: 5′-GCCCAT-TACTTGGTCCATCAGTCAT-3′; forward: 5′-TCAAGA-GTCTGAATGTGCATGT-3′ and reverse: 5′-AGTAGAAA CAAGGAGTTTTTT-3′).

Sequence analysis

The RT-PCR products were subjected to electrophoresis in agarose gel, and specific DNA was excised and purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and then sequenced at the Weizmann Institute of Science, Rehovot, Israel, with a Model 3700 DNA Analyzer (Perkin Elmer, California) by means of capillary electrophoresis. All the primers used are available in the Division of Avian and Aquatic Diseases, at the Kimron Veterinary Institute.

Analysis of nucleotide and deduced amino acid sequences

Nucleotide and deduced amino acid sequences were aligned and edited by means of the profile-based progressive alignment procedure, ClustalW, Version 1.83 using the DNASTAR and BioEdit Package, Version 5 software (Ibis Therapeutics, a division of Isis Pharmaceuticals, Inc.).

Phylogenetic analysis

The sequences of Israeli H5N1 isolates were compared with sequences of H5N1 influenza viruses isolated in the Gaza Strip and with H5N1 sequences from the GeneBank database. Nucleotide sequences of HA and NA genes were used to study and construct the respective phylogenetic trees. The phylogenetic analysis was performed with the PHYLIP (Phylogeny Inference Package) software, Version 3.65 (Department of Genome Sciences and the Department of Biology at the University of Washington). The nucleotide sequences of the Israeli and Palestinian isolates are available from GenBank under the accession numbers EF532622–EF532643.

Results

The general picture of the outbreaks in Israel and Gaza and the associated problems were described by Leventhal et al. [27]. The first case of H5N1 flu in Israel was diagnosed on 16th March 2006, and a total of nine H5N1-affected flocks were recorded in industrial poultry coops in Israel between the 16th and 31st March. The majority of them were in coops adjacent to the Gaza Strip. During these outbreaks, nine H5N1 viruses were isolated in Israel, and an additional five viruses were isolated from materials obtained in Gaza

and delivered by the Palestinian Veterinary Service. The virus caused fatal disease after inoculation into chickens and mice, and induced cytopathogenic effects in chicken corneal cell culture. The genes coding HA and NA proteins were sequenced in six Israeli and five Palestinian isolates.

Sequence analysis of the HA genes

Sequence analysis of the HA genes from the six Israeli and five Gaza isolates showed that the protein coding portion of all these HA genes consisted of 1,707 nucleotides from start to stop codons. The HA gene nucleotide sequences of these viruses showed high homology: 99.59–100% (average—99.82%). Point mutations were discovered in 10 nucleotide positions, and only two nucleotide substitutions caused amino acid changes: N72S (A/chicken/Gaza/714/2006) and S288N (A/chicken/Gaza/713/2006). The first 48 nucleotides encoded the signal peptide; the next 1,656 bases encoded uncleft precursor (HA0), and the last three nucleotides (stop codon) caused the ending of the protein synthesis. All the Israeli and Gaza isolates had identical signal peptides comprising the 16 following amino acids: MEKIVLLLAIVSLVKS. All the HA0 from the Israeli and Gaza isolates contained multiple basic amino acids at the cleavage site, RRRKKR/G, which connects two polypeptide chains, HA1, and HA2. All the isolates had HA proteins containing seven potential glycosylation sites, five on the HA$_1$ chain (10, 11, 23, 165, and 286), and two on the HA$_2$ chain (484 and 543). Analysis of the amino acid sequences associated with receptor binding activity showed that all the studied isolates had identical receptor binding sites: Y98, S136, W153, H183, E190, R193, L194, K218, S221, K222, G225, Q226, S227, G228 (in H3 numbering).

Phylogenetic analysis of the HA genes

The phylogenetic analysis of the HA genes showed that H5N1 viruses isolated in the Middle East, including the Israeli and Gaza isolates in the present study, could be included in a single subgroup which, in turn, fell into the same group as H5N1 viruses isolated in other countries in 2005–2006 (Fig. 1). All of the HA genes of the Israeli isolates sequenced in the present study exhibited some differences from the H5N1 viruses those have been isolated from poultry and humans in several other countries, including Vietnam, Japan, Korea, China, and Indonesia during 1997–2006.

Sequence analysis of the NA genes

Study of the nucleotide sequence of the NA gene showed that the genes of all the Israeli isolates were 1,350 nucleotides in length and encoded a polypeptide of 449 residues.
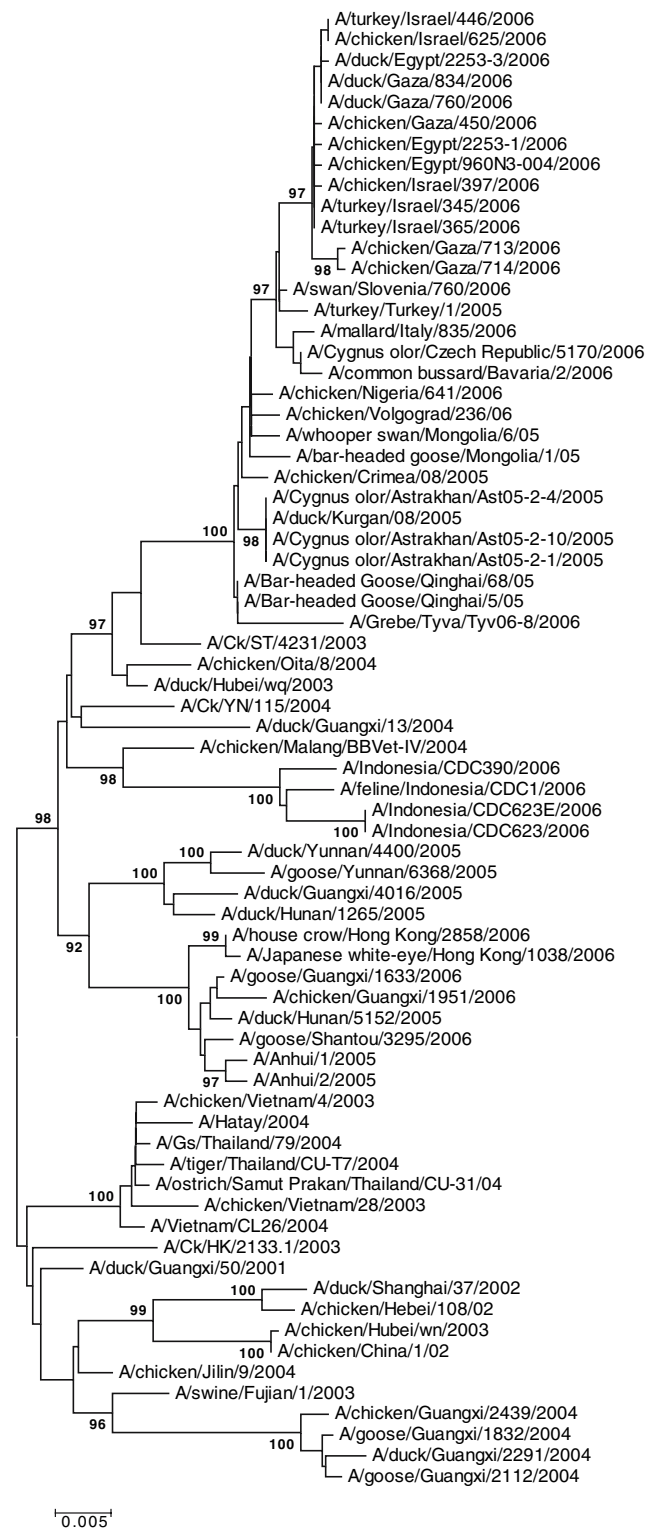

Fig. 1 Phylogenetic trees for the H5 HA genes of influenza A viruses. The nucleotide sequences were analyzed with MEDA 3.1 by using a neighbour joining algorithm. The HA1 tree is rooted to A/duck/Guangxi/50/2001 (H5N1)

As compared with early H5N1 isolates, e.g., A/duck/Mongolia/54/01, all the studied viruses had NA proteins

with deletions 20 amino acids in length. The NA polypeptide contained one hydrophobic domain located near its N-terminus, which is typical for a class II integral membrane protein. There were three sites (68, 126, and 215) for the potential addition of an N-linked carbohydrate. The NA genes from the Israeli and Gaza isolates are closely related. Nucleotide point mutations were detected in 11 sites, only two of which caused amino acid substitutions (All studied H5N1 viruses had NA proteins with typical catalytic site (R118, D151, R152, R224, E276, R292, R371, and Y406 – in N2 numbering) and framework site (E119, R156, W178, S179, D198, I222, E227, H274, E277, N294, E425 – in N2 numbering).

Phylogenetic analysis of the NA genes

Phylogenetic analysis of the NA genes showed that the Israeli isolates might be included in a single group together with viruses recently isolated in Gaza and Egypt. Moreover, the Israeli isolates clustered with the majority of the H5N1 viruses isolated in European, Asian, and African regions during 2005–2006. In contrast, earlier isolated H5N1 viruses fell into different groups from the Israeli strains (Fig. 2).

Discussion

The outbreaks of highly pathogenic avian influenza in birds occurred in Israel and Gaza in March and April 2006. A sum of 14 viruses were isolated from poultry and were identified as the highly pathogenic H5N1 subtype. The molecular characterization and an analysis of phylogenetic relationships indicated that all the Israeli and Gaza isolates belonged to a single cluster, and that their genetic characterizations were practically identical with those of H5N1 viruses isolated in Egypt in 2006, e.g., A/duck/Egypt/2253-3/2006, and were closely related to other H5N1 strains isolated during this period in European, Asian, and African countries. However, the isolates in the present study were different from H5N1 viruses recently isolated in Indonesia. It is reasonable to suppose that the viruses that caused the Israeli and Gaza outbreaks had Egyptian parentage, or that the Egyptian and Israeli isolates originated from a single unknown virus.

Analysis of the amino acid sequences of the Israeli H5N1 HA proteins revealed a pattern typical of HPAI. The signal peptide consisted of 16 amino acids, including 11 nonpolar residues, so that the peptide possessed the high level of hydrophobicity that is necessary for initiation of HA protein synthesis and successful virus propagation [28, 29]. The matured HA of the H5N1 Israeli and Gaza isolates contained the connected segment between HA1 and HA2
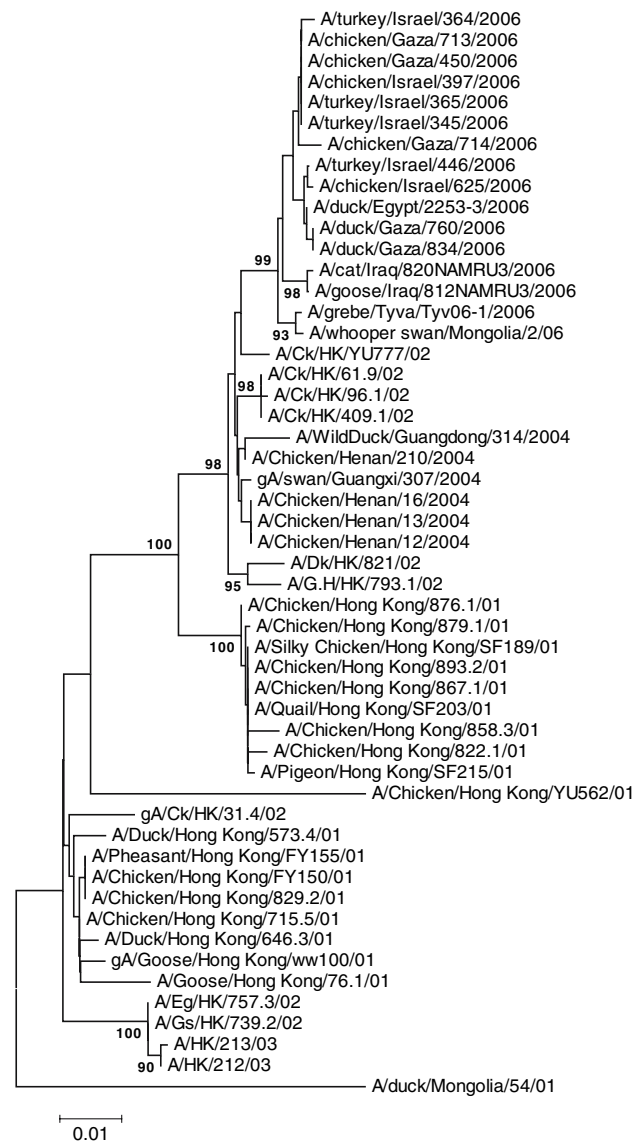

Fig. 2 Phylogenetic trees for the N1 NA genes of influenza A viruses. The nucleotide sequences were analyzed with MEDA 3.1 by using a neighbour joining algorithm. The NA tree is rooted to A/duck/Mongolia/54/01 (H5N1)

chains, the so-called cleavage site, which consists of the six basic amino acids: RRRKKR. The multi-basic cleavage site is one of the important characteristics of the highly pathogenic influenza virus strains [30, 31]. It is known, that this structure may be cleaved by both tripsin-like extracellular and furin-like cellular proteases. Moreover, this sequence possesses high level of hydrophilicity that, in turn, promotes the high accessibility of this site for proteolytic enzyme action. The Israeli and Gaza isolates differed from the majority of other H5N1 isolates in that phenylalanine occupied position 553, the first amino acid residue of the cytoplasmic tail; usually, H5N1 viruses have serine

in this position. Apart from the presently studied viruses, F553 was present in HA of several recently isolated Egyptian and Nigerian H5N1 viruses: A/chicken/Egypt/2253-1/2006, A/duck/Egypt/2253-3/2006, A/chicken/Egypt/960N3-004/2006, A/chicken/Nigeria/SO493/2006, and A/chicken/Nigeria/SO300/2006. In the present study, alignment of the HA amino acid sequences revealed that all of the Israeli and Gaza isolates possessed glutamine at position 226 and glycine at position 228 (in H3 numbering), and these are known to be associated with binding to typical for avian a(2,3)-linked sialic acids [32–37]. In addition, some other amino acids (Y98, S136, W153, H183, E190, and L194) of the receptor-binding pocket that were identified among the isolates are known to bind preferentially to a(2,3)-linked but not to a(2,6)-linked sialic acids [38, 39].

The H5N1 viruses isolated in Israel and Gaza had the NA protein with deletion of 20 amino acids, that is typical of this subtype strains isolated from poultry [40, 41]. All the presently studied isolates contained amino acid residues in the catalytic and framework sites which have been shown elsewhere to be inherent in viruses sensitive to NA-inhibitor drugs [42–45].

In the present study, we have demonstrated that a single group of highly pathogenic H5N1 avian influenza viruses caused outbreaks in Egypt, Gaza, and Israel. How these viruses were introduced into Israeli poultry remains unknown. Several routes of introduction are possible: by wild birds, by virus-contaminated materials, or by humans working in poultry farms. Rapid diagnosis and immediate application of preventive measures enabled the Israeli outbreaks to be eliminated within the minimal time frame. Our experience confirms the conclusions of Chen et al. [46] that "the best approach to avert the threat is to control H5N1 virus infection at its source, domestic poultry."

With regard to the risk of human infection, it should be noted that all the human pandemics of AIV were caused by viruses with the HA receptor binding pocket preferentially bound to receptors that terminate in an a(2,6)-linked sialic acid [47–51]. Thus, none of the HA proteins of the H5N1 isolates examined in the present study have the characteristics that are necessary to initiate a human pandemic.

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